Gene Amplification in *Bacillus subtilis*

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A strain of *Bacillus subtilis* that carries in its genome a staphylococcal chloramphenicol acetyltransferase gene (from pC194) responds to growth at different concentrations of chloramphenicol by an alteration in the number of copies per genome of the sequences encoding the gene. Growth at 20 µg chloramphenicol ml⁻¹ results in a 15-fold amplification of the sequences, whereas growth in the absence of chloramphenicol results in their loss. The mechanism of *in situ* amplification probably has much in common with that involved in 'R factor transitioning'. The hybridization procedures that have been used for accurately determining the number of copies of the amplified DNA sequences are potentially useful for plasmid copy number determination. The findings reported here also provide a potentially useful alternative to more conventional cloning strategies that are based on autonomous plasmids in *B. subtilis*. The particular advantages that can be envisaged include enhanced stability of the cloned sequences and control of the number of copies that are present.

INTRODUCTION

Gene amplification is of widespread occurrence in eukaryotic organisms (Schimke, 1982). The gene that encodes the mammalian dihydrofolate reductase provides one of the best-known examples. The enzyme is inhibited by methotrexate, and drug-resistant cell lines arise by the amplification (up to 200-fold) of sequences containing the dihydrofolate reductase gene (Alt *et al.*, 1978; Nunberg *et al.*, 1978). Presumably, the elevated gene dosage effectively satisfies an increased demand for the enzyme. Gene amplification also satisfies fluctuations in the demand for certain gene products during development (Rochaix *et al.*, 1974; Spradling & Mahowald, 1981; Chisholm, 1982), and it has been implicated in oncogenesis (Dalla-Favera *et al.*, 1982; Collins & Groudine, 1982; Little *et al.*, 1983). The multigene families that commonly occur in eukaryotic organisms are thought to be the relics of gene amplification events that occurred in the evolutionary past (Hood *et al.*, 1975).

Gene amplification in prokaryotic organisms has received comparatively less attention. Nevertheless, many examples are known. In their review in 1977, Anderson & Roth described several procedures that have been used to promote the duplication of specific segments of the chromosome of *Escherichia coli* and *Salmonella typhimurium*. In common with eukaryotes, selection for increased gene dosage can be applied to generate strains that carry multiple copies of a gene. For example, Novick and his colleagues obtained an approximately fourfold amplification of the *lac* operon after growth of *E. coli* for many generations on a limiting concentration of lactose as sole carbon source (Horiuchi *et al.*, 1962, 1963). Growth of a *Proteus mirabilis* strain containing an R plasmid in the presence of chloramphenicol, to which the plasmid conferred resistance, resulted in the tenfold amplification of the plasmid-borne r determinant (Perlman & Rownd, 1975). This process has been termed 'R factor transitioning'. The chromosomes of *E. coli* and *Bacillus subtilis* contain multiple copies of the rRNA genes (Nomura *et al.*, 1977; Stewart *et al.*, 1982). There are numerous other examples of low-level gene amplification in prokaryotes (Anderson & Roth, 1977). In the streptomycetes, high-level amplification (up to 500 copies per genome) of specific chromosomal sequences has been

described. Members of the genus *Streptomyces* commonly show genetic instability and in several cases variants with altered abilities to elaborate 'secondary products' have been shown to harbour tandemly repeated arrays of short DNA sequences in their genomes (Ono *et al.*, 1982; Schrepf, 1982; Fishman & Hersherberger, 1983). In an earlier report, this phenomenon was noted amongst the progeny arising from interspecific protoplast fusions (Robinson *et al.*, 1981).

Recently, there have been reports of the amplification of antibiotic-resistance determinants of plasmid origin when they are forced to integrate into the chromosome of *E. coli* (Gutterson & Koshland, 1983), *Streptococcus pneumoniae* (Vasseghi & Claverys, 1983) and *B. subtilis* (Young, 1983). The last of these reports concerned a plasmid, p1949, that lacked a functional replication origin for *B. subtilis*, although it carried a chloramphenicol-resistance determinant that is expressed in this organism (Haldenwang *et al.*, 1980). Integration of the plasmid into the chromosome was facilitated by inserting into it a 5.7 kb *EcoRI*-*Bam*HI fragment of *B. subtilis* DNA that was able to transform *pheA1* and *nic-38* recipients to prototrophy (Young, 1983). This paper confirms that the amplified plasmid sequences are organized as a tandemly repeated array. The number of copies of the reiterated sequences has been accurately determined. It has been found that the degree of amplification can be controlled by manipulation of the concentration of chloramphenicol in the growth medium.

METHODS

Strains. *Bacillus subtilis* 168 *trpC2* is the laboratory wild-type strain. Strain CL104 *trpC2 spo0B136 cat* has been described previously (Young, 1983) and strain GSY860 *argA11 nic-38* was obtained from C. Anagnostopoulos, Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France.

Preparation of DNA. Plasmid pSC5 (Young, 1983), which was used as the source of the *phe-nic* fragment of *B. subtilis* DNA for nick-translation, was extracted from a $r_k^- m_k^- recBC$ derivative of *E. coli* C600 by preparing a cleared lysate (Clewell & Helinski, 1969) and subjecting it to two cycles of caesium chloride density gradient centrifugation. Chromosomal DNA from strains 168 and CL104 was prepared by caesium chloride density gradient centrifugation, essentially as described by Harris-Warwick *et al.* (1975). All DNA preparations were finally dialysed against Tris (10 mM)/EDTA (1 mM) buffer, pH 8; DNA concentrations were estimated from the A_{260} of the preparations.

Restriction endonucleases. *EcoRI*, *Bam*HI and *Sal*GI were obtained from BRL; *Bgl*II and *Hind*III were obtained from Cambridge Biotechnology Laboratories. Enzymes were used in twofold excess for digesting plasmid DNA samples. In order to ensure that digestions of chromosomal DNA samples were complete, the reaction was started by adding 5 units of the appropriate enzyme, 60 min later a further 5 units of enzyme was added, and after 120 min had elapsed the samples were used for electrophoresis. The reaction conditions (i.e. pH, salt concentration, etc.) were as recommended by the supplier.

Agarose gel electrophoresis. A horizontal gel electrophoresis apparatus was employed to separate DNA fragments according to their size. For routine analysis 0.6% (w/v) agarose gels were employed.

Nick-translation. A sample (about 0.5 µg) of the 5.7 kb *phe-nic* fragment was prepared by restricting 1 µg of plasmid pSC5 (Young, 1983) with *EcoRI* and *Bam*HI and separating the two restriction fragments obtained by electrophoresis through 0.6% agarose. DNA was recovered from the gel by electrophoresis onto DEAE-paper (Dretzen *et al.*, 1981). It was radioactively labelled to a specific activity of about 10^7 d.p.m. (µg DNA)⁻¹ using ³²P-labelled dCTP (Amersham) according to the procedure of Jeffreys & Flavell (1977).

Southern hybridization. DNA restriction fragments were applied to agarose gels and electrophoretically separated according to their size. The DNA was depurinated *in situ* as described by Wahl *et al.* (1979) to promote the quantitative transfer of large DNA fragments in the next stage of the procedure. The method of Southern (1979) was used to transfer DNA fragments to a nitrocellulose membrane (Schleicher & Schüll, Dassel, FRG), after which the hybridization and washing protocol of Sager *et al.* (1981) was employed, except that dextran sulphate was omitted from the solutions. Autoradiography was carried out at -20 °C using prefogged Kodak X-Omat RP film and an intensifying screen.

In the quantitative hybridization experiments, samples (1 µg) of *Bam*HI- or *Sal*GI-digested CL104 DNA were separated by electrophoresis through 0.35-0.4% (w/v) agarose (Miles Laboratories, Stoke Poges, UK). The gels were fragile and had to be handled carefully. After transfer to nitrocellulose, autoradiography was used to localize the radioactively labelled DNA fragments. Segments A, B and C (see Fig. 1) of the nitrocellulose membrane, corresponding to the single-copy border fragment (18 kb in *Bam*HI-digested samples), a background sample and the 11 kb reiterated sequences, respectively, were excised and counted to an accuracy of $\pm 2-3\%$ (two SD) using a Beckman liquid scintillation spectrometer. The number of copies of the reiterated *phe-nic* sequences is given by $[(c - b)/(a - b)] + 1$ where *a*, *b* and *c* are the activities of the nitrocellulose segments A, B and C, respectively.

Fig. 1. Hybridization of the *phe-nic* fragment to endonuclease-digested DNA from strains 168 and CL104. DNA samples (1 µg) were digested with *Bam*HI, *Bg*II and *Hind*III. After electrophoresis through 0.35% agarose (a) or 0.6% agarose (b) the DNA fragments were transferred to a nitrocellulose filter. Hybridizations were carried out as described in Methods. The sizes of the fragments were determined with reference to standard samples of undigested λ DNA and λ DNA digested with *Hind*III. The portions of the filter labelled A, B and C correspond to the segments that were excised from *Bam*HI-digested samples for quantitative measurement of hybridization. (See Table 1 and Fig. 3.)

This method was employed instead of direct scanning of the autoradiograms, since this latter approach has two potential drawbacks. First, the image intensity depends on the extent to which the film has been prefogged and second, it is linearly related to the activity present only over a relatively narrow range (Laskey, 1980).

RESULTS

Structure of the pheA region of the chromosome in strain CL104

Strain CL104 of *B. subtilis* harbours multiple copies of a chloramphenicol-resistance plasmid comprising 5.6 kb of vector (p1949) sequences and 5.7 kb of host sequences (the *phe-nic* fragment) that have become integrated into the *pheA* region of the chromosome (Young, 1983).

Fig. 2. Restriction map of the *pheA* region of the *B. subtilis* chromosome. The 5.7 kb *EcoRI*-*BamHI* fragment (stippled) located on the map between about 18 and 24 kb includes part of the *pheA* locus at the *EcoRI* end, and part, at least, of the *nic* locus. Two copies of the reiterated DNA segment are shown inserted at the *EcoRI* site (at about 18 kb). Between eight and nine copies are present per cell in strain CL104; it is absent from strain 168. The reiterated DNA forms a tandemly repeated array of 11.3 kb units, comprising 5.7 kb of host sequences (the *phe-nic* fragment) plus 5.6 kb of plasmid p1949 sequences, including those that encode the *cat* gene. The plasmid DNA is shown as a single line. The sizes (kb) of the *HindIII* fragments that hybridize to the *phe-nic* fragment (see Fig. 1) are as indicated. The positions of the *HindIII* sites that lie outside the region from about 15 to 26 kb on the map have not been determined. The restriction sites are represented as follows: B, *BamHI*; E, *EcoRI*; H, *HindIII*; S, *SalGI*.

Chromosomal DNA from strains 168 and CL104 was cleaved with a variety of restriction endonucleases and the DNA fragments were separated according to their size using agarose gel electrophoresis. After transfer to nitrocellulose, hybridization was carried out using a radioactively labelled sample of the *phe-nic* fragment as probe (Fig. 1). The DNA from both strains yielded an 18 kb *BamHI* fragment to which hybridization occurred at equivalent intensity. An additional 11 kb fragment to which much more intense hybridization occurred was seen in the sample of strain CL104 DNA; this represents the entire integrated plasmid which has a single *BamHI* site (Young, 1983). After digestion of the chromosomal DNA with *HindIII*, the probe hybridized with three fragments of DNA from strain 168. The largest and smallest of these (4.9 kb and 1.7 kb) are also seen at equivalent intensity in the *HindIII*-digested sample of strain CL104 DNA. The remaining 3.2 kb fragment shows a much greater intensity of hybridization in the CL104 sample than in the 168 sample. Two additional intensely hybridizing fragments (6.0 kb and 1.9 kb) are also present in the *HindIII*-digested sample of CL104 DNA; together with the 3.2 kb fragment mentioned earlier, these sequences comprise the amplified plasmid that has been inserted into the chromosome of strain CL104. These data are summarized in Fig. 2.

Plasmid DNA is amplified approximately eightfold in strain CL104

The integrated plasmid lacks a restriction site for the enzyme *BglII*. It had been hoped that information concerning the copy number of the amplified DNA could be obtained by comparison of the mobilities of the *BglII* DNA fragments from strains 168 and CL104 that hybridize to the *phe-nic* probe (see Fig. 1). However, the *BglII* fragment from strain 168 DNA was comparatively large (25 kb); it was replaced in the equivalent sample of CL104 DNA by an intense band of hybridization corresponding to about 50 kb of DNA. This method did not

Table 1. *Determination of the number of copies of the amplified DNA sequences in strain CL104 by quantitative hybridization*

Samples (1 µg) of CL104 DNA were completely digested with *Bam*HI or *Sal*GI, subjected to electrophoresis through 0.4% agarose and transferred to a nitrocellulose membrane. They were hybridized against a nick-translated sample of the *phe-nic* fragment (5×10^5 d.p.m.) for 49 h and then the positions of the radioactively labelled DNA fragments were determined by autoradiography. Three portions of equivalent size were excised from the membrane (labelled A, B and C in Fig. 1) and their radioactivities determined. Sample B, which comprises a region of the filter between the upper single-copy fragment (A), and the lower multiple-copy fragment (C) served as a background control. Each sample was counted three times for a total of 120 min.

Sample	c.p.m.	Background subtracted	Ratio A/C
<i>Bam</i> A	87	23	8.7
<i>Bam</i> B	64	—	
<i>Bam</i> C	263	199	
<i>Sal</i> A	86	22	8.6
<i>Sal</i> B	64	—	
<i>Sal</i> C	254	190	

therefore provide adequate resolution for the determination of copy numbers in excess of two per genome. Accordingly, the quantitative hybridization procedure described in Methods was employed. It is based on the fact that after digestion of CL104 DNA with *Bam*HI and hybridization, the 18 kb fragment (A) is present as a single copy per genome, whereas the 11 kb fragment (C) corresponds to the amplified DNA itself (Fig. 2). A similar result is obtained when CL104 DNA is digested with *Sal*GI (Young, 1983). The results in Table 1 indicate that an eight- to ninefold amplification of the integrated plasmid sequences has occurred in strain CL104.

Several attempts were made to obtain independent confirmation of this value by measuring the relative efficiencies with which *Eco*RI + *Bam*HI-digested samples of DNA from strains 168 and CL104 gave *Nic*⁺ and *Arg*⁺ transformants of strain GSY860. After double digestion with *Eco*RI + *Bam*HI, the transforming activity was so low that reliable values could not be obtained. However, with the undigested control samples the *Nic*⁺ transforming activity was 2.8 times greater in strain CL104 DNA than in strain 168 DNA.

Manipulation of the number of copies of the amplified DNA segment in strain CL104

After overnight growth in nutrient broth in the absence of chloramphenicol, 30 out of 210 single-colony isolates had lost the ability to grow on chloramphenicol-supplemented nutrient agar. Southern hybridization experiments using DNA isolated from six representative chloramphenicol-sensitive segregants revealed that in all cases the amplified 11 kb *Bam*HI fragment (see Fig. 1) had been lost. It was surmised that the excised copies of the reiterated sequences that had been lost in this fashion from one chromosome might reintegrate into the sister chromosome in a cell containing two copies of the genome. This would lead to amplification of the chloramphenicol acetyltransferase gene, which would presumably be reflected in subsequent generations of bacteria by an enhanced chloramphenicol tolerance. Bacteria were serially subcultured on nutrient agar containing chloramphenicol at a range of concentrations. Growth occurred at all chloramphenicol concentrations up to 20 µg ml⁻¹ after the first subculture. Bacteria were repeatedly subcultured onto media containing successively higher concentrations of chloramphenicol until, after five subcultures, isolates were obtained that could grow on media containing 50 µg chloramphenicol ml⁻¹. DNA was prepared from these strains and quantitative hybridization was used to determine the number of copies of the amplified sequences (Fig. 3). As expected, after growth in the absence of chloramphenicol the amplified segment was lost. At a concentration of 2 µg chloramphenicol ml⁻¹, at which the wild-type strain (168) was still able to form colonies, there appears to be some selective advantage in the retention of about three copies of the reiterated sequences per genome. At a chloramphenicol concentration of 20 µg ml⁻¹ there were about 15 copies of the reiterated sequences per genome. Unfortunately, the autoradiograms of the DNA sample from the strain growing at 50 µg chlor-

Fig. 3. Amplification of the reiterated sequences in strain CL104 in response to elevated concentrations of chloramphenicol. Strain CL104 was serially subcultured on nutrient agar at a range of concentrations of chloramphenicol. DNA was isolated from the bacteria and the number of copies of the *phe-nic* fragment per genome was determined by quantitative hybridization.

amphenicol ml⁻¹ showed smearing and it was not possible to obtain a quantitative estimate of copy number for this sample. At concentrations of chloramphenicol of 20 µg ml⁻¹ and above, the amplified sequences were evident from visual inspection of ethidium bromide-stained agarose gels (Fig. 4). Hybridization experiments with undigested DNA samples (data not shown) indicated that a significant proportion of the amplified sequences were extra-chromosomal.

DISCUSSION

In a strain of *Bacillus subtilis* that carries as an integral part of its chromosome the chloramphenicol acetyl transferase (*cat*) gene of the *Staphylococcus aureus* plasmid, pC194, it has been shown that selective amplification of the plasmid sequences will occur *in situ* in response to an elevated demand for the *cat* gene product. When the selective pressure was reduced, the degree of amplification was reduced accordingly; if it was removed altogether, the amplified sequences were lost. The plasmid sequences alternate with *B. subtilis* chromosomal sequences to form a tandemly duplicated array, and a copy of the chromosomal sequences is found at either extremity of the array (Fig. 2). The recombination processes involved in amplification probably have much in common mechanistically with those that occur during 'R factor transitioning' (Perlman & Rownd, 1975; Peterson & Rownd, 1983).

It is not yet possible to say whether amplification commonly occurs when insertion vectors such as p1949 are used in *B. subtilis*. However, it is clearly not of universal occurrence, since Haldenwang *et al.* (1980) did not detect amplification when they used a 2 kb *EcoRI** fragment of *B. subtilis* DNA to direct the insertion of this plasmid into the *cysA* region of the chromosome. In other experiments using a different insertion vector, it appears that extensive amplification does not occur (Piggot *et al.*, 1984). On the other hand, similar results to those reported here have been obtained with a plasmid comprising a 0.4 kb *EcoRI*-*Bam*HI fragment from the *hisA* region of the chromosome, together with the 5.6 kb *EcoRI*-*Bam*HI fragment of p1949 (M. Young & P. Brazier, unpublished). It is possible that plasmid p1949 is particularly prone to amplification when both the *EcoRI* and *Bam*HI sites are used for cloning. However, amplification does not seem to be restricted to this particular plasmid, since it has been reported for a recombinant plasmid derived from pUB110 (Saito *et al.*, 1983). It may also explain the results obtained by Wilson *et al.* (1981), although the authors tentatively attributed the presence of a disproportionately intense band of hybridization (see their Fig. 2, lane C) to free plasmid DNA. It would seem plausible to propose that amplification of the *cat* gene will depend, in part at least, on the efficiency with which it is transcribed. If the gene is placed in an environment where expression

Fig. 4. Agarose gel electrophoretogram of *Bam*HI-digested samples of strain CL104 DNA. Strain CL104 was grown in nutrient broth containing a range of concentrations of chloramphenicol: lane 1, none; lane 2, $0.2 \mu\text{g ml}^{-1}$; lane 3, $0.5 \mu\text{g ml}^{-1}$; lane 4, $2.0 \mu\text{g ml}^{-1}$; lane 5, $5.0 \mu\text{g ml}^{-1}$; lane 6, $20 \mu\text{g ml}^{-1}$; lane 7, $50 \mu\text{g ml}^{-1}$. The position of the 11 kb amplified fragment is marked with an arrow.

occurs through the agency of a relatively weak promoter, then a high level of gene expression will not be possible unless amplification takes place. Further work will be required to define precisely the factors that predispose chromosomal sequences to undergo amplification.

The number of copies of the amplified DNA was determined by hybridization. Quantitative treatment of the data was possible because the radioactively labelled probe had homology with the single copy border fragment and the multicopy amplified DNA that were conveniently separated by agarose gel electrophoresis of *Bam*HI-digested DNA. This approach is potentially useful for the determination of plasmid copy number, and is directly applicable to recombinant plasmids that carry a fragment of the host chromosome, since the fragment can be used as a convenient hybridization probe. It is of interest that the number of copies of the amplified DNA in strain CL104 appeared to be much lower when measured by transformation than when measured by hybridization – the values obtained were 2.8 and 8.6, respectively. Similar results, based on assays of the transforming activity of strain CL104 DNA using a *pheA1* recipient

strain, were obtained previously (Young, 1983). This may be attributed to the fact that the multiple copies of the amplified DNA are tandemly repeated. Hence, several copies will tend to be carried on the same piece of transforming DNA. Since the tandemly repeated array comprises some 100 kb (8.6×11.3 kb) of DNA, and the apparent amplification as measured by transformation was only 2.8-fold, it may be deduced that the average size of the transforming fragment in these experiments was about 35 kb. This value is similar to that calculated by Henner & Hoch (1980), which is interesting in view of the fact that the amplified DNA comprises alternating blocks of plasmid and chromosomal sequences, which will tend to reduce homology between the recipient chromosome and the incoming DNA.

Evidence was obtained to suggest the presence of significant numbers of excised plasmid molecules in strains grown at high concentrations of chloramphenicol (data not shown). Very careful inspection of Fig. 1 will reveal extremely weak hybridization to the very small numbers of these molecules in the undigested and *Bgl*II-digested samples of strain CL104 DNA. The presence of these excision products raises the interesting possibility that the procedures reported here might prove useful as an alternative to conventional cloning strategies in *B. subtilis*. Propagation of cloned material as an integral part of the chromosome rather than as an autonomous plasmid may help to circumvent the well-known problem of plasmid instability in *B. subtilis* (Ostroff & Pène, 1983). The tendency for excision to occur at high copy numbers is potentially useful, since it provides a convenient source of the cloned sequences for transfer to an alternative host in which the vector can replicate autonomously. Since the number of copies of the cloned DNA can be manipulated experimentally, the effects of altered gene dosage can readily be explored. If sequences were inserted relatively close to the chromosomal replication origin, the dichotomous replication that is associated with rapid growth (Yoshikawa *et al.*, 1964) might add another dimension to the gene amplification.

We are currently exploring the possibility that transfer of the *recE4* mutation into an amplified strain may stabilize the reiterated sequences, such that they can then be perpetuated in the absence of antibiotic selection. This intriguing possibility is of potential interest industrially, since the cost of providing a high concentration of an antibiotic in an industrial-scale process is not insignificant.

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