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Analysis of a commercially improved *Penicillium chrysogenum* strain series: involvement of recombinogenic regions in amplification and deletion of the penicillin biosynthesis gene cluster

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Several commercially improved strains of *Penicillium chrysogenum* have been shown to carry amplifications of the entire penicillin biosynthesis gene cluster. Analysis previously carried out using the strain BW 1890 has here been extended to the characterisation of other members of the SmithKline Beecham strain improvement series. We have determined the length of the amplicon to be 57.4 kb and shown a general increase in copy number and penicillin titre through the series. Sequence analyses of the promoter regions of the *acvA*, *ipnA* and *aat* genes in the high titre strain BW 1901, and comparisons with wild-type sequences have not identified any potentially titre-enhancing mutations. In addition, cDNA screening has failed to identify any further transcribed elements within the co-amplified region. The homogeneity of hybridisation patterns and the identification and analysis of a single copy revertant has shown that the amplification is of a direct tandem nature and we propose a model of chromatid misalignment and recombination as its mode of generation. Hybridisation analysis of penicillin non-producing mutants has indicated the loss, in all those investigated, of the entire penicillin biosynthesis gene cluster, similarities between the deletion junctions in these strains and comparison with previously published data indicating the presence of recombinogenic regions flanking the penicillin biosynthesis gene cluster.

Keywords: amplification; β-lactam; penicillin; recombination; strain improvement

Introduction

Commercial production of penicillin began in 1941 using *Penicillium notatum* NRRL 1249-B21 in surface culture fermentation. Despite the identification later that year, of a new strain, NRRL 832, which produced good yields in aerated submerged culture, the search continued for high titre natural isolates. This quest was rewarded in 1943 with the isolation of a strain of *Penicillium chrysogenum* which yielded higher titres of penicillin than *P. notatum* NRRL 832. This strain, designated NRRL 1951, was reported as being '. . . a loose textured strain of *P. chrysogenum* which has produced satisfactory yields in submerged culture and which appears to offer considerable promise as a source of more productive sub-strains' [17]. This prediction proved well founded, research headed by the University of Wisconsin resulting in the generation of a family of strains producing ever higher titres of penicillin. Strains descended from members of this series continue to be isolated by pharmaceutical companies. Figure 1, compiled from several sources [1,5,11,16], represents the most complete record of strain improvement series thus far published, though incomplete records and industrial confidentiality prohibit the construction of a fully comprehensive chart.

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Concerted cloning programmes in the late 1980s resulted in the isolation, from *P. chrysogenum*, of three genes responsible for the production of penicillin. The *acvA*, *ipnA* and *aat* genes encoding δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) [22], isopenicillin N synthase (IPNS) [3,8], and acylcoenzyme A: 6-aminopenicillanic-acid-acyltransferase (ACT) [4,10], respectively, were found to be tightly clustered within the 39-kb genomic insert of the cosmid pCX3.2 [23].

Slot blot and densitometry analysis of genomic DNA from the SmithKline Beecham high producer strain BW 1890 probed with the *ipnA* gene, indicated that between eight and 16 copies are present in BW 1890. In addition, Southern analysis demonstrated that the entire 39-kb insert of pCX3.2 is amplified in the high titre production strain. To establish the effect of the amplification on transcription levels, mRNA was isolated from BW 1890 and NRRL 1951 after 16 h of fermentation. It was found that the IPNS mRNA level in BW 1890 was 32 to 64-fold that of NRRL 1951, an increase too great to be due to the amplification alone [21].

Concurrent research demonstrated the existence of amplified penicillin biosynthesis genes in two further strains which exhibit elevated production levels [5]. Using overlapping phage clones, a total of 35 kb contiguous DNA was cloned and mapped, probes from either end establishing that the entire contig is amplified in the high titre *P. chrysogenum* strains P-2 and AS-P-78. Dot blots revealed that the copy numbers of penicillin genes in these strains are approximately 9- and 6-fold respectively [5,12].

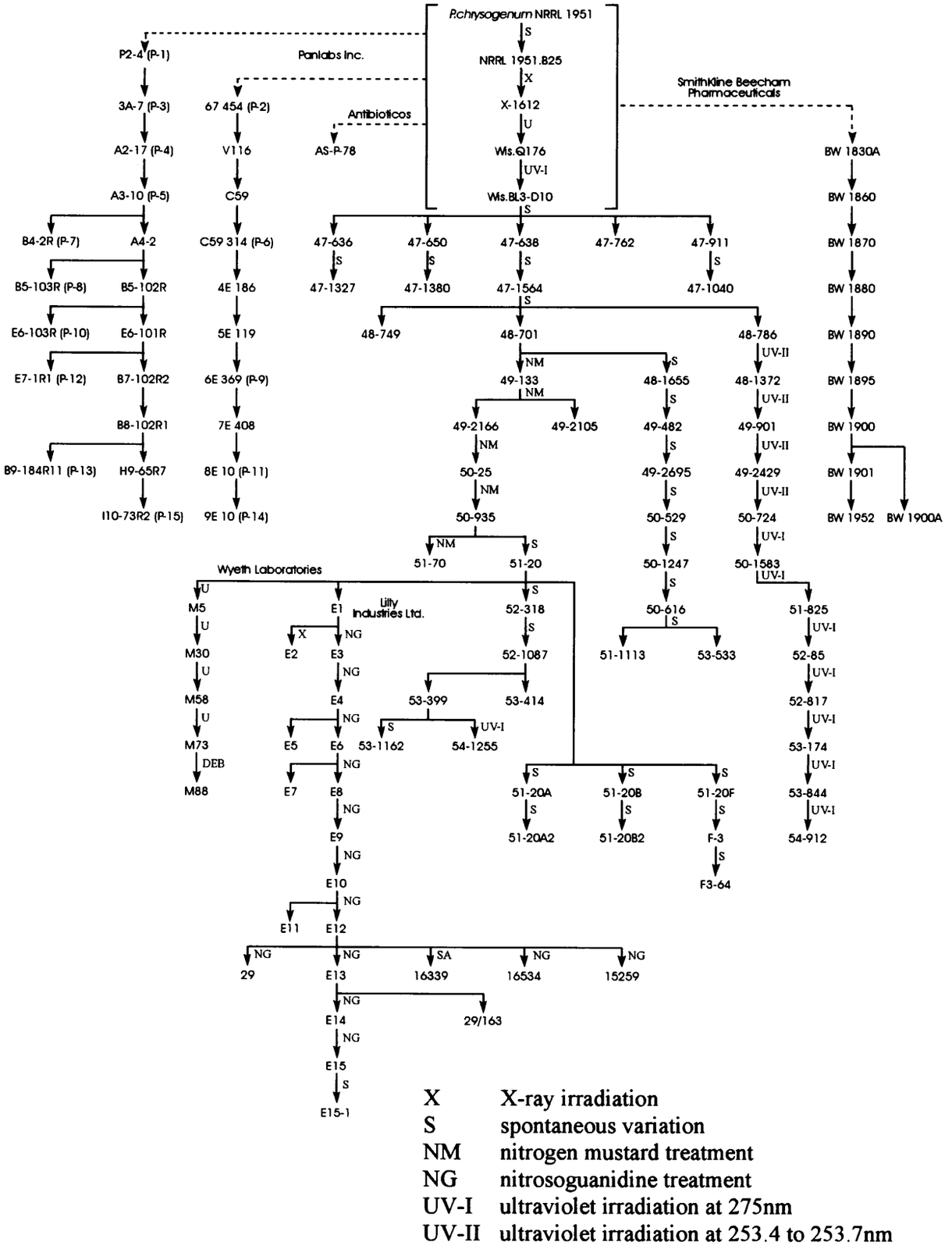

Figure 1 Lineages of improved strains derived from *Penicillium chrysogenum* NRRL 1951 (after Backus and Stauffer [1], Barredo *et al* [5], Elander [11] and Lein [16]).

More recently it has been shown that the amplifications in the high titre *P. chrysogenum* strains E1 and AS-P-78 are tandem, and that a conserved TTTACA hexanucleotide sequence may be involved in their generation [12].

On the basis of simultaneous research involving the SmithKline Beecham high producing strains, we here present additional evidence that amplification of the penicillin biosynthesis gene cluster is generated by unequal sister chromatid exchange, and that closely linked regions may possess recombinogenic properties. Evidence is provided for a tandem array of copies arranged head-to-tail. We also report the general increase in copy number throughout the series and draw comparisons with penicillin titre analyses.

Materials and methods

Strains, media and growth conditions

Penicillium chrysogenum strains are detailed in Table 1. *Escherichia coli* DH5α (Gibco BRL Life Technologies Ltd, Paisley, UK) was used for the propagation of plasmids and cosmids.

Solid medium for growth of *P. chrysogenum* was glycerol molasses (GM) as detailed by Smith *et al* [21]. Liquid medium for growth of *P. chrysogenum* was a defined complete medium (DCM) essentially as detailed by Cove [9] with the addition of 10 ml L^{-1} of CM supplement, comprising (g L^{-1}): adenine 3.75, L-methionine 2.5, lysine 18.25, riboflavin 0.5. Standard solid and liquid media were used for the growth of *E. coli*, supplemented where necessary with ampicillin to a concentration of 100 μg ml^{-1}. Penicillin V seed medium contained (g L^{-1}): corn steep liquor 35, glucose 15, $CaCO_3$ 5; and (ml L^{-1}): rape seed oil 8 (pH adjusted to 5.9 with NaOH). C5 final medium for penicillin titre assays contained (g L^{-1}): corn steep liquor 35, $MgSO_4 \cdot 7H_2O$ 4, Na_2SO_4 4, phenoxyacetic acid 6; and (ml L^{-1}): rape seed oil 6 (pH adjusted to 6.0 with NaOH). Medium for the selection of spontaneous variants was minimal medium (MM) previously described by Rowlands and Turner [19].

Table 1 Strains of *Penicillium chrysogenum* used for analysis

P. chrysogenum strains	Characteristics
NRRL 1951	Wild-type isolate [17]
BW 1830A, BW 1860, BW 1870, BW 1880, BW 1890, BW 1895, BW 1900, BW 1901, BW 1952	SmithKline Beecham strain improvement series
BW 1900A	Single copy derivative of BW 1900
M 1951	Sub-isolate of NRRL 1951 and mother strain of H2, H8, M1/13 and A1
H2, H8, M1/13, A1	Mutants blocked in penicillin biosynthesis

Isolation and manipulation of DNA

Total genomic DNA extraction from *P. chrysogenum* was by the method described by Ballance *et al* [2]. Small-scale preparations of plasmid and cosmid DNA were performed as described by Holmes and Quigley [14]. Large-scale preparations of plasmid and cosmid DNA were performed by CsCl gradient ultra-centrifugation as detailed by Sambrook *et al* [20]. Gene disruption was carried out using a similar strategy to Brakhage *et al* [6]. Colony hybridisations and Southern analyses were performed as detailed by Sambrook *et al* [20]. Standard PCR conditions according to Perkin Elmer GeneAmp PCR Reagent Kit were followed and the PCR cycle started with the addition of AmpliTaq DNA polymerase. Conditions for amplification of the promoter regions P*acvA*-P*ipnA* and P*aat* were: 95°C hot start for 7 min followed by 25 cycles of 1 min at 94°C, 1 min at 37°C and 3 min at 72°C. On completion the reaction was held at 30°C. DNA was sequenced using Pharmacia T7 and USB™ Sequenase®Version 2.0 ^{35}S-dATP systems according to manufacturer's instructions. Conditions for amplification of the 'switch fragment' were: 95°C hot start for 5 min followed by a cycle of 1 min at 94°C, 1 min at 52°C, 4 min at 72°C for 30 cycles. On completion the reaction was held at 4°C.

To assay for penicillin titre, 15 ml of seed medium was inoculated with a loopful of spores and grown for 2 days at 25°C, shaking at 250 rpm. One millilitre of seed culture was transferred to 10-ml aliquots of C5 medium and the cultures grown for 4 days as above. After centrifuging at $3000 \times g$ at 4°C for 20 min, the broth supernatant was collected and the pencillin titre determined by imidazole derivatisation and spectrophotometric determination (COBAS BIO centrifugal analyser, Roche Products Ltd, Heanor, UK) [7].

Nucleic acids

Cosmids used were pCX3.2, pIPS3 [21,22]. Plasmid vectors used were pUC19 [24]; pBSSK+ (Stratagene, Cambridge, UK); pRAJ275 (Clontech, Cambridge Bioscience, Cambridge, UK); p3SR2 [15]. Oligonucleotide primers for PCR and sequencing were: Pair A (P*acvA*-P*ipnA*); 5′-ggccttggggtggaagccatgg-3′, 5′-cccactgcattggtgcatgctgtc-3′: Pair B (P*acvA*-P*ipnA*); 5′-cttgggggtggaaggcatgctgtc-3′, 5′-cgcattcccactgcattggtccatgg-3′: Pair C (P*aat*); 5′-atccatggctctgagctaaaccga-3′, 5′-gccttgacagaccatgggaagcat-3′: Pair D (switch fragment); 5′-ttgctgcgttgaacaga-3′, 5′-tggttgtgatatgttgg-3′.

Results

Mapping of pCX3.2

The existing partial restriction map of pCX3.2 was extended, with respect to *Bam*HI, using standard techniques (Figure 2). In the process, construct pROG1 was produced by *Bam*HI restriction of pCX3.2 followed by religation. This was mapped with respect to *Eco*RI and *Bgl*II, and found to comprise the vector, pCAP2, linked to 7.6 kb of genomic insert from the region 3′ to the *aat* gene (Figure 2).

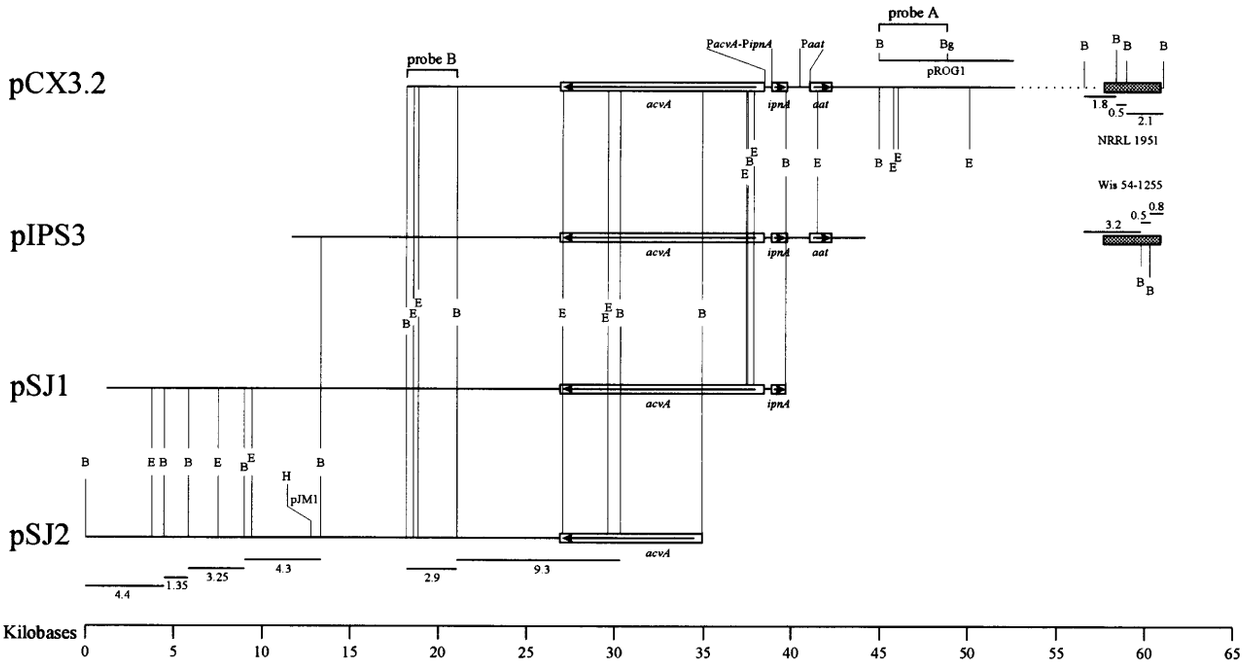

Figure 2 Aligned restriction maps of cosmids pCX3.2, pIPS3, pSJ1 and pSJ2. B, *Bam*HI; E, *Eco*RI; Bg, *Bgl*II; H, *Hin*dIII. Solid lines represent cloned regions, dotted lines represent non-cloned regions and shaded boxes represent the 3.2-kb switch fragment.

Two suitable fragments of pCX3.2 flanking the penicillin biosynthesis gene cluster were chosen for use as probes against a *P. chrysogenum* cosmid library. A 3.9-kb *Bam*HI/*Bgl*II fragment of pROG1 was selected to initiate the walking process 3′ to the *aat* gene (probe A, Figure 2), and the 2.9-kb *Bam*HI fragment of pCX3.2 was chosen from the 3′ flanking region of the *acvA* gene (probe B, Figure 2).

Chromosome walking

A cosmid bank of *P. chrysogenum* Oli13 [21] was screened by colony hybridisation using probe A (Figure 2). Though cosmids identical to pCX3.2 were identified, the screening was unsuccessful in isolating new overlapping clones and probing with probe A was suspended.

The screening was repeated using probe B (Figure 2) and eighteen clones were isolated. Of these, eight were pCX3.2, and four were pIPS3, a cosmid already previously isolated [22]. The remaining six clones were of two types, each of which clearly overlap pCX3.2, but extend further 3′ to the *acvA* gene. These were named pSJ1 and pSJ2.

Analysis of overlapping clones

In order to determine whether the end of the amplification had been reached, *Bam*HI-digested NRRL 1951 and BW 1890 genomic DNAs were probed with the cosmids isolated. Autoradiographs indicated that the cloned region extended outside the amplified region, both probes separately identifying the same 4.4-kb fragment that is not amplified in BW 1890 (Figure 3). We believe this fragment to be the same as that estimated as 4.6 kb by Fierro *et al* [12]. For both pSJ1 and pSJ2 probes, a notable difference between the banding patterns of NRRL 1951 and BW 1890 is a band indicating a 1.5-kb *Bam*HI fragment identifiable only in the amplified strain (Figure 4). It is proposed that this band represents the junction fragment between adjacent amplicons, and that it is constituted in part by DNA from the unamplified 4.4-kb fragment. The 4.4-kb *Bam*HI fragment was subsequently subcloned into the cosmid vector pWE15, instability problems having been encountered with smaller vectors in this case. Insert DNA from the resulting construct, pSAN4.4, was then used to verify homology with the novel 1.5-kb *Bam*HI fragment. As an aid to further analysis of the region 3′ to the *acvA* gene, the 4.3-, 3.25- and 1.35-kb *Bam*HI fragments of pSJ2 were subcloned, the former into pUC19 and the latter two into pBSSK+ (Bluescript) to yield the plasmids pMJ4.3, pVCN3.25 and pVCN1.35, respectively.

Identification of repeated sequences

Using conventional techniques, pCX3.2, pIPS3, pSJ1 and pSJ2 were mapped with respect to the enzymes *Bam*HI and *Eco*RI (Figure 2). However, the mapping of pSJ1 and pSJ2 was hindered by a high degree of cross-hybridisation between non-adjacent fragments within the cosmids (Figure 5). Under high stringency conditions, the 1.35-kb fragment was shown to cross-hybridise with both the 4.3-kb and 9.3-kb fragments whilst the 4.3-kb fragment cross-hybridised to only the 1.35-kb fragment.

Southern analysis of pMJ4.3 using the 1.35-kb probe, identified a 0.56-kb *Bam*HI/*Hin*dIII fragment which was subcloned into pUC19 to yield the plasmid pJM1 (Figure 2). The inserts of pJM1 and pVCN1.35 were sequenced and shown to share a 79-bp sequence displaying 93.7% identity (Figure 6).

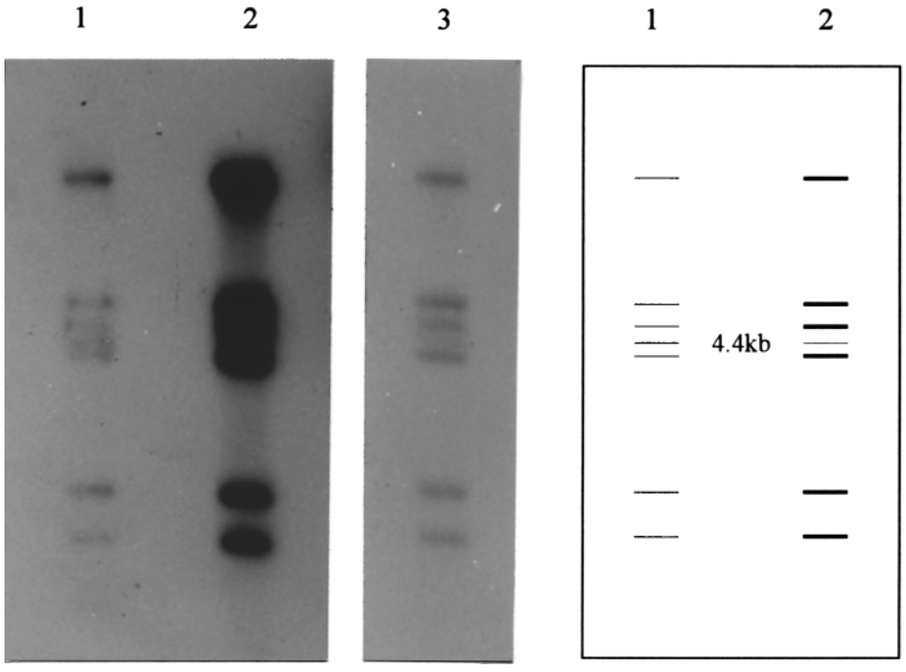

Figure 3 Southern analysis of *Bam*HI-digested NRRL 1951 and BW 1890 genomic DNAs using pSJ2 as a probe template. Disparity in band intensity defines the end of the unit of amplification. The window of resolution of the gel excludes the 1.35-kb fragment shown in Figure 4. *Lane 1*, NRRL 1951; *2*, BW 1890; *3*, BW 1890 shorter exposure.

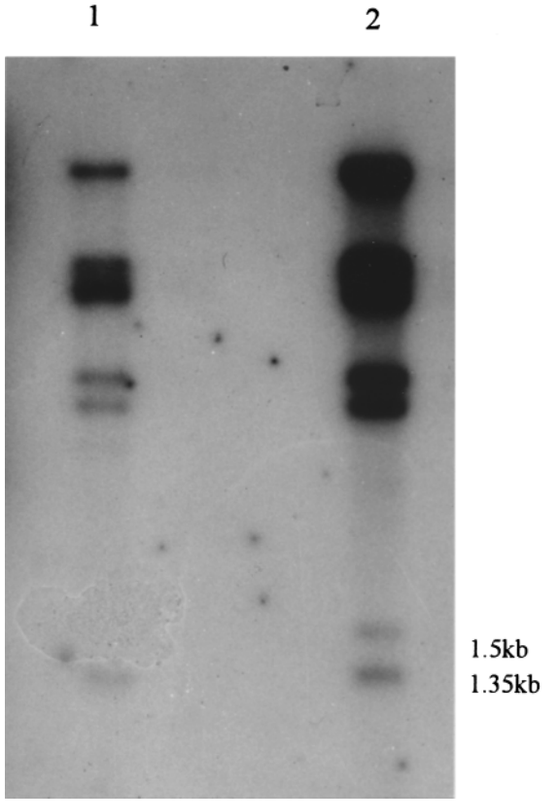

Figure 4 Southern analysis of *Bam*HI-digested *P. chrysogenum* BW 1890 genomic DNA using pSJ2 as a probe template. Identification of a 1.5-kb fragment spanning the junction between adjacent amplicons in BW 1890. *Lane 1*, NRRL 1951; *2*, BW 1890.

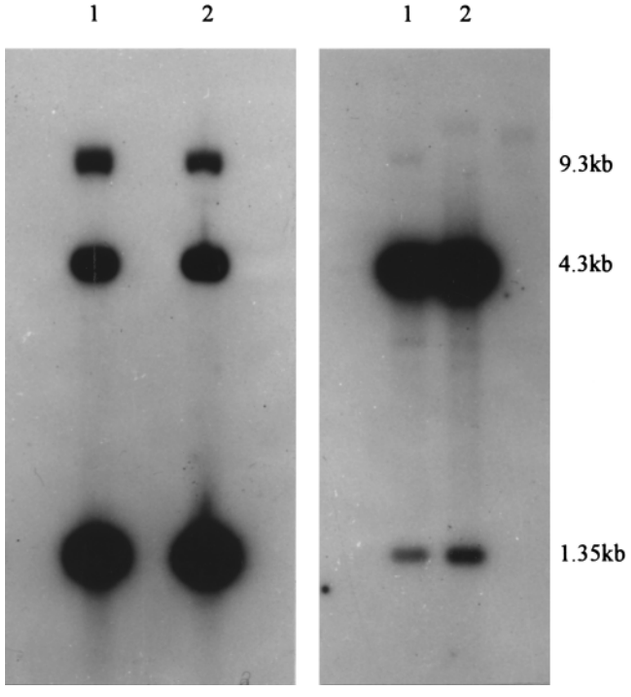

Figure 5 Southern hybridisation of *Bam*HI-digested pSJ1 and pSJ2 DNA, using the 1.35-kb (left) and 4.3-kb (right) *Bam*HI fragments of pSJ2 as probe templates. *Lane 1*, pSJ1; *2*, pSJ2.

Strain series analysis

The detection of a 1.5-kb junction fragment in BW 1890 provided a basis for the analysis of other high titre strains of *Penicillium chrysogenum*, in particular those previous to and succeeding BW 1890 in the SmithKline Beecham

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pVCN1.35    CCGATCCCAG CAGTGACACG CAACGGTAAG
            |||||||||| |||||||||| ||||||||||
pJM1        CCGATCCCAG CAGTGACACG CAACGGTAAG

pVCN1.35    CCTCTGCTCC GAATCCTTCT CCACCAAGTC
            |||||||||| |||||||||| |||||||| |
pJM1        CCTCTGCTCC GAATCCTTCT CCACCAAGAC

pVCN1.35    CCTAGCGGAG ACAGGATCC
            ||  |||||||   ||| ||  |
pJM1        CCCAGCGGAG GCAGCATGC
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Figure 6 Region of identity between the 1.35-kb and 4.3-kb *Bam*HI fragments of pSJ2: 93.7% identity over 79 bp.

Table 2 Table of densitometrically derived junction fragment copy numbers, their corresponding cluster copy numbers and percentage penicillin titres

Strain	Junction fragment copy number	Cluster copy number	Penicillin titre in C5 medium (% of BW 1901)
NRRL 1951	0	1	1
BW 1900A	0	1	11
BW 1830A	2	3	30
BW 1860	3	4	33
BW 1870	3	4	29
BW 1880	4	5	41
BW 1890	13	14	55
BW 1895	14	15	44
BW 1900	20	21	48
BW 1901	33	35	100
BW 1952	49	50	111

Strain improvement series. *Bam*HI-digested genomic DNAs from each of the strains available were blotted and probed with the 3.25-kb *Bam*HI fragment, internal to the amplicon (Figure 2), in order to reveal the magnitude of the amplification. Simultaneous probing with the 4.4-kb fragment provided a base level, single copy signal and revealed the junction fragment bands (Figure 7). It is interesting to note that the 1.63-kb λ kb ladder fragment was always detected when probing with the 4.4-kb fragment. Densitometric analysis of the autoradiograph indicates a general increase in copy number from NRRL 1951 through to BW 1952. The wild-type strain, NRRL 1951, exhibits the expected single copy pattern, as does BW 1900A, a low titre derivative of BW 1900. Two transects of the autoradiograph encompassing the 4.4- and 1.5-kb fragment bands were scanned and interpreted as 3D optical density profiles. The values determined for the assumed single copy 4.4-kb fragment bands were used to correct for differences in DNA loadings across the series, the conversion factor then being applied to the 1.5-kb junction fragment values. From the resulting data were derived approximate junction fragment numbers and hence estimated cluster copy number (Table 2).

The single copy nature of the BW 1900A cluster region was confirmed by gene disruption experiments. The *acvA* gene was disrupted by transformation with the construct

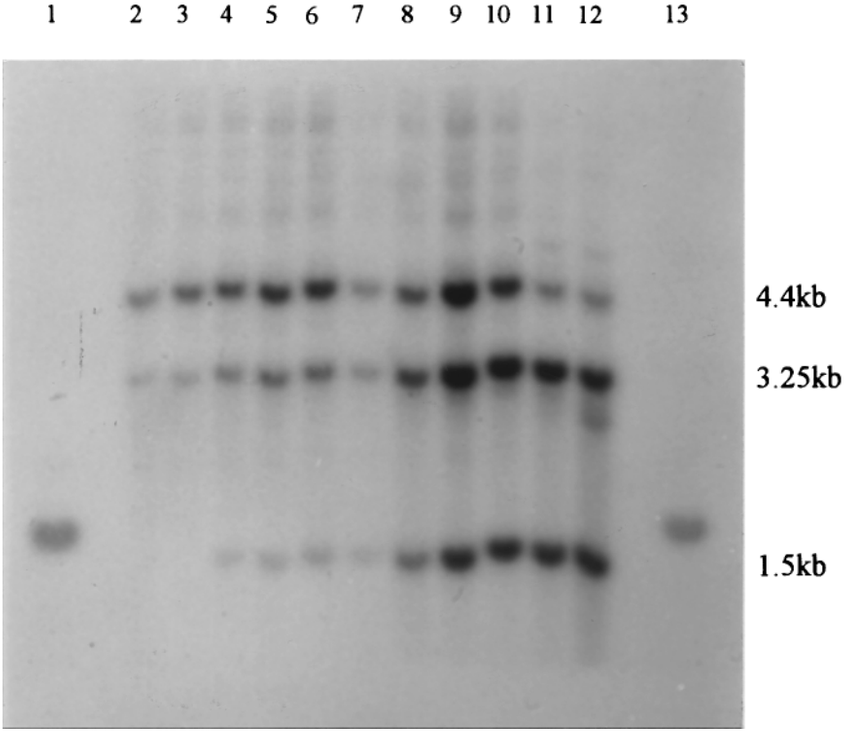

Figure 7 Southern analysis of the SmithKline Beecham production strain series using the 4.4-kb and 3.25-kb *Bam*HI fragments of pSJ2 as probe templates. *Lane 1*, λ kb ladder; *2*, NRRL 1951; *3*, BW 1900A; *4*, BW 1830A; *5*, BW 1860; *6*, BW 1870; *7*, BW 1880; *8*, BW 1890; *9*, BW 1895; *10*, BW 1900; *11*, BW 1901; *12*, BW 1952; *l3*, λ kb ladder.

pPEN3 which consists of the 8-kb internal *Eco*RI fragment of *acvA* cloned into p3SR2 [15]. Transformants were selected for their ability to grow on acetamide and then screened for penicillin titre. A number were found to have lost penicillin titre and the disruption of the *acvA* gene was confirmed by Southern hybridisation (not shown).

To relate the copy number data with penicillin biosynthesis, shake-flask fermentations were carried out in C5 fermentation media. The penicillin titres were determined by spectrophotometric analysis and are expressed as a percentage of that of BW 1901 (Table 2).

Analysis of BW 1901 promoter regions

The percentage titre of 11% obtained for BW 1900A (Table 2) indicates that titre-enhancing mutations other than cluster amplification have occurred during the strain improvement process. Possible sites are the intergenic promoter region between the divergently transcribed *acvA* and *ipnA* genes (P*acvA*-P*ipnA*) and the promoter of the *aat* gene (P*aat*) (Figure 2). Using BW 1901 genomic DNA as template, the P*acvA*-P*ipnA* and P*aat* regions were amplified by PCR (see materials and methods for primers). The P*acvA*-P*ipnA* PCR products were cut with *Sph*I and *Nco*I, the fragment gel purified and cloned into pRAJ275 using standard methods. Similarly the P*aat* PCR products were cut with *Hin*dIII and *Nco*I and cloned into pRAJ275.

Sixteen independent PCR clones of the P*acvA*-P*ipnA* region and 16 PCR clones of the P*aat* region from BW 1901 were completely sequenced alongside PCR clones from NRRL 1951. No sequence differences were observed in the BW 1901 clones as compared to the NRRL 1951 control PCR clones.

The above method was repeated using BW 1900A DNA as the PCR template. Six clones from each promoter region were sequenced but no changes found with respect to NRRL 1951.

Identification of right-hand end of amplified region

Using sequence data already published [12,13], primers were designed with which to PCR amplify the so-called switch-fragment 3′ to the *aat* gene. Though estimated at 3.4 kb by Fierro *et al*, our data indicate this fragment to be of 3.2 kb. The switch-fragment is found in different orientations in NRRL 1951 and Wis 54-1255, and in a combination of orientations in the amplified strain E1 [12]. This phenomenon has been attributed to recombination between two conserved hexanucleotide sequences which border the region [12]. The PCR product was used as a probe against a Southern blot of wild-type, improved and non-producing strains of *P. chrysogenum* (Figure 8). The hybridisation patterns obtained indicate that the orientations of the switch fragment in NRRL 1951 and Wis 54-1255 match those already resolved for these strains by Fierro *et al* [12]. BW 1890 carries the switch fragment in the same orientation as in Wis 54-1255, the high intensity of the 3.2-kb *Bam*HI fragment of BW 1890, when compared to that of Wis 54-1255, showing that this fragment lies within the amplified region. Detection of the 1.5-kb junction fragment indicates that the right-hand end of the amplicon lies within the switch fragment. Further, a comparison of the intensity of the 0.8- and 0.5-kb bands indicates that the former fragment is unamplified and contains the point involved in the original recombination event which resulted in duplication of the gene cluster.

Analysis of non-penicillin-producing mutants

During initial research into the genetic basis of penicillin biosynthesis, SmithKline Beecham Pharmaceuticals isolated *P. chrysogenum* mutants deficient in this characteristic. Recent Southern analysis of the non-penicillin-producing mutants H2, H8 and M1/13, using the 4.4-kb *Bam*HI fragment as a probe, revealed that in all three strains the 4.4-kb restriction fragment has been lost and a novel 3.6-kb fragment generated. The non-penicillin-producing mutant A1 exhibits a recombinant *Bam*HI fragment of 11.7 kb (Figure 9). Southern analysis also showed that in each case the entire cluster of penicillin biosynthesis genes had been deleted (data not shown), and that all sequences internal to the switch fragment are absent from the non-producing strains (Figure 8).

It is conceivable that transcribed or regulatory sequences may lie within the co-amplified region 3′ to the *acvA* gene. Indeed, under low stringency conditions, the 4.3-kb *Bam*HI fragment of pSJ2 has been shown to hybridise with genomic DNAs from both *Penicillium italicum* and *Metarrhizium anisopliae* (data not shown). An attempt has been made to detect additional transcribed genes in the region downstream of the *acvA* gene by probing a *P. chrysogenum* cDNA library with the 1.35-, 3.25- and 4.3-kb *Bam*HI frag-

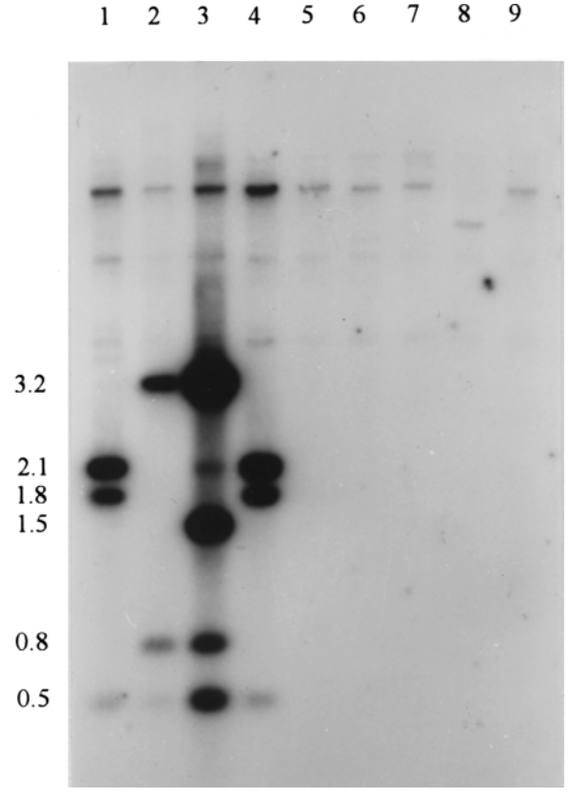

Figure 8 Southern analysis of wild-type, high-producing and non-producing strains of *P. chrysogenum* using the 3.2-kb switch fragment as a probe template. *Lane 1*, NRRL 1951; *2*, Wis 54-1255; *3*, BW 1890; *4*, M1951; *5*, H2; *6*, H8; *7*, A1; *8*, E13; *9*, M1/13.

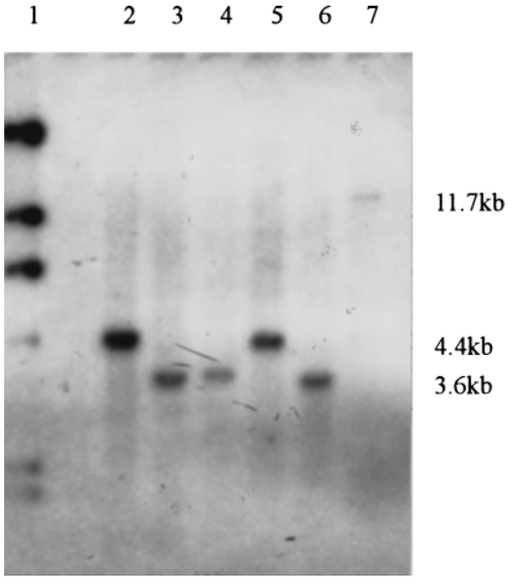

Figure 9 Southern analysis of *P. chrysogenum* mutants blocked in penicillin biosynthesis, using the 4.4-kb *Bam*HI fragment of pSJ2 as a probe template. *Lane 1*, λ *Hin*dIII size marker; *2*, NRRL 1951; *3*, H2; *4*, H8; *5*, M1951; *6*, M1/13; *7*, A1.

ments of pSJ2. Though control screenings were successful in detecting *ipnA* cDNA, no clones were detected using the above fragments.

Isolation and analysis of spontaneous variants

P. chrysogenum BW 1890 grows as small crinkled colonies, a morphology often associated with increased penicillin titre [1,16,18]. Incubation on minimal medium, however, resulted in the generation of faster growing sectors, two of which, BW 1890P and BW 1890F, were isolated. Genomic DNAs from these two strains were run alongside BW 1890 and probed with the 4.4-kb *Bam*HI fragment in order to detect any drop in copy number which may be associated with the change in morphology (Figure 10). Both BW

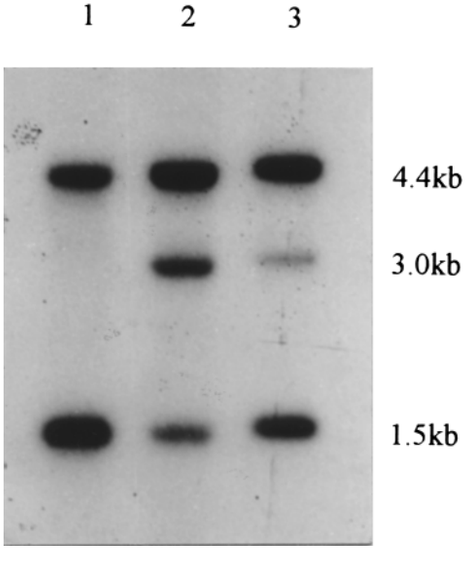

Figure 10 Southern analysis of genomic DNA from spontaneous variants of BW 1890, using the 4.4-kb *Bam*HI fragment of pSJ2 as a probe template. *Lane 1*, BW 1890; *2*, BW 1890P; *3*, BW 1890F.

1890P and BW 1890F exhibited a decrease in the intensity of the junction fragment band when compared to BW 1890. However, the appearance of a novel 3.0-kb fragment indicates that the rearrangement involved is not that of simple chromatid misalignment and recombination. It is interesting to note that a decrease in the intensity of the 1.5-kb band was accompanied by an increase in the intensity of the 3.0-kb band.

Discussion

The SmithKline Beecham strain improvement series was developed from the Wisconsin strain lineage over a period of many years by a process of random mutagenesis, screening and selection. Previous analysis had shown that in one member of this series, BW 1890, the entire penicillin biosynthesis gene cluster is amplified to between eight and 16 copies [21]. Further investigation has indicated that the amplified region is approximately 57.4 kb in length. The apparent homogeneity of the amplification throughout the series, together with the detection of a 1.5-kb junction fragment, has confirmed that the copies are tandemly arranged, rather than dispersed, and suggests generation through chromatid misalignment and recombination (Figure 11). Whether or not the initial recombination event was homologous, once duplication had been achieved, a large stretch of homologous DNA was available for misalignment and hence further amplification or indeed deletion. It is proposed that the single copy strain BW 1900A was derived from the high copy strain BW 1900 by just such a mechanism, thus providing additional evidence for the tandem nature of the amplification. Disruption of the *acvA* gene in BW 1900A yielded a penicillin non-producer, verifying that this strain is indeed single copy with respect to the penicillin biosynthesis gene cluster.

Data have been published detailing nucleotide base sequences across the junctions between consecutive units of amplification in the improved *P. chrysogenum* strains E1 and AS-P-78 [12]. Though we have not yet characterised the junction in such detail, it is perhaps significant that in both the SmithKline Beecham strain series and strain E1, the amplified regions are bounded by the same non-amplified *Bam*HI fragments. However, it is evident from the disparity in the sizes of the *Bam*HI fragments spanning the junctions, that the amplicons are not identical.

Our analysis of the penicillin non-producing mutants A1, H2, H8 and M1/13 (Figure 9), and that of Wis 54-1255 npe10, Bb-1/125, Bb-1/168 and Bb-1/759 by Fierro *et al* [13], are also significantly similar. In all but A1, deletion of the penicillin biosynthesis cluster region has occurred through a recombination event within the 4.4-kb fragment resulting in the generation of a novel *Bam*HI fragment of approximately 3.6 kb. The 11.7-kb recombinant *Bam*HI fragment exhibited by strain A1 would further indicate the existence of an alternative site of recombination 3′ to the *aat* gene. Additional evidence for the existence of a recombinogenic region within the 4.4-kb fragment was obtained from the Southern analysis of spontaneous variants of BW 1890 (Figure 10). A decrease in the copy number of the 1.5-kb junction fragment is accompanied by an apparent increase in that of the 3.0-kb fragment. These data, though

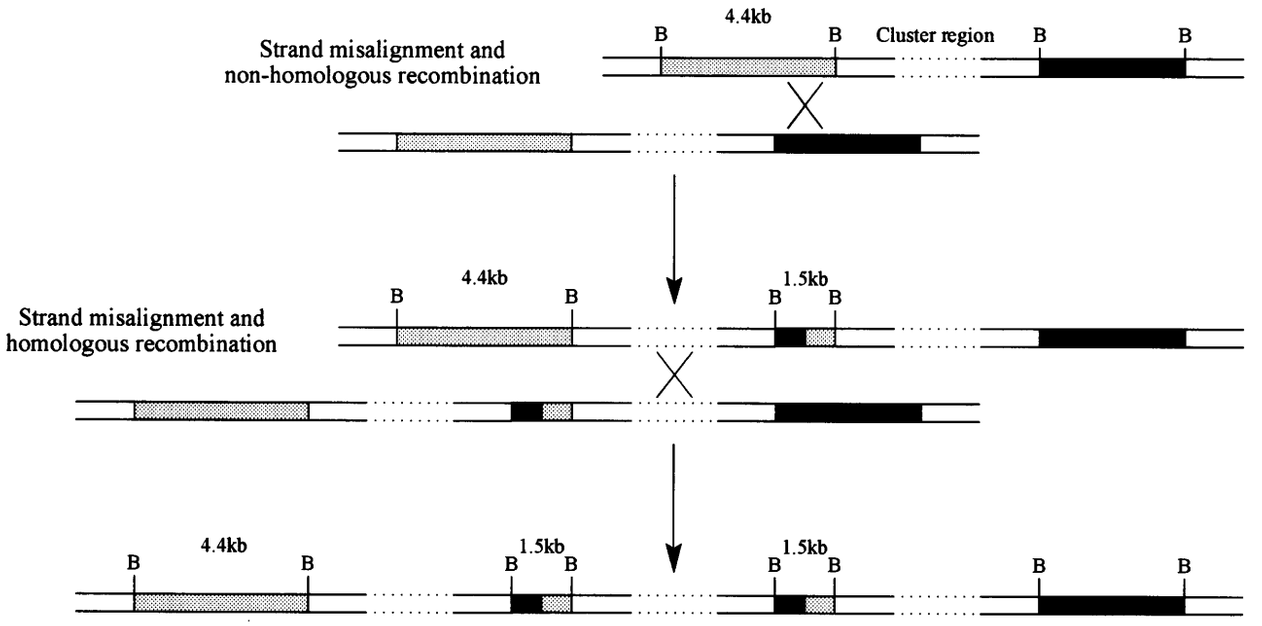

Figure 11 Theoretical model for the amplification of the penicillin biosynthesis gene cluster in the SmithKline Beecham series of improved *P. chrysogenum* strains.

insufficient to propose a hypothetical model, suggest the existence of a repeated or progressive recombinational event in which sequences internal to the junction fragment play a key role.

Using densitometric and titre analyses, we have estimated copy numbers for each strain of the SmithKline Beecham series, and draw comparison with penicillin production (Table 2). General increases in both titre and copy number are evident through the series, this trend being repeated in tests with two further fermentation media (data not shown).

That other titre-enhancing mutations have occurred through repeated random mutagenesis is demonstrated by the enhanced penicillin titre and IPNS mRNA levels, with respect to NRRL 1951, of BW 1990A, the single copy derivative of BW 1900 (Table 2). However, sequence analyses of BW 1901 and BW 1900A have shown that no mutations have been generated within the promoter regions of the penicillin biosynthesis structural genes. These data suggest that the increased penicillin production in amplified strains may be due to altered regulation of the biosynthesis pathway through changes in trans-acting regulatory factors. It would be expected that, as cluster copy number becomes no longer a limitation, mutations other than amplification will become increasingly important in the selection of high titre variants. Data indicate that BW 1952 titres are not proportionally increased with copy number, indicating the presence of other limiting factors, such as trans-acting regulators or primary metabolic pathways, which must be alleviated before the full potential of the extra copies may be realised. A change in background non-specific hybridisation pattern is observed with the generation of BW 1901 and persists in BW 1952. The latter also exhibits a band representing a novel 2.7-kb *Bam*HI fragment present in low copy number and yet to be explained.

Though the co-amplified DNA 3′ to the *acvA* gene is large enough to encompass further genetic elements, such as regulatory genes, instrumental in penicillin biosynthesis, neither cDNA screening nor sequence database searches have provided any evidence for such.

In conclusion, the results detailed and discussed above provide an insight into the nature, generation, extent and effect of the penicillin biosynthesis gene cluster amplification in SmithKline Beecham production strains. Research by Fierro *et al* has shown that crossing over resulting in amplification or deletion has occurred within the hexanucleotide sequence TTTACA in all cases they have investigated [13]. They have suggested that this sequence may be a hotspot for site-specific recombination after mutation with nitrosoguanidine, the process possibly being part of a fungal SOS system similar to that found in *E. coli*. Our results obtained through analysis of high titre strains, non-penicillin-producing mutants and spontaneous variants, also suggest the existence of recombinogenic sequences which may predispose the region to mutagen-induced unequal interchromatid exchange.

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