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Elicitation of Penicillin Biosynthesis by Alginate in *Penicillium chrysogenum*, Exerted on *pcbAB*, *pcbC*, and *penDE* Genes at the Transcriptional Level

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Abstract Alginate and alginate-derived oligomannuronate enhanced penicillin production in shake flask and fermentor cultures of *Penicillium chrysogenum* Wis 54-1255 (containing a single copy of the penicillin gene cluster) and in the high producer strain *P. chrysogenum* AS-P-99 (containing multiple copies of the penicillin gene cluster). Alginate was not used as a single carbon source by *P. chrysogenum*. The stimulatory effect on penicillin production was observed in a defined medium and, to a lower extent, in a complex production medium containing corn steep liquor. Alginate-supplemented cells showed higher transcript levels of the three penicillin biosynthetic genes, *pcbAB*, *pcbC*, and *penDE*, than cells grown in the absence of alginate. The promoters of the *pcbAB*, *pcbC*, and *pen*DE genes were coupled to the reporter *lacZ* gene and introduced as monocopy constructions in *P. chrysogenum* Wis 54-1255 *npe*10 by targeted integration in the *pyrG* locus; the reporter β-galactosidase activity expressed from the three promoters was stimulated by alginate added to the culture medium of the transformants. These results indicate that the stimulation of penicillin production by alginate was derived from an increase in the transcriptional activity of the penicillin biosynthesis genes. The induction by alginate of the transcription of the three penicillin biosynthetic genes is a good example of the coordinated induction of secondary metabolism genes by elicitors of plant (or microbial) origin.

Key words: Alginate, elicitation, mRNA activation

In natural environments, filamentous fungi often compete for nutrients with other microorganisms. Secondary metabolites including antibiotics may function as antagonist agents inhibiting the growth of bacteria and other microorganisms [8, 20], or as communication molecules signaling cell differentiation [15]. Intercellular communication between plant or bacteria and filamentous fungi mediated by secreted molecules probably plays an important role in the induction (elicitation) of secondary metabolism. Alginates are produced by algae (kelp) and some bacteria [6, 16, 32].

Antibiotic biosynthesis by *Penicillium chrysogenum* has been investigated on the molecular level over the past few decades [7, 22, 23]. Three penicillin biosynthetic genes (*pcbAB*, *pcbC*, and *penDE*) have been cloned and characterized [4, 9, 29]; *pcbAB* encodes a large multifunctional nonribosomal peptide synthetase that is involved in the formation of the first intermediate δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV); *pcb*C encodes isopenicillin N synthase which converts LLD-ACV to isopenicillin N; and *penDE* encodes acyl-CoA:isopenicillin N acyltransferase which catalyzes the last step in penicillin biosynthesis [1]. However, the regulatory mechanisms of penicillin biosynthesis in *P. chrysogenum* are still poorly understood [19]. The transcript levels of the three penicillin biosynthetic genes (*pcbAB*, *pcbC*, and *penDE*) correlate well with the production of penicillin, thereby suggesting that the expression of the three genes is regulated at the level of mRNA [22, 26]. Some components of complex media used in the industrial production of penicillin (e.g. corn steep liquor) are known to increase the levels of penicillin gene transcripts (Gutiérrez, S. and J. F. Martín, unpublished results) yet the molecules responsible for this stimulation remain unknown.

Elicitation by alginate and other oligosaccharides in the production of secondary metabolites has been reported in plant cell cultures [28, 30, 31]. Ariyo *et al.* [2, 3] found that alginate oligosaccharides cause a significant increase in the yield of penicillin. They also suggested that alginate oligosaccharides may function as activators of defense processes in *P. chrysogenum*.

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Interest in the elicitation mechanism of secondary metabolites in fungi and the availability of the different genes of the penicillin biosynthetic pathway prompted the current study on the effect of alginate at the molecular level on penicillin gene expression. The alginate was found to affect penicillin biosynthesis at the level of transcription of the three penicillin biosynthetic genes (*pcbAB*, *pcbC*, and *penDE*) in both a low penicillin-producing strain, *P. chrysogenum* Wis 54-1255, and a very high penicillin producer, *P. chrysogenum* AS-P-99.

MATERIALS AND METHODS

Strains and Growth Conditions

Penicillium chrysogenum Wis 54-1255, a low penicillin production strain, and AS-P-99, a very high penicillin production strain with multiple copies of the penicillin gene cluster (Antibioticos, S.A., León, Spain), were used in all the fermentation experiments. *P. chrysogenum* Wis 54-1255 was used in the transcriptional analysis. *P. chrysogenum* Wis 54-1255 *npe*10 *pyrG*, a deletion mutant that lacks the penicillin gene cluster (to avoid integration at the *pen* cluster site), was used as the host strain for the targeted integration of expression cassettes at the *pyrG* locus. *E. coli* DH5α was used for the high frequency plasmid transformation. *Micrococcus luteus* ATCC 9341 was used for the bioassays of penicillin production.

Both defined and complex media were used for the seed culture development and penicillin production. The Defined Inoculum (DI) medium contained (in g per liter) citric acid, 3; acetic acid, 25; ethanolamine, 3; $(NH_4)_2SO_4$, 5; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.05; $ZnSO_4 \cdot 7H_2O$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.01; $MnSO_4 \cdot 4H_2O$, 0.01; $CoSO_4$, 0.005; NaCl, 0.001; and glucose, 2. The pH of the medium was adjusted to 5.5. The Defined Production (DP) medium was the same as above but contained 30 g/l lactose and 10 g/l sucrose instead of glucose. For benzylpenicillin production, phenylacetic acid was added to a final concentration of 1 g/l.

The fermentations were also performed in a complex medium to investigate the effect of elicitors under industrial conditions. The Complex Inoculum (CI) medium contained (in g per liter) corn steep solids, 20; yeast extract, 10; sucrose, 20; and $CaCO_3$, 5. The pH of the medium was adjusted to 5.7. The Complex Production (CP) medium for fermentation was composed of (in g per liter) corn steep solids, 35; lactose, 55; $CaCO_3$, 10; $MgSO_4 \cdot 7H_2O$, 3; KH_2PO_4, 7; and phenylacetic acid, 4. The pH of the medium was adjusted to 6.8.

Preparation of Oligomannuronate

The oligomannuronate was prepared by the partial hydrolysis of alginate from *Macrocystis pyrifera* (Sigma, St. Louis, MO, U.S.A.) in 0.3 M HCl as described previously [2]. The oligomannuronate or alginate was dissolved in distilled water and adjusted to pH 6.8. The solutions were autoclaved at 121°C for 15 min. Appropriate amounts of oligomannuronate or alginate solutions were then added to the defined or complex fermentation medium. The oligomannuronate contained 7-10 mannuronate units, as determined by thin-layer chromatography.

Penicillin Production Cultures

Fungal spores were scraped off from agar slants, and inoculated into 100-ml aliquots of the growth medium (either DI or CI) in 500-ml flasks. The flasks were incubated at 25°C with 250 rpm in a rotary incubator (New Brunswick Scientific, U.S.A.) for 36 h. For penicillin production, 10 ml of the seed culture was added to 100-ml aliquots of the production medium (either DP or CP) in 500-ml flasks and incubated under the same conditions (25°C and 250 rpm).

The penicillin concentrations in each culture were determined by a bioassay, as described previously [18]. Three fermentation batches were carried out in each case and duplicate samples were used in all assays. The reported values are the average of six determinations.

Isolation of *P. chrysogenum* RNA

The mycelia of *P. chrysogenum*, grown in a defined medium with or without alginate, were collected after 24, 48, 72, 96, 120, and 144 h by filtration through Nytal (Nytal, Switzerland) filters (30 μm-pore size) and washed with 1 volume of 0.9% NaCl. The washed mycelia were frozen at −70°C and then stored until use. The total RNA was extracted from samples (0.5 g) of different mycelia using the RNeasy/procedure (Promega, U.S.A.).

Probes and Northern Hybridization

Four probes were used to quantify the expression of each of the indicated genes: 1) a 7.9-kb *Eco*RI fragment of *P. chrysogenum* DNA internal to the *pcbAB* gene [9]; 2) a 1.0-kb *Nco*I fragment containing the entire *pcbC* gene [4]; 3) a 1.65-kb *Xba*I-*Xho*I fragment carrying the complete *penDE* gene [5]; and 4) a 0.42-kb *Sac*I fragment internal to the *P. chrysogenum* actin A gene. The probes were labeled by nick translation with [α-^{32}P] dCTP using standard methods [27]. The same amount of total RNA from each sample was transferred to Hybond N (Amersham Pharmacia-Biotech, Barcelona, Spain) nylon membranes. The membranes were prehybridized for 3 h at 42°C in 50% formamide-5× Denhardt's solution-5× SSPE-0.1% sodium dodecyl sulfate (SDS)-salmon sperm DNA (500 μg/ml) [27]. The hybridizations were carried out overnight at the same temperature and with the same buffer as that used for prehybridization except that 100 μg/ml salmon sperm DNA was used. After the hybridization, the membranes

were washed first with 2× SSC-0.1% SDS for 20 min at 42°C, followed by 0.1× SSC-0.1% SDS for 20 min at 42°C, and finally with 0.1× SSC-0.1% SDS for 10 min at 65°C. The membranes were exposed to X-ray film for 7 days at -70°C.

The intensity of the hybridization bands was determined by using a phosphorimager scanner (Instant-Imager, Packard). Hybridizations with the actin A probe (0.42 kb *Sac*I fragment) were used as the internal control.

Monocopy Constructions Expressing the *lacZ* Reporter Gene Under the Control of *pcbAB*, *pcbC*, and *penDE* Promoters

Monocopy transformants of *P. chrysogenum* Wis 54-1255 *npe*10 *pyrG* were obtained by targeted integration at the *pyrG* locus of the promoters fused to the *lacZ* reporter in plasmids pZ2bAB, pZ2C, and pZ3bDE [12]. β-Galactosidase was determined in the extracts of the disrupted *P. chrysogenum* cells, as described previously [12, 17].

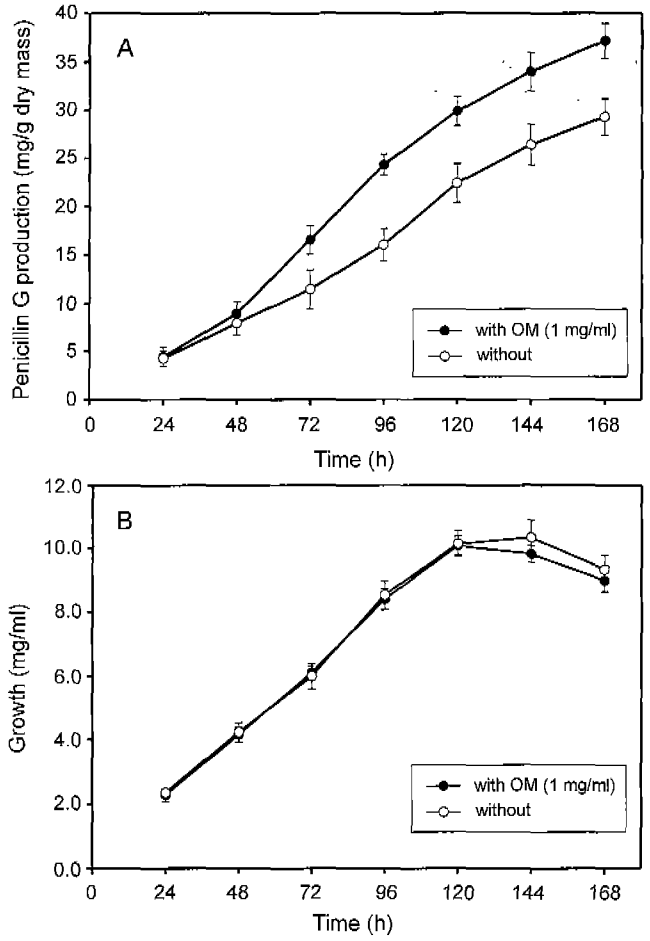

Fig. 1. (A) Effect of oligomannuronate (1 mg/ml) on penicillin production and (B) growth of *P. chrysogenum* Wis 54-1255 in DP medium.
Vertical bars indicate standard deviation in triplicate flasks.

RESULTS

Oligomannuronate Enhanced Penicillin Production by *P. chrysogenum* Wis 54-1255 in Defined Medium

Oligomannuronate at a final concentration of 1 mg/ml (Fig. 1) enhanced the penicillin production by *P. chrysogenum* Wis 54-1255 in the DP (defined) medium. There was an approximately 50% maximum increase in penicillin production after 72, 96, and 120 h without any significant changes in the biomass when compared to the control cultures without oligomannuronate. These results were repeated in five different experiments, and in all cases, a stimulation of 35 to 50% of the specific penicillin production were observed. The degree of polymerization of oligomannuronate was tested by thin-layer chromatography. The results showed that the oligomannuronate was larger than 7 mannuronate units and usually within a range of 7 to 10 residues.

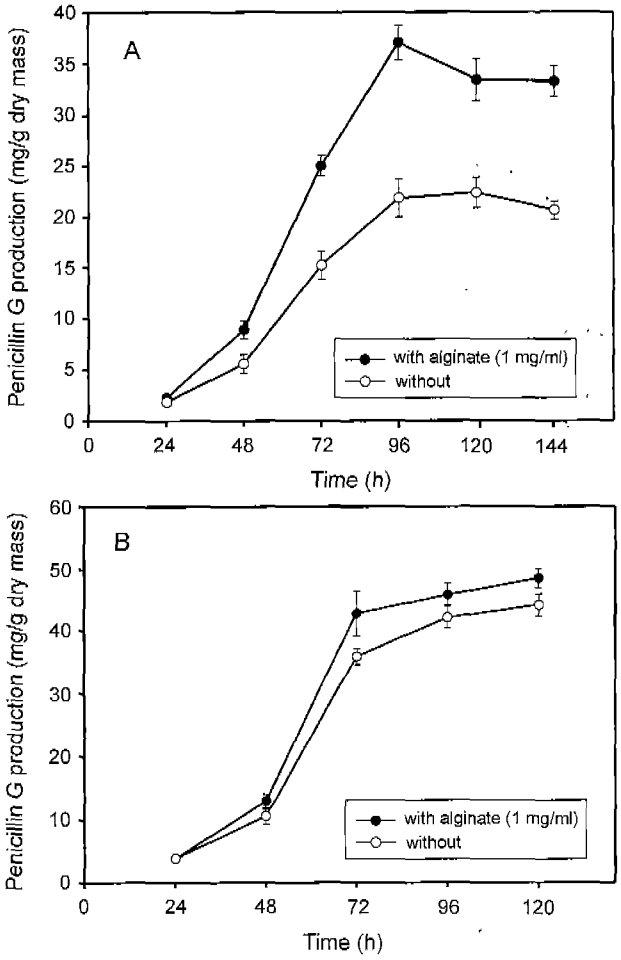

Fig. 2. Comparative effect of alginate (1 mg/ml) on specific penicillin production by *P. chrysogenum* Wis 54-1255 in (A) DP medium or (B) complex medium.
Note that the scale values in B are higher than in A. Vertical bars indicate standard deviation.

Alginate *Versus* Oligomannuronate as Elicitor of Penicillin Biosynthesis

Since the oligomannuronate stimulatory effect was found to be highly dependent on the batch of oligomannuronate (ranging from a 25 to 50% increase in penicillin production) and its use on a large scale would be difficult, the effect of alginate (which is commercially available) on penicillin production by *P. chrysogenum* Wis 54-1255 was investigated in the DP medium. The addition of alginate at a final concentration of 1 mg/ml to cultures of *P. chrysogenum* Wis 54-1255 caused a significant increase in penicillin production (Fig. 2A), similar to that obtained with oligomannuronate at the same final concentration. The penicillin production profiles were similar for both the alginate and oligomannuronate-supplemented cultures. Alginate showed the maximum effect on penicillin levels after 96 h of culturing, which was about 70% higher than the control cultures without alginate.

Alginate had a lower stimulation effect in the complex medium than in the defined medium. When added to CP cultures, alginate increased the penicillin production, although with a lower stimulatory effect than in the DP medium (Fig. 2B). The growth of *P. chrysogenum* Wis 54-1255 in the CP medium was faster than in the DP medium during the initial 48 h, resulting in trophophase-idiophase kinetics. The specific rate of penicillin biosynthesis was higher than in the DP medium (up to 50 mg of penicillin per g of dry weight as compared to 30–35 mg/g of dry weight in the DP medium) (Fig. 2B).

P. chrysogenum did not use Alginate as the Single Carbon Source

The fermentation data showed that alginate did not affect the growth of *P. chrysogenum* in the defined medium (containing 30 g/l lactose and 10 g/l sucrose as the carbon source). *P. chrysogenum* Wis 54-1255 was unable to grow in the defined medium with alginate as the single carbon source in either liquid or solid cultures.

Comparative Effect on High Penicillin-Producing Strain *P. chrysogenum* AS-P-99 *versus* Low Producer

The high producing strain AS-P-99 accumulated very high levels of penicillin in the DP medium (up to 120 mg per g of dry weight). The kinetics of the antibiotic accumulation were different since the penicillin synthesis was kept active for at least 168 h. The addition of alginate to cultures of *P. chrysogenum* AS-P-99 stimulated early penicillin accumulation after 48 and 72 h (Fig. 3). After 72 h, the stimulatory effect became less intense, and yet with the same magnitude as in the lower producer Wis 54-1255 (about a 50% increase).

Alginate Induced Transcription of *pcbAB*, *pcbC*, and *penDE* Genes

Both oligomannuronate and alginate increased the specific penicillin production rate. To determine if alginate was acting on the transcriptional level, the steady state levels of the mRNAs of *pcbAB*, *pcbC*, and *penDE* were investigated. *P. chrysogenum* Wis 54-1255 cells grown in the DP medium with alginate showed higher levels of the 11.4 kb (*pcbAB*), 1.1 kb (*pcbC*), and 1.15 kb (*penDE*) transcripts than the unsupplemented cultures (Fig. 4A) at different times throughout the fermentation in three independent experiments (Fig. 4). The maximum increases in the transcript levels of *pcbAB* and *pcbC* were 40% and 20%, respectively, after 48 h of culturing with alginate, and about 50% increase after 96 h for *penDE*, as compared to the control cultures without the elicitor. These results agree with the transcription profile of penicillin biosynthetic genes, in which the transcription of the *penDE* gene follows that of the *pcbAB* and *pcbC* genes [11, 12, 26].

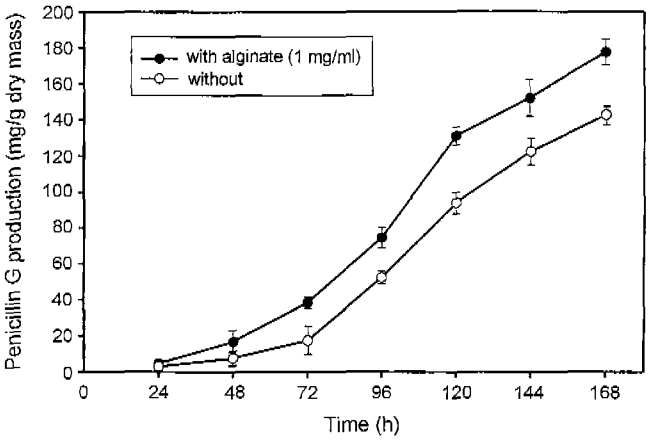

Fig. 3. Stimulatory effect of alginate (1 mg/ml) on penicillin production by high-producing strain, *P. chrysogenum* AS-P-99, in DP medium.
Note that this strain maintained a very high rate of penicillin biosynthesis for more than 168 h. Vertical bars indicate standard deviation in triplicate flasks.

These findings were confirmed by determining the β-galactosidase activity when the three promoters were coupled to the reporter *lacZ* gene in monocopy constructions. The results of the reporter studies (Table 1) showed that in the samples taken after 48, 72, and 96 h, there were increments in the expression of the *pcbAB* and *pcbC* genes in the alginate-supplemented medium. The *penDE* gene was not significantly expressed in the pZ3bDE constructions, thereby suggesting that the coupling of this promoter did not allow the correct expression of the reporter gene.

These results clearly show that alginate stimulated the transcription of the *pcbAB*, *pcbC*, and *penDE* genes. The transcript (1.6 kb) level of the actin A gene (used as the internal control) changed very little during fermentation in either the alginate-supplemented or unsupplemented medium.

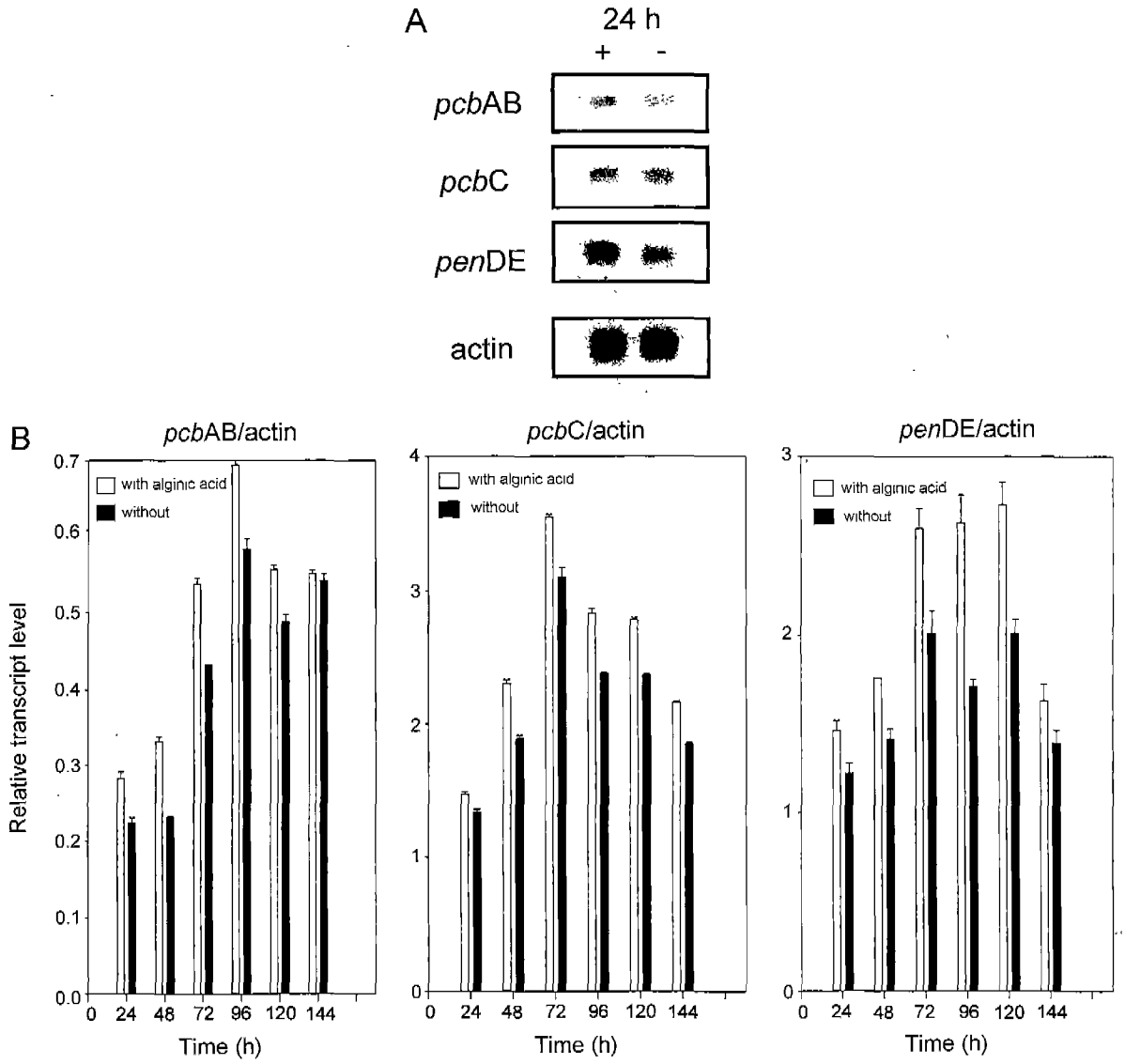

Fig. 4. (A) Steady-state transcript levels of *pcbAB*, *pcbC*, *penDE*, and *actA* genes with and without alginate.
Four parallel filters were hybridized with probes internal to the *pcbAB*, *pcbC*, *penDE*, and *actA* genes (see Materials and Methods). The same amount of total RNA was applied to all lanes in the filters. The filters corresponding to the *pcbAB*, *pcbC*, and *penDE* probes were exposed to X-ray film for one week and the hybridization with the actin probe for two weeks. (B) Quantification of the intensity of hybridization with *pcbAB*, *pcbC*, and *penDE* probes relative to the amount of label in the control hybridization with the actin A probe, using a phosphorimager counter (Instant Imager Packard). Clear bars: indicate cultures with alginate; dark bars: indicate cultures without alginate.

DISCUSSION

Alginate from *Macrocystis pyrifera* and its derivative oligomannuronate both increased penicillin production in two different strains of *P. chrysogenum*. Although it is possible that alginate may have been partially hydrolyzed to oligomannuronate, *P. chrysogenum* was unable to grow with alginate or oligomannuronate as the sole carbon source.

The stimulatory effect of alginate on penicillin biosynthesis is a finding of industrial relevance, because it is a cheap elicitor that can be easily obtained commercially. Alginate increased penicillin production in cultures of *P. chrysogenum* Wis 54-1255 and AS-P-99. These strains differ in the number of copies of the penicillin gene cluster they contain (growth characteristics are similar in both strains although other genetic differences cannot be excluded). The current results suggest that the stimulatory effect of alginate was not influenced by the gene copy number. In both strains, alginate increased the rate of penicillin biosynthesis during the early penicillin production phase.

Alginate also stimulated penicillin production in the complex medium, although the stimulation was lower than in the defined medium. This may have been due to the presence of elicitors in the corn steep in the CP medium. Corn steep is a complex

Table 1. β-Galactosidase acitivity expressed from the *pcbAB*, *pcbC*, and *penDE* promoters coupled to the *lacZ* reporter gene in monocopy transformants. β-Galactosidase acitivity (units/mg cell mass)

Constructions	48 h		72 h		96 h	
	without alginate	with alginate	without alginate	with alginate	without alginate	with alginate
pcbAB-lacZ	1.4	1.5	16.0	23.0	12.7	23.0
pcbC-lacZ	0.9	1.0	40.0	183.0	117.8	134.0
*penDE-lacZ**	0	0	1.0	2.4	5.6	7.0

*The endogenous β-galactosidase activity of *P. chrysogenum npe10 pyrG* were between 0 and 5.3 units/mg of cell mass and has been subtracted in all cases. Note that the construction with the *penDE* promoter is non-functional in the *pyrG* locus.

mixture of compounds, including amino acids, oligosaccharides, and growth factors, released by the acid hydrolysis of corn [10, 14]; some of these compounds could have served as elicitors, thereby partially masking the effect of alginate.

The current results showed that the stimulatory effect of alginate on penicillin biosynthesis was consistent with an increase in the steady-state levels of the transcripts of the *pcbAB*, *pcbC*, and *penDE* genes. The expression of the penicillin biosynthesis genes is coordinated [21] to ensure that the formation of all gene products occurs in the proper amount; the expression of the genes is activated by the same regulatory factors [22]. Accordingly, the simultaneous induction of the three penicillin biosynthesis genes suggests that the effect of alginate on penicillin biosynthesis was possibly mediated through the action of a regulatory protein that interacted with the *pcbAB-pcbC* bidirectional promoter [19] and *penDE* promoter.

The elicitation of plant secondary metabolism by alginate-derived oligosaccharides [25, 28] and other molecules secreted by phytopathogens [24] is well known. Ariyo and coworkers [2, 3] previously reported that alginate-derived oligosaccharides also play a role in the elicitation of penicillin production. In the current study, since alginate was not used as a carbon source by *P. chrysogenum*, and there was a high concentration of lactose in the medium, it is unlikely that its stimulatory effect on penicillin biosynthesis was due to the nutritional effects of the monosaccharides derived from alginate. Ariyo and coworkers also reported that the stimulatory effect appeared to be due to oligosaccharides rather than monosaccharides [2, 3]. Our results confirm the observations of Ariyo and coworkers, and indicate that the induction effect is exerted at the transcriptional level on the three penicillin biosynthesis genes.

Thus, the observations reported in this work are consistent with the proposal that alginate oligosaccharides seem to function as activators of secondary metabolism processes in *P. chrysogenum* [2]. An interesting point is why alginate or oligomannuronate enhance penicillin biosynthesis. Alginates are synthesized by several *Pseudomonas* species that are normal inhabitants of soil and compete with filamentous fungi [13]. As such, the presence of alginate molecules may signal the need to enter secondary metabolism, thereby resulting in antibiotic production and morphological differentiation. It is unknown whether alginate is partially hydrolyzed by *P. chrysogenum*. The induction is probably exerted by a signal transduction cascade initiated by alginate or oligomannuronate at the membrane level and may not require transport of the inducer.

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