JOURNAL OF BACTERIOLOGY, Nov. 1992, p. 7063–7067
0021-9193/92/217063-05$02.00/0

Vol. 174, No. 21

Resolution of Chromosomes III and VI of *Aspergillus nidulans* by Pulsed-Field Gel Electrophoresis Shows that the Penicillin Biosynthetic Pathway Genes *pcbAB*, *pcbC*, and *penDE* Are Clustered on Chromosome VI (3.0 Megabases)

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Received 13 March 1992/Accepted 5 August 1992

An improved electrophoretic molecular karyotype of *Aspergillus nidulans* ATCC 28901 has been obtained by contour-clamped electric field gel electrophoresis, which separates seven chromosomal bands and allows resolution of chromosomes III and VI. The three genes of the penicillin biosynthetic pathway, *pcbAB*, *pcbC*, and *penDE*, encoding α-aminoadipyl-cysteinyl-valine synthetase, isopenicillin N synthase, and isopenicillin N acyltransferase, respectively, are clustered together on a chromosome of 3.0 Mb, corresponding to linkage group VI, whereas the *argB* gene was located on a chromosome of 3.4 Mb, corresponding to linkage group III. Three other strains of *A. nidulans* contained a modified chromosome III of about 3.1 Mb that overlaps with chromosome VI, forming a doublet. Resolution of chromosomes III and VI in strain ATCC 28901 allowed unequivocal mapping of the penicillin gene cluster on chromosome VI of *A. nidulans*.

The genes encoding the biosynthesis of different antibiotics, pigments and other secondary metabolites in *Streptomyces* spp., filamentous fungi, and other producer organisms are frequently clustered together (see review by Martín and Liras [18]). The three genes encoding the penicillin biosynthetic pathway in *Penicillium chrysogenum* are known to be clustered in the genome of *P. chrysogenum* (3, 6, 7, 25). The DNA region encoding the penicillin gene cluster is greatly (10- to 13-fold) amplified in some strains of *P. chrysogenum*, probably as the result of tandem reiteration of a large DNA fragment (2). Similarly, the genes encoding cephalosporin biosynthesis in *Acremonium chrysogenum* are linked in at least two subclusters located on different chromosomes (9, 24).

Aspergillus nidulans produces very low levels of penicillin. However, this filamentous fungus has the advantage of a well-developed genetic map that facilitates molecular genetic studies on the mechanisms of control of antibiotic biosynthesis (1, 11). Four mutations which result in the absence of penicillin production, *npeA*, *npeB*, *npeC*, and *npeD*, were located in four linkage groups and were mapped relative to other loci on linkage groups VI, III, IV, and II, respectively (10, 15, 16). The *npeA* locus probably encodes enzymes involved in the biosynthesis of the tripeptide δ-(L-α-aminoadipyl-L-cysteinyl-D-valine) (ACV), since strains bearing mutations at this locus recovered penicillin G production when supplemented with ACV (14, 15).

We cloned the *penDE* gene of *A. nidulans* that encodes isopenicillin N acyltransferase, which catalyzes the last step of the penicillin biosynthetic pathway (19), and observed that it is linked to the *pcbC* gene (encoding isopenicillin N synthase), which catalyzes the second step of the biosynthetic pathway. MacCabe et al. (12) reported that the *npeA* locus might be the location of the entire cluster of the penicillin pathway. To study this hypothesis it was of interest to clone the gene (*pcbAB*) encoding ACV synthetase.

The development of pulsed-field gel electrophoresis technology has opened the possibility of separating linear double-stranded DNA molecules up to 10 Mb (21). A refinement of this technique, contour-clamped electric field (CHEF) gel electrophoresis (5), has been used to resolve the genomes of several organisms into defined chromosomal bands (23, 26, 29). Using this technique, Brody and Carbon (4) resolved the genome of *A. nidulans* into six chromosomal bands. The availability in our laboratory of the three genes of the penicillin biosynthetic pathway prompted us to study the location of the penicillin biosynthetic genes in *A. nidulans* chromosomes.

Linkage of the *pcbAB*, *pcbC*, and *penDE* genes of *A. nidulans*. The DNA of phage λAn*aat*1 (Fig. 1) (19) was digested separately with four restriction enzymes (*Bam*HI, *Hin*dIII, *Eco*RI, and *Sal*I) and hybridized with probes corresponding to the *penDE* and *pcbC* genes of *P. chrysogenum* (Fig. 2, probes A and B, respectively) and to the 5′ and 3′ regions of the *pcbAB* gene of *A. chrysogenum* (9) (Fig. 2, probes D and C, respectively) labeled by nick translation.

The *penDE* gene (encoding isopenicillin N acyltransferase) of *P. chrysogenum* hybridized with a *Bam*HI band of 2.1 kb, a *Hin*dIII band of 4.2 kb, two *Eco*RI fragments of 7.0 and 1.95 kb (in Fig. 3 the latter is seen as an 11-kb band since it is fused to the arm of phage λEMBL3), and a 7.6-kb *Sal*I fragment (Fig. 3). The *pcbC* gene of *P. chrysogenum* hybridized with two *Bam*HI fragments of 0.67 and 0.9 kb, a *Hin*dIII band of 4.2 kb, a 7.0-kb *Eco*RI fragment, and a 7.6-kb *Sal*I fragment.

The 5′ region of the *pcbAB* gene of *A. chrysogenum* (probe D) gave hybridization with three *Bam*HI bands (3.2, 3.0, and 2.3 kb), two *Hin*dIII bands (5.2 and 2.3 kb), two *Eco*RI bands (7.0 and 2.8 kb), and three *Sal*I bands (7.6, 1.5, and 1.0 kb). Finally, the 3′ region of the *pcbAB* gene (probe C) hybridized with two *Bam*HI bands (3.0 and 2.3 kb), two

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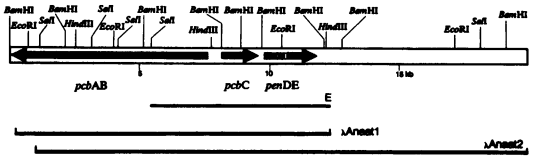

FIG. 1. Restriction map of the 19.7-kb *A. nidulans* DNA region carrying the genes *pcbAB*, *pcbC*, and *penDE* (arrows). The arrowheads indicate the direction of transcription of the three genes. The *penDE* gene contains three introns (open boxes within the arrow). The DNA fragments indicated by the brackets have been cloned in vector EMBL3 as described previously (19), originating phages λAn*aat*1 and λAn*aat*2. The 7.6-kb *Sal*I fragment (E in the figure) was used as a probe in hybridization studies with the chromosomes separated by CHEF gel electrophoresis.

*Hin*dIII bands (5.2 and 2.3 kb, both weak signals), two *Eco*RI bands (7.0 and 2.8 kb, the last one with a weak signal), and three *Sal*I bands (7.6, 1.0 [weak signal], and 0.8 kb; the distal *Sal*I site of the last band belongs to the EMBL3 phage DNA). The *pcbAB* genes of *P. chrysogenum* (7), *A. chrysogenum* (9), and *A. nidulans* (13) are unusually large genes (about 11.2 kb) that contain three repeated nucleotide sequences which appear to correspond to the domains of the protein involved in activation of each of the three amino acids (17). This homology between the three regions of this unusually long gene explains the complex pattern of hybridization with probes C and D (3′ and 5′ regions, respectively, of the ACV synthetase gene) since each probe hybridizes with the homologous region and also with DNA fragments carrying each of the two other repeated domains.

These results indicated that the three penicillin biosynthetic genes are closely linked in a 19.5-kb region (Fig. 1). A 7.6-kb *Sal*I fragment (probe E, Fig. 1) includes the *pcbC* and *penDE* genes and part of the *pcbAB* and was, therefore, used as a probe to locate the cluster of genes in the *A. nidulans* chromosomes separated by CHEF gel electrophoresis. This *Sal*I fragment corresponds to a 12.7-kb fragment in the

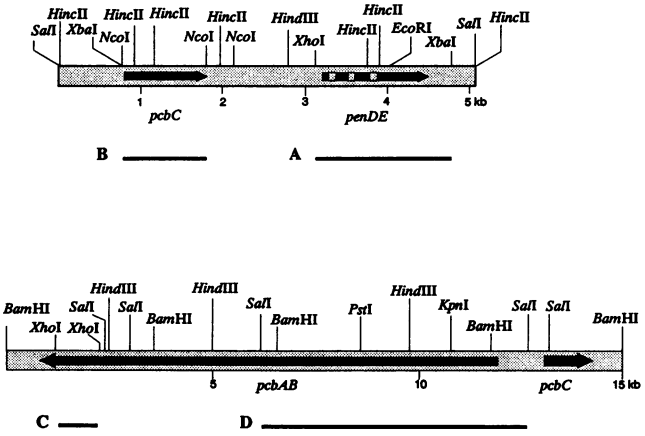

FIG. 2. Fragments of the DNA of *P. chrysogenum* (top) (5.1-kb *Sal*I) carrying the late penicillin biosynthetic genes (*pcbC*, *penDE*) and *A. chrysogenum* (bottom) (15-kb *Bam*HI) carrying the early (*pcbAB*, *pcbC*) cephalosporin biosynthetic genes used as probes to detect the linkage of the three genes in the genome of *A. nidulans*. Probe A, *penDE* gene; probe B, *pcbC* gene; probe C, 3′ region of the *pcbAB* gene; probe D, 5′ region of the *pcbAB* gene. All probes were labeled by nick translation with [α-^{32}P]dCTP as described previously (3, 6, 19).

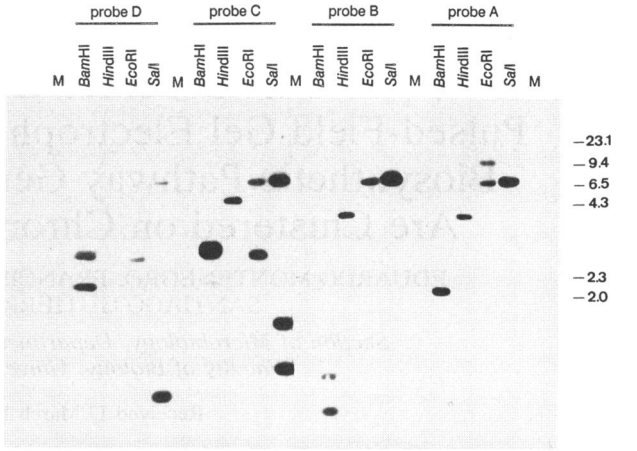

FIG. 3. Linkage of the genes *pcbAB*, *pcbC*, and *penDE* is shown by hybridization with probes A, B, C, and D (Fig. 2). Phage λAn*aat*1 was digested with *Bam*HI (lanes 1, 5, 9, and 13), *Hin*dIII (lanes 2, 6, 10, and 14), *Eco*RI (lanes 3, 7, 11, and 15), or *Sal*I (lanes 4, 8, 12, and 16). Lanes M were loaded with size markers (*Hin*dIII-digested λDNA and *Hin*fI–*Bgl*II-digested pBR328) (no hybridization). Lanes 1 to 4 were hybridized as described previously (19) with probe D; lanes 5 to 8 were hybridized with probe C; lanes 9 to 12 were hybridized with probe B; and lanes 13 to 16 were hybridized with probe A. Size markers (in kilobases) are indicated by bars on the right.

chromosomal DNA of *A. nidulans*, since one of the *Sal*I sites in phage λAn*aat*1 belongs to the *Sal*I site of the polylinker.

The *pcbC* and *penDE* genes of *A. nidulans* have been previously sequenced (19, 22, 28, 32), and they are known to be biologically active by complementation studies. Hybridization studies (Fig. 3) suggest that the *pcbAB* gene of *A. nidulans* has a structure (i.e., repeated domains) similar to those of *P. chrysogenum* (11.37 kb) and *A. chrysogenum* (11.13 kb). Initial determination of the sequence of the 5′ region revealed a high degree of similarity with the sequence of the *pcbAB* genes of *P. chrysogenum* and *A. chrysogenum* and showed that the orientation of the *pcbAB* gene of *A. nidulans* is the same as in *P. chrysogenum* and *A. chrysogenum*, i.e., it is transcriptionally divergent with respect to the *pcbC* gene. These results agree with the recent findings of MacCabe and coworkers (13).

Electrophoretic karyotype of *A. nidulans*: location of the penicillin gene cluster. The intact chromosomal DNA of *A. nidulans* ATCC 28901 was released from mycelial protoplasts directly in agarose plugs. By using consecutive switching intervals of 45 min at 45 V for 72 h and 35 min at 40 V for 277 h in a CHEF electrophoresis system for a total time of 349 h, seven chromosomal bands were resolved and observed by spectrodensitometry of the gel (Fig. 4). The relative intensity of the bands after ethidium bromide staining suggested that one of the bands was probably a doublet. This result is in agreement with eight linkage groups reported for *A. nidulans* by genetic and cytological determinations (11) and improves the resolution reported recently by Brody and Carbon (4), who managed to separate six chromosomal bands. By using as controls the chromosomes of *Schizosaccharomyces pombe* (estimated sizes of 3.5, 4.6, and 5.7 Mb) (31) and *Saccharomyces cerevisiae* (2.2, 1.6, and 1.125 Mb for the biggest chromosomes) (20), the sizes of the *A. nidulans* chromosomes determined by the position of the peaks after integration with the spectrodensitometer were

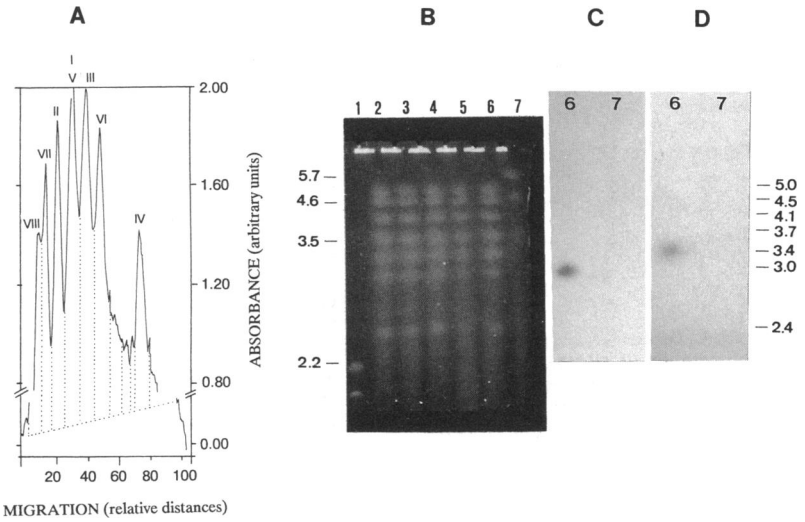

FIG. 4. (A) Densitometry of the photographic film of the gels in panels B to D to determine the mobility and the size of each band, carried out with a Shimadzu scanning spectrodensitometer at 400 nm. (B to D) Separation by CHEF gel electrophoresis of the chromosomes of *A. nidulans* (panel B) and hybridization of lanes 6 and 7 with probe E (Fig. 1) (panel C) or with the *argB* probe (see text) (panel D) labeled by nick translation. Protoplasts of *A. nidulans* were obtained as described by Yelton et al. (33) except that MM was supplemented with yeast extract (2 g/liter). To obtain protoplasts the washed mycelia were suspended in buffer supplemented with dithiothreitol (10 mM). The protoplasts (4×10^8 protoplasts per ml) were embedded in 0.7% low-melting-point agarose (Sigma)–0.7 M KCl–50 mM EDTA (pH 8.0). After solidifying at 4°C for 1 h, the agarose plugs were incubated at 50°C with ESP (0.5 M EDTA [pH 9.2], 1% lauroyl sarcosine, 1 mg of proteinase K per ml) for 48 h with gentle shaking to lyse the protoplasts in situ and washed twice with 50 mM EDTA (pH 8.0) at room temperature. Electrophoresis was carried out in 0.65% agarose (chromosomal grade; BioRad) in 0.5× Tris-borate-EDTA buffer at 10 to 11°C with buffer changes at 72, 144, 216, and 288 h. Consecutive switching intervals of 45 min at 45 V for 72 h were followed by switching intervals of 35 min at 40 V for 277 h in a CHEF electrophoresis system (DRII; BioRad). After staining with ethidium bromide and destaining in 0.5× Tris-borate-EDTA buffer for 3 h, the DNA was denatured and fragmented by soaking the gel consecutively in HCl (0.25 M, 20 min), 1.5 M NaCl–0.5 M NaOH (45 min), and 1 M Tris HCl (pH 8.0)–1.5 M NaCl (60 min). The fragmented DNA was transferred to nylon (Hybond-N) in 15× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 40 h. The filter was washed with 3× SSC for 5 min and fixed by UV irradiation for 10 min. Lanes: 1, large chromosomes of *S. cerevisiae* (2.2 and 1.6 Mb; BioRad); 2 to 6, different preparations of *A. nidulans* ATCC 28901 DNA; 7, chromosomes of *Schizosaccharomyces pombe* (5.7, 4.6, and 3.5 Mb; BioRad). The sizes of yeast chromosomal markers (in megabases) are indicated on the left of the gels. The estimated sizes (in megabases) of the *A. nidulans* chromosomes are indicated on the right of the gels.

5.0, 4.5, 4.1, 3.7, 3.4, 3.0, and 2.4 kb. The sizes of the six large bands are similar but not exactly identical to those reported by Brody and Carbon (4) for strain FGSC4. However, we have in our strain a clear seventh band of 2.4 Mb that moves ahead of the other chromosomes and behind the largest chromosome of *S. cerevisiae* (chromosome XII, 2.2 Mb). The total size of the genome estimated by addition of the sizes of the seven bands is 29.9 Mb and by DNA-DNA reassociation analysis has been reported as 26 Mb (27).

By comparison of the data in Fig. 4 with those reported by Brody and Carbon (4), it seemed that we had resolved the doublet of linkage groups III and VI reported by these authors. Therefore, we carried out hybridization studies with probes internal to the *pen* gene cluster and also with the *argB* gene, which is known to map in linkage group III of *A. nidulans* (4). A. 3.1-kb *Xba*I fragment carrying the entire *argB* gene was isolated from plasmid pAN923-41A (30) provided by C. van den Hondel. Studies using the 7.6-kb *Sal*I fragment (which contains *pcbC*, *penDE*, and part of the *pcbAB* gene) as a probe showed a clear hybridization with the chromosome of 3.0 Mb (Fig. 4) that corresponds to linkage group VI, whereas hybridization with the *argB* probe (linkage group III) showed that this gene is located on the chromosome of 3.4 Mb, which is different from the chromosome on which the *pen* gene cluster is found. These findings support the observation of MacCabe et al. (12), who suggested that the *npeA* locus, previously mapped in linkage group VI (10, 15, 16), corresponds to the location of the cluster of penicillin biosynthetic genes.

In the past, the *A. nidulans* chromosomes were identified by cytological or genetic analysis. Genetic linkage groups were assigned roman numerals in order of their discovery (11). Brody and Carbon (4) have established that chromosomes 1 to 8, as determined electrophoretically, correspond in order of decreasing size to linkage groups VIII, VII, II, and I and V (doublet), III and VI (doublet), and IV. Our results agree with the sizes estimated for linkage groups VIII, VII, II, and I and V (doublet) (Fig. 4). In our experimental conditions the second doublet was resolved into two bands of 3.4 and 3.0 Mb, corresponding to linkage groups III and VI (the latter carrying the *pen* gene cluster). Finally, the smallest chromosome (linkage group IV according to Brody and Carbon [4]) appears to have a size of 2.4 Mb in our gels.

The strain that we used in this study (ATCC 28901) is a white conidial biotinless mutant with increased penicillin production obtained after UV irradiation (8). Differences in chromosome size between this strain and those used by Brody and Carbon (4) may have arisen as a result of the mutation of the strain. Shifts in gel migration are known to occur in the chromosomal DNAs from several *Aspergillus* strains (4). Therefore, we compared this strain with the wild type (*A. nidulans* NRRL 194) and two other strains of *A. nidulans* (R153 and O^R31). As shown in Fig. 5, these three strains of *A. nidulans* showed karyotypes in which the

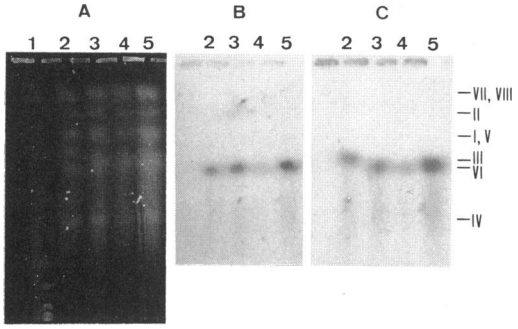

FIG. 5. (A) Separation of chromosomes of strains *A. nidulans* ATCC 28901 (lane 2), *A. nidulans* NRRL 194 (wild type) (lane 3), *A. nidulans* R153 (lane 4), and *A. nidulans* O^R31 (lane 5). Lane 1, control chromosomes of *Schizosaccharomyces pombe* and *S. cerevisiae*. (B) Hybridization with the *pen* cluster probe (linkage group VI). (C) Hybridization with the *argB* probe (linkage group III). CHEF gel electrophoresis was carried out as described in the legend to Fig. 4.

chromosomes corresponding to linkage groups III and VI were not resolved, whereas in the same gel, they were clearly separated in *A. nidulans* ATCC 28901. Studies using the *pen* cluster and the *argB* gene as probes showed that the hybridization signals of chromosomes VI and III overlap in the wild type and also in strains R153 and O^R31. The hybridization band with *argB* in strain ATCC 28901 is clearly retarded and moves behind the hybridizing band in the other *A. nidulans* strains. Apparently the mutagenic treatment has produced a rearrangement, probably involving some fragment duplication or translocation, resulting in an increased size of chromosome III in this particular strain. This phenomenon has allowed a clear resolution of linkage groups III and VI in strain ATCC 28901 and unequivocal mapping of the *pen* gene cluster to linkage group VI (3.0 Mb).

This work was supported by a grant of Gist-Brocades, Delft, Holland.

We thank J. L. Barredo and B. Díez for fruitful discussions and M. I. Corrales, M. González, R. Barrientos, and M. A. Liébana for excellent technical assistance.

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