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ORIGINAL PAPER

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Mutants blocked in penicillin biosynthesis show a deletion of the entire penicillin gene cluster at a specific site within a conserved hexanucleotide sequence

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Abstract The organization of the genes of the penicillin cluster has been studied in three different mutants of *P. chrysogenum* impaired in penicillin biosynthesis. The three blocked mutants (derived from the parental strain *P. chrysogenum* Bb-1) lacked the genes *pcbAB*, *pcbC* and *penDE* of the penicillin biosynthetic pathway and were unable to form isopenicillin N synthase and isopenicillin N acyltransferase. All strains were identified as *P. chrysogenum* derivatives by fingerprinting analysis with $(GTG)_n$ as a probe. The borders of the deleted region were cloned and sequenced, showing the same junction point in the three mutants. The deleted DNA region was found to be identical to that described in *P. chrysogenum npe10*. The frequent deletion of the *pen* gene cluster at this point may indicate that this cluster is located in an unstable genetic region, flanked by hot spots of recombination, that is easily lost by mutagen-induced recombination.

Introduction

The cluster of genes *pcbAB*, *pcbC* and *penDE* encoding the enzymes that carry out the three steps of the penicillin pathway (the so-called *pen* cluster) (Martín 1992) has been fully characterized in the last few years both in *Penicillium chrysogenum* (Barredo et al. 1989a, b; Díez et al. 1989, 1990) and *Aspergillus nidulans* (McCabe et al. 1990; Montenegro et al. 1992). In *A. nidulans* the *pen* cluster is located in chromosome VI of 3×10^6 bases (3.0 Mb) (Montenegro et al. 1992) whereas in *P. chrysogenum* is located in chromosome I of 10.4 Mb (Fierro et al. 1993). The entire cluster of genes has been shown to be amplified in high-penicillin-producing strains of *P. chrysogenum* (Barredo et al. 1989c; Smith et al. 1989).

Mutants impaired in the different steps of antibiotic biosynthesis are very useful instruments for studying gene expression and gene/enzyme relationships (Queener et al. 1978). Frederiksen and Emborg (1984) described three mutants of *P. chrysogenum* Bb-1 blocked in penicillin biosynthesis (obtained at Leo Pharmaceutical Products, Ballerup, Denmark), but none of these mutants has been characterized at the enzymatic or genetic level. Since we had available probes internal to the three penicillin biosynthetic genes, it was of interest to study the organization of the penicillin gene cluster in these non-producer mutants as compared to the parental strain, and to elucidate whether the *pen* genes are expressed by determining the enzymes encoded by them.

We describe in this article that the three non-producer mutants lack the entire region carrying the penicillin gene cluster. The three mutants arose by a deletion at the same point, probably by recombination, which suggests that this genomic region may be easily lost in *P. chrysogenum.*

Materials and methods

Strains

All the *P. chrysogenum* strains used in this work and their characteristics are listed in Table 1.

The parental *P. chrysogenum* Bb-1 from Leo Pharmaceuticals and the three blocked mutants derived from it were kindly provided by H. B. Nielsen, Leo Pharmaceutical Products Ltd., Ballerup, Denmark. *P. chrysogenum* Wis 54-1255, a penicillin-producing strain, was obtained from A.L. Demain and *P. chrysogenum* Wis 54-1255 *npe10* was isolated in our laboratory (Cantoral et al. 1993).

Culture conditions

Spores of the different strains were collected from cultures grown in plates of Power medium [obtained by mixing (1:1 v/v) standard

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Table 1 Strains of *P. chrysogenum* and *A. nidulans* used in this work

Strains	Characteristics	Origin
P. chrysogenum npe10	Deletion mutant blocked in penicillin production. Derived from Wis 54-1255	J. M. Cantoral and S. Gutiérrez This laboratory (Cantoral et al. 1993)
P. chrysogenum Bb-1	Penicillin producer, Parental strain of three blocked mutants	Leo Pharmaceuticals (Frederiksen and Emborg 1984)
P. chrysogenum Bb-1/125	Mutant blocked in penicillin biosynthesis	Leo Pharmaceuticals (Frederiksen and Emborg 1984)
P. chrysogenum Bb-1/168	Mutant blocked in penicillin biosynthesis	Leo Pharmaceuticals (Frederiksen and Emborg 1984)
P. chrysogenum Bb-1/759	Mutant blocked in penicillin biosynthesis	Leo Pharmaceuticals (Frederiksen and Emborg 1984)
P. chrysogenum NRRL 1951	Wild type isolated from a cantaloupe	ATCC 9480
P. chrysogenum Wis 54-1255	Penicillin producer. Derived from the wild-type *P. chrysogenum*	A. L. Demain
A. nidulans ATCC 28901	White conidial biotinless mutant; increased penicillin yield	ATCC 28901

Czapek medium and PM1 medium (30 g lactose, 5 g Bacto Peptone, 0.5 g corn steep solids, 4 g NaCl, 1 mg $CuSO_4 \cdot 7H_2O$, 3 mg $FeCl_3 \cdot 6H_2O$, 60 mg KH_2PO_4, 50 mg $MgSO_4 \cdot 7H_2O$, 30 g agar, 1 l distilled water, pH 6.5)] at 27° C for 5–7 days. Seed cultures in MCI2 medium (20 g corn steep solids, 20 g sucrose, 5 g yeast extract, 5 g $CaCO_3$, 1 l distilled water, pH 5.7) were inoculated with about 10^6 spores/ml and grown at 25° C in a rotary G10 incubator (New Brunswick Scientific, New Brunswick, N.J.) at 25 °C for 36 h. Complex production medium CPM1 (55 g lactose, 35 g corn steep solids, 12.5 ml of 20% phenylacetic acid, 0.3 g $MgSO_4 \cdot 7H_2O$, 10 g $CaCO_3$, 7 g KH_2PO_4, 1 l distilled water, pH 6.8, 100 ml in 500-ml flasks) was inoculated with 5 ml seed culture. The production cultures were grown at 25° C for 96 h in a rotary shaker as described above.

Antibiotic assays

Penicillin in the cultures was routinely determined by bioassay against *Micrococcus luteus* or *Bacillus subtilis* using dilutions of culture broth supernatants as described previously (Revilla et al. 1986). The levels of penicillin produced were confirmed by HPLC analysis using a μBondapack C18 column and the solvent system reported by Alvarez et al. (1993).

Cell-free extracts

Cells were collected at 48 h of incubation, washed three times with 0.9% NaCl to remove adhering penicillin and frozen at −20° C. Frozen mycelium was thawed and suspended in TD buffer (50 mM TRIS/HCl, 5 mM dithiothreitol, pH 8.0) at 0.25 mg wet mycelium/ml. The cells were disrupted by sonication in a Branson sonifier using pulses of 10 s for a total time of 2.5 min until all mycelium was observed under the microscope to be disintegrated. Cell debris was removed by centrifugation at 18 000 *g* for 20 min at 4° C. The enzyme extracts of the non-producer mutants were concentrated tenfold by precipitating the protein with ammonium sulphate (80% saturation) and resuspending it in one-tenth of the initial volume. The cell-free extracts were filtered through Sephadex G-25 to remove small molecules before the enzyme assays.

Isopenicillin N synthase and isopenicillin N acyltransferase assays

Isopenicillin N synthase activity in extracts of the different strains was assayed by determining the conversion of δ(L-α-aminoadipyl)-L-cysteinyl-D-valine into isopenicillin N as described by Ramos et al. (1985).

The acyl-CoA: 6-aminopenicillanic acid(6-APA) acyltransferase activity of isopenicillin N acyltransferase (one of the five related activities of this enzyme; Alvarez et al. 1993) was determined by measuring the conversion of 6-APA and phenylacetyl-CoA into benzylpenicillin, as described by Alvarez et al. (1987, 1993).

The activities of both enzymes are given as pkat (i.e. picomoles of product formed per second)/mg protein in the extracts. Total protein was determined using the Bradford (1976) method.

Preparation of total *P. chrysogenum* DNA

Total DNA of *P. chrysogenum* was obtained by a modification of the method of Specht et al. (1982), essentially as described by Gutiérrez et al. (1991) and Cantoral et al. (1993). Separation of DNA fragments by electrophoresis in 0.7% agarose gels was done by standard methods (Sambrook et al. 1989).

Southern blotting and hybridizations

After separation in agarose gels, the DNA was transferred to nylon filters (Hybond N, Amersham) and fixed to the filter by UV light irradiation (7–10 min) as described by Sambrook et al. (1989). Probes were labelled by nick translation and purified in an elutip-D minicolumn (Sigma). Hybridizations were carried out by standard methods (Sambrook et al. 1989).

PCR cloning and sequencing

The DNA region around the expected deletion site (according to restriction endonuclease analysis) was amplified by the polymerase chain reaction (PCR) using oligonucleotides A, 5′CTAGGTACTTG TAAGCTT3′ and B, 5′CGGGTTAAATTTGGATCC3′, deduced from the nucleotide sequence of the *P. chrysogenum npe10* strain.

Adequate DNA fragments were sequenced on both strands by the dideoxy-nucleotide chain-termination method by standard procedures.

Fingerprinting analysis of total DNA

Total DNA of *P. chrysogenum* was digested with *Eco*RI and electrophoresed in 0.7% agarose. The DNA was denatured by treatment with 0.5 M NaOH and 0.15 M NaCl for 35 min, followed by washing with 0.5 M TRIS/HCl, pH 8.0, 0.15 M NaCl for 45 min, and finally with 6 × standard saline citrate for 35 min. The DNA was fixed in the gel under vacuum at room temperature for 45 min and then at 60° C for 60 min. Prehybridization, labelling with [γ-^{32}P]ATP of the poly(GTG) oligonucleotide, and hybridization were essentially as described by Meyer et al. (1991).

Results

Penicillin production by the different strains

Production of penicillin by the different strains was studied in submerged cultures grown in CPM1 medium (see Materials and methods). Results showed that *P. chrysogenum* Wis 54-1255 produced about 500 μg/ml at 96 h of incubation and the parental Bb-1 strain of Leo Laboratories reached 632 μg/ml under the same experimental conditions, whereas mutants Bb-1/125, Bb-1/168 and Bb-1/759 did not synthesize detectable amounts of penicillin (Fig. 1).

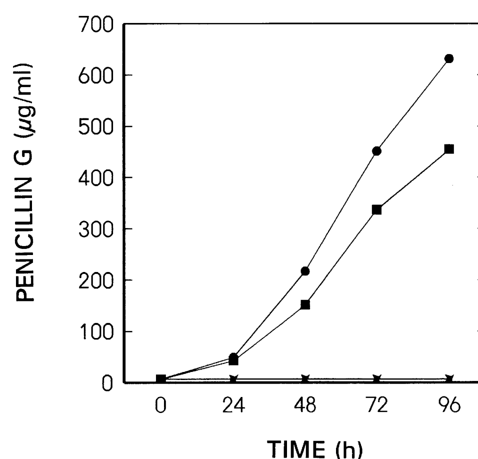


Fig. 1 Production of penicillin by different mutants of *P. chrysogenum* and the parental strains Wis 54-1255 and Bb-1 in complex production medium CPM1. ● *P. chrysogenum* Bb-1, ■ Wis 54-125, × Bb-1/125, Bb-1/168 and Bb-1/759

Isopenicillin N synthase and acyl-CoA: 6-APA acyltransferase activities in the mutants

The isopenicillin N synthase and acyl-CoA: 6-APA acyltransferase activities were determined in extracts of all strains. Since initial results suggested that the Bb-1-derived mutants had little or no isopenicillin N synthase and 6-APA acyltransferase activities, the total protein in their extracts was precipitated with ammonium sulphate and suspended in one-tenth of the initial volume. Results (Table 2) showed that strain Bb-1 had a high isopenicillin N synthase activity whereas the activity was slightly lower in Wis 54-1255, and the Bb-1-derived blocked mutants showed no isopenicillin N synthase activity.

Similar results were obtained for the acyl-CoA: 6-APA acyltransferase activity (Table 2). The three Bb-1-derived mutants showed no detectable acyl-CoA: 6-APA acyltransferase activity in the crude extract or after Sephadex G-25 gel filtration.

Table 2 Isopenicillin N synthase and isopenicillin N acyltransferase activites in the different mutants. Results are averages of two different experiments; 1 pkat = 1 pmol product formed/s. The enzyme activities were assayed in extracts of cells grown for 48 h in CPM-1 medium, as described in Materials and methods. Mutants Bb-1/125, Bb-1/168 and Bb-1/759 showed no enzyme activities when the extracts were precipitated with ammonium sulphate and suspended in one-tenth of the initial volume

Strains	Isopenicillin N synthase (pkat/mg protein)	Isopenicillin N acyltransferase (pkat/mg protein)
P. chrysogenum Bb-1	19.5	24.4
P. chrysogenum Bb-1/125	0	0
P. chrysogenum Bb-1/168	0	0
P. chrysogenum Bb-1/759	0	0
P. chrysogenum Wis 54-1255	13.3	19.7

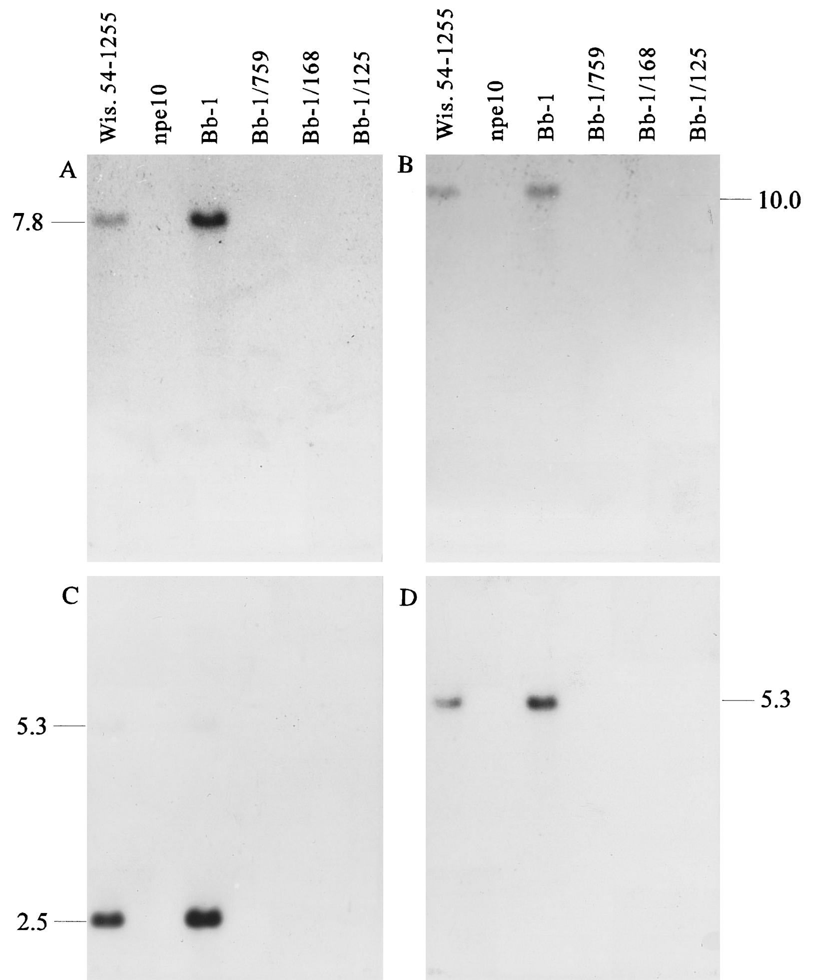
The Bb-1-derived mutants lack the penicillin gene cluster

The presence and organization of the three penicillin biosynthetic genes was tested in the parental strain Bb-1 and the three blocked mutants derived from it. As an additional control, *P. chrysogenum npe10*, a previously described deletion mutant (Cantoral et al. 1993) was included in the hybridization studies.

Since the *pcbAB* is an unusually large gene (11.4 kb) which might have suffered deletions, the blots were hybridized with probes from the 5′ region of the gene (2.2 kb *Sal*I fragment) and from the 3′ region (445 bp *Eco*RI fragment). Results (Fig. 2A, B) showed that the same 7.8-kb *Eco*RI bands and 10.0-kb *Bam*HI bands hybridized with each probe in strain Bb-1, but no hybridization signals were observed in strains Bb-1/125 Bb-1/168 and Bb-1/759 or in the control deletion mutant *npe10.*

Similar results were obtained when hybridizations were done with a probe carrying the *pcbC* gene (a 1.0-kb *Nco*I fragment that corresponds exactly to the open-reading frame of this gene; Barredo et al. 1989a) (Fig. 2C) or with a 1.6-kb *Xho*I-*Xba*I fragment of the

Fig. 2A–D Hybridization of total DNA of the different *P. chrysogenum* strains digested with *Eco*RI (**A**) or *Bam*HI (**B, C, D**) with probes corresponding to the 5′region of *pcbAB* (**A**), 3′region of *pcbAB* (**B**), pcbC (**C**) and *penDE* (**D**) (see text for details). The size of the hybridizing bands is shown by *bars* at the *left* and the *right* of the panels (kb)



pen gene cluster that carries the *penDE* gene (Fig. 2D). Both the *pcbC* and *penDE* genes were present in strain Bb-1, but not in the three blocked mutants derived from it.

Using the four probes it was concluded that strain Bb-1, which exhibits positive hybridization, has retained the same organization of the penicillin gene cluster region as other known *P. chrysogenum* strains, e.g. Wis 54-1255 or AS-P-78.

Fingerprint analysis

To confirm that the three Bb-1-derived mutants were really *P. chrysogenum* strains a fingerprint analysis of the Bb-1 parental strain and the three Bb-1-derived blocked mutants was carried out. As shown in Fig. 3, the fingerprint of mutants Bb-1/125, Bb-1/168 and Bb-1/759 was identical to that of the parental Bb-1 strain, and also very similar to the pattern of bands of the wild-type *P. chrysogenum* NRRL 1951 and strain Wis 54-1255, but clearly different from the fingerprint of

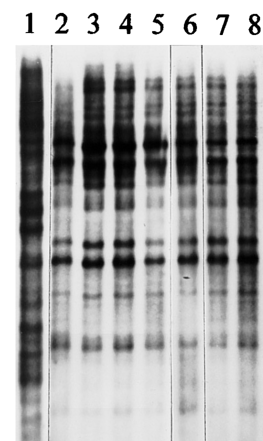


Fig. 3 Fingerprint analysis of *Eco*RI-digested total DNA of *A. nidulans* ATCC 28901 (*lane 1*) and several strains of *P. chrysogenum*: Bb-1 (*lane 2*), Bb-1/759 (*lane 3*), Bb-1/168 (*lane 4*), Bb-1/125 (*lane 5*), *npe10* (*lane 6*), Wis 54-1255 (*lane 7*) and NRRL 1951 (*lane 8*). Note that all *P. chrysogenum* strains including the Bb-1 derivatives have the same fingerprint pattern, which is different from that of *A. nidulans*

A. nidulans. No differences were observed in the pattern of bands of the deletion mutants Bb-1/125, Bb-1/168 and Bb-1/759 as compared to the parental strain, suggesting that the deletion is probably small and is not reflected in the pattern of bands observed in the fingerprints.

These results clearly indicate that strains Bb-1/125, Bb-1/168 and Bb-1/759 are *P. chrysogenum* derivatives as reported previously by Frederiksen and Emborg (1984), and that the lack of the penicillin biosynthetic genes is due to a deletion of the region carrying the penicillin gene cluster.

Location of the borders of the deleted region

Using the technique of chromosome walking, phages from the wild-type strain of *Penicillium chrysogenum* were obtained downstream from the *pcbAB* and the *penDE* genes (F. Fierro and J. F. Martín, unpublished). Total DNA of the four mutants and their parental strains was hybridized with several probes (obtained from the phages) more and more distant from the *pen* cluster. Finally two probes were found (probe A and probe D, Fig. 5) which gave positive hybridization with the DNA of the four mutants (Fig. 4). Since the sizes of the hybridization bands were exactly the same in all the mutants, it was possible that the three Bb-1 derivatives had the same deletion points as the strain *npe10*, the deletion borders of which had been previously cloned and characterized (F. Fierro and J. F. Martín, unpublished). Using two oligonucleotides (A and B in Materials and methods), based on the sequence of the strain *npe10*, each situated at one side of the junction point in this strain, a DNA fragment of 350 bp was cloned by PCR and sequenced. Results indicated that the sequence of this fragment in the three Bb-1 derivatives was identical and the same as in strain *npe10*. Comparison of the nucleotide sequence of the wild-type strain with the three Bb-1-derived mutants showed that the junction point coincided in all mutants and was situated within the hexanucleotide TGTAAT (Fig. 6).

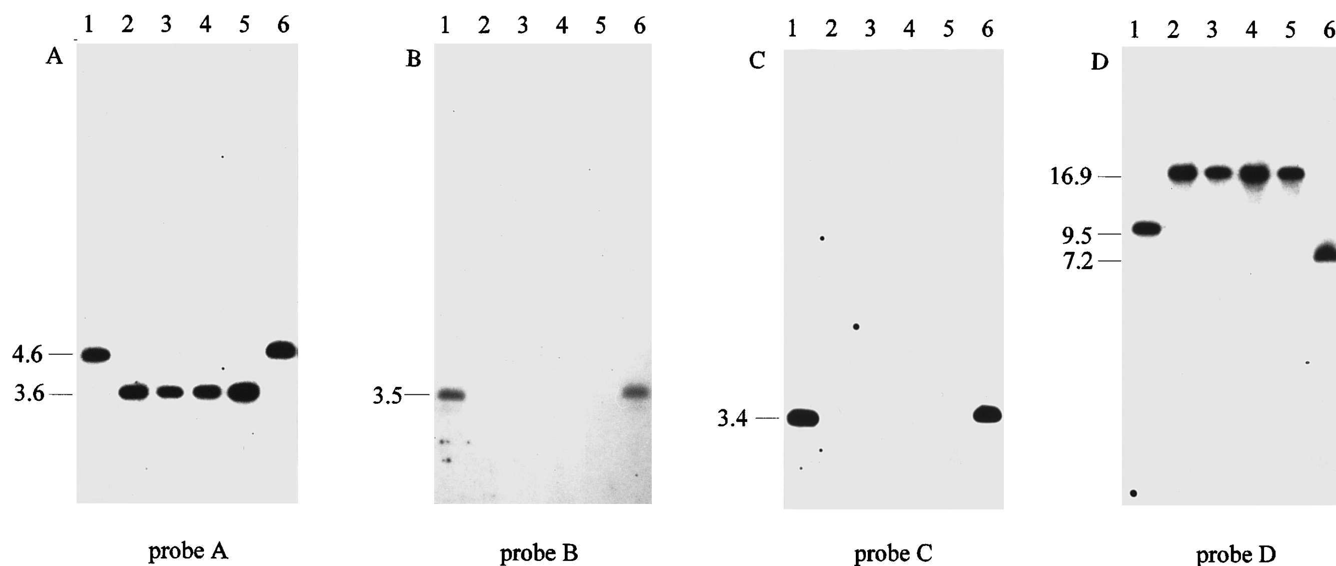
The deleted region was 57.9 kb in the three Bb-1-derived mutants, the same size as in the previously studied strain *npe10* (Fig. 5).

Discussion

Many mutants of *P. chrysogenum* with improved penicillin yields have been obtained using a variety of mutagenic agents (Elander 1983; Lein 1986). However little attention has been paid to mutants impaired in penicillin biosynthesis. These mutants are very useful for complementation analysis and gene/enzyme relationship studies (Queener et al. 1978). Some of the mutagenic treatments have resulted in point mutations or deletions, which may affect either the structural or positively acting regulatory genes, which may lead to impairment of penicillin biosynthesis.

As shown in Fig. 2, the three derivatives of *P. chrysogenum* Bb-1 obtained at Leo Pharmaceutical laboratories did not show hybridizations with any of the probes internal to the three penicillin biosynthetic genes, although all of them showed, by hybridization with the $(GTG)_n$ probe, an identical fingerprint to that of the parental Bb-1 and other *P. chrysogenum* strains including the wild-type isolate NRRL 1951. Cantoral et al. (1993) reported that one out of nine *P. chrysogenum*

Fig. 4A–D Hybridization of total DNA of the different strains digested with *Bam*HI (**A**) or *Eco*RI (**B**, **C**, **D**) with probes from the wild type corresponding to the borders of the deleted region; outside it (*probes A*, *D*) or inside the deleted region (*probes B*, *C*). The exact situation of these probes is shown in Fig. 5. The sizes of the hybridizing bands are indicated by *bars* on the *left* (kb). *Lane 1* Bb-1; *2* Bb-1/125; *3* Bb-1/168; *4* Bb-1/759; *5 npe10*; 6 Wis 54-1255



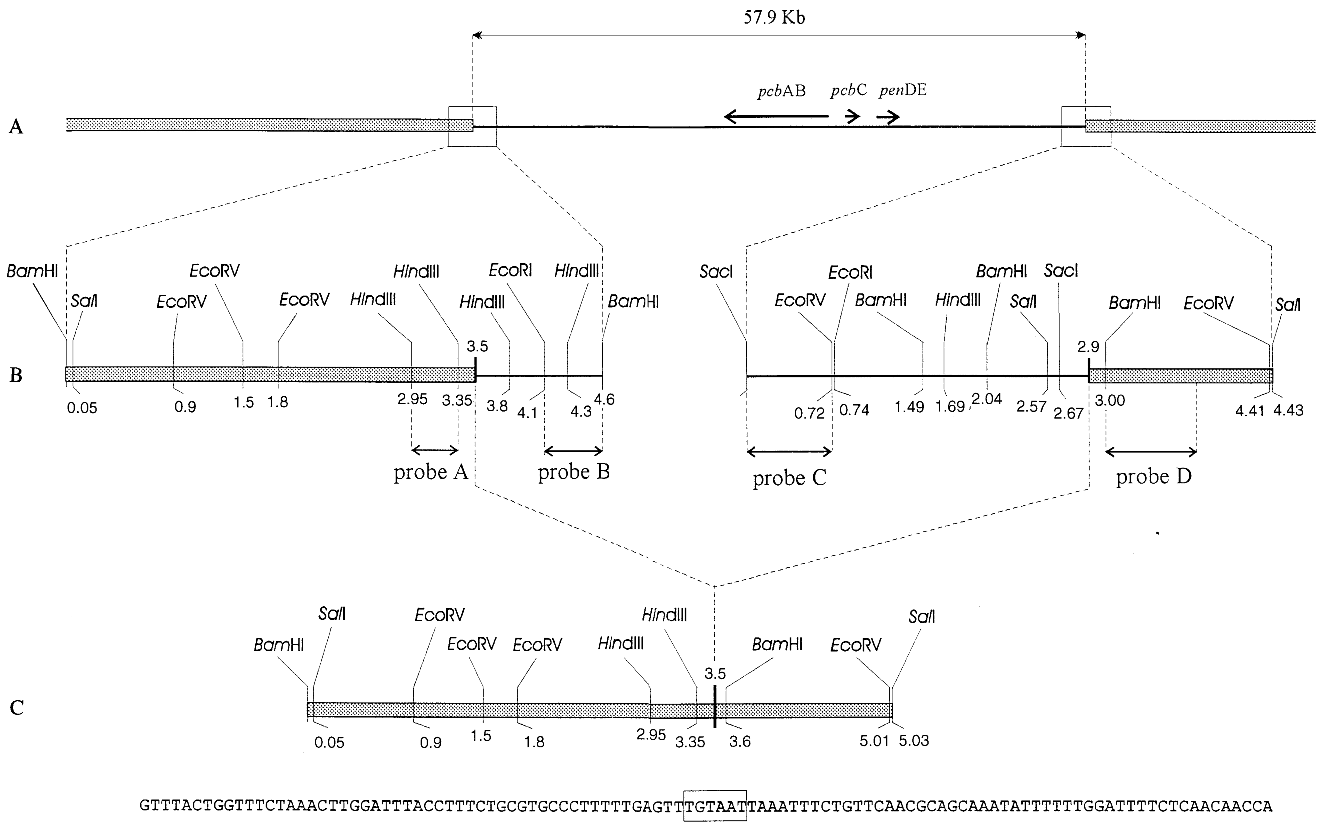
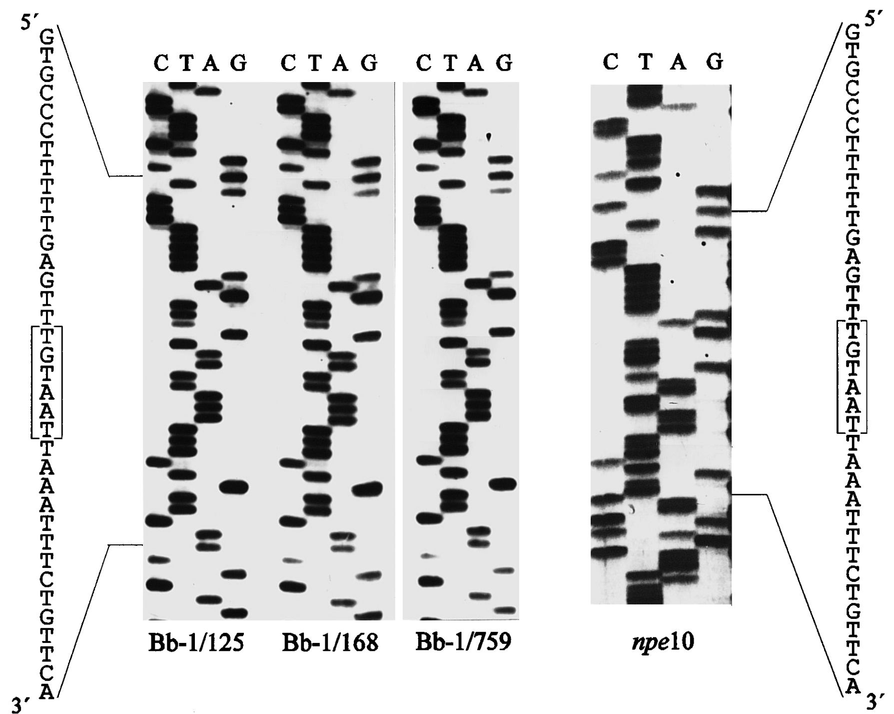


Fig. 5A–C Scheme of the *pen* DNA region in strain Wis 54-1255 (**A, B**) and in the four deletion mutants (**C**). **A** The *pen* cluster and the total size of the deleted region are indicated. **B** A more detailed restriction map of the borders; probes A and B are shown in the *left border*; probes C and D in the *right border*. **C** DNA region and nucleotide sequence resulting from the deletion process in the four mutants. The sequence at the junction point is *boxed*. The deleted DNA sequences are indicated by a single line and those remaining (non-deleted) are shown as stippled boxes

Fig. 6 Sequencing gels corresponding to the four mutants showing the sequence at the junction zone with the hexanucleotide TGTAAT



Wis 54-1255 derivatives impaired in penicillin biosynthesis had lost the entire penicillin cluster.

It seems, therefore, that the loss of the penicillin gene cluster following mutation is a relatively frequent phenomenon. However the *pen* gene cluster is not located in a plasmid although it may form part of a genetic element that may have been transfered to filamentous fungi from bacteria (Ramón et al. 1987; Aharonowitz et al. 1992). The *pen* gene cluster has been mapped by hybridization studies in the largest chromosome (chromosome I) of *P. chrysogenum* as resolved by pulsed-field electrophoresis (Fierro et al. 1993).

The presence of a conserved hexanucleotide TGTAAT at the deletion site in all three Bb-1-derived mutants and also in *npe10*, indicates that this sequence plays an important role in the deletion. As shown in Fig. 5, this sequence has probably been formed by recombination between similar sequences occurring at two sites separated by the deleted fragment. It is interesting that the ends of the deletions in the blocked mutants correspond to the border of the amplified regions in some high-producing mutants (F. Fierro and J. F. Martín, unpublished), which suggests that rearrangement of the region carrying the *pen* gene cluster may occur following mutagenic treatments resulting in either deletions or amplifications. Amplifications of the *pen* cluster are probably unstable, reverting upon repeated subculturing to the single-copy parental-type strain, which may explain the well-known degeneration of some high-producing strains.

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References

Aharonowitz Y, Cohen G, Martín JF (1992) Penicillin and cephalosporin biosynthetic genes: structure, organization, regulation, and evolution. Annu Rev Microbiol 46:461–495

Alvarez E, Cantoral JM, Barredo JL, Díez B, Martín JF (1987) Purification to homegeneity and characterization of acyl coenzyme A:6-aminopenicillanic acid acyltransferase of *P. chrysogenum*. Antimicrob Agents Chemother 31:1675–1682

Alvarez E, Meesschaert B, Montenegro E, Gutiérrez S, Díez B, Barredo JL, Martín JF (1993) The isopenicillin N acyltransferase of *P. chrysogenum* has isopenicillin N amidohydrolase, 6-aminopenicillanic acid acyltransferase and penicillin amidase activities, all of which are encoded by the single *pen*DE gene. Eur J Biochem 215:323–332

Barredo JL, Cantoral JM, Alvarez E, Díez B, Martín JF (1989a) Cloning, sequence analysis and transcriptional study of the isopenicillin N synthase of *Penicillium chrysogenum* AS-P-78. Mol Gen Genet 216:91–98

Barredo JL, Solingen P van, Díez B, Alvarez E, Cantoral JM, Kattevilder A, Smaal EB, Groenen MAM, Veenstra AE, Martín JF (1989b) Cloning and characterization of acyl-CoA:6-APA acyltransferase gene of *Penicillium chrysogenum*. Gene 83:291–300

Barredo JL, Díez B, Alvarez E, Martín JF (1989c) Large amplification of a 35-kb DNA fragment carrying two penicillin biosynthetic genes in high penicillin producing strains of *Penicillium chrysogenum*. Curr Genet 16:453–459

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Cantoral JM, Gutiérrez S, Fierro F, Gil-Espinosa S, Liempt M van, Martín JF (1993) Biochemical characterization and molecular genetics of nine mutants of *Penicillium chrysogenum* impaired in penicillin biosynthesis. J Biol Chem 268:737–744

Díez B, Barredo JL, Alvarez E, Cantoral JM, Solingen P van, Groenen MAM, Veenstra AE, Martín JF (1989) Two genes involved in penicillin biosynthesis are linked in a 5.1 kb *Sal*I fragment in the genome of *Penicillium chrysogenum*. Mol Gen Genet 218:572–576

Díez B, Gutiérrez S, Barredo JL, Solingen P van, Voort LHM van der, Martín F (1990) The cluster of penicillin biosynthetic genes. Identification and characterization of the *pcb*AB gene encoding the α-aminoadipyl-cysteinyl-valine synthetase and linkage to the *pcb*C and *pen*DE genes. J Biol Chem 265:16358–16365

Elander R (1983) Strain improvement and preservation of β-lactam producing microorgansims. In: Demain AL, Solomon NA (eds) Antibiotics containing the beta-lactam structure I. Springer, Berlin Heidelberg New York pp 97–146

Fierro F, Gutiérrez S, Díez B, Martín JF (1993) Resolution of four large chromosomes in penicillin-producing filamentous fungi: the penicillin gene cluster is located on chromosome II (9.6 mb) in *Penicillium notatum* and chromosome I (10.4 mb) in *Penicillium chrysogenum*. Mol Gen Genet 241:573–579

Frederiksen RB, Emborg C (1984) Conversion of cephalosporin-C into 7-phenoxy-acetamido-cephalosporanic acid by acyltransferase of mutants of *Penicillium chrysogenum*. Biotechnol Lett 6:549–554

Gutiérrez S, Díez B, Montenegro E, Martín JF (1991) Characterization of the *Cephalosporium acremonium pcb*AB gene encoding α-aminoadipyl-cysteinyl-valine synthetase, a large multidomain peptide synthetase: linkage to the *pcb*C gene as a cluster of early cephalosporin-biosynthetic genes and evidence of multiple functional domains. J Bacteriol 173:2354–2365

Lein J (1986) The panlabs penicillin strain improvement program. In: Vanek Z, Hostalek Z (eds) Overproduction of microbial metabolites. Strain improvement and process control strategies. Butterworth, Boston, pp 105–139

Martín JF (1992) Clusters of genes for the biosynthesis of antibiotics: regulatory genes and overproduction of pharmaceuticals. J Ind Microbiol 9:73–90

MacCabe AP, Riach MBR, Unkles SE, Kinghorn JR (1990) The *Aspergillus nidulans npe*A locus consists of three contiguous genes required for penicillin biosynthesis. EMBO J 9:279–287

Meyer W, Koch A, Niemann C, Beyermann B, Epplen JT, Börner T (1991) Differentiation of species and strains among filamentous fungi by DNA fingerprinting. Curr Genet 19:239–242

Montenegro E, Fierro F, Fernández FJ, Gutiérrez S, Martín JF (1992) Resolution of chromosomes III and VI of *Aspergillus nidulans* by pulsed-field gel electrophoresis shows that the penicillin biosynthetic pathway genes *pcb*AB, *pcb*C, and *pen*DE are clusterd on chromosome VI (3.0 megabases). J Bacteriol 174:7063–7067

Queener SW, Sebek OK, Vezina C (1978) Mutants blocked in antibiotic synthesis. Annu Rev Microbiol 32:593–636

Ramón D, Carramolino L, Patino C, Sánchez F, Peñalva MA (1987) Cloning and characterization of the isopenicillin N synthetase gen mediating the formation of the β-lactam ring in *Aspergillus nidulans*. Gene 57:171–181

Ramos FR, López-Nieto MJ, Martín JF (1985) Isopenicillin N synthase of *Penicillium chrysogenum*, an enzyme that converts δ-(L-α-aminoadipyl)-L-cysteinyl)-D-valine to isopenicillin N. Antimicrob Agents Chemother 27:380–387

Revilla G, Ramos FR, López-Nieto MJ, Alvarez E, Martín JF (1986) Glucose represses formation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine and isopenicillin N synthase but not penicillin acyltransferase in *Penicillium chrysogenum.* J Bacteriol 168: 947–952

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Smith DJ, Bull JH, Edwards J, Turner G (1989) Amplification of the isopenicillin N synthetase gene in a strain of *Penicillium chrysogenum* producing high levels of penicillin. Mol Gen Genet 216:492–497

Specht CA, Dirusso CC, Novotny CP, Ullrich RC (1982) A method for extracting high-molecular-weight deoxyribonucleic acid from fungi. Anal Biochem 119:158–163

Note added in proof

The deleted region of the *npe10* mutant has been published recently (Fierro F, Barredo JL, Díez B, Gutiérrez S, Fernández FJ, Martin JF (1995) Proc Natl Acad Sci USA 92:6200–6204