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Biosynthesis and phosphate control of candicidin by *Streptomyces acrimycini* JI2236: effect of amplification of the *pab*AB gene

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SUMMARY

Biosynthesis of candicidin by *Streptomyces acrimycini* JI2236 was strongly inhibited by phosphate. *p*-Aminobenzoic acid (PABA) synthase activity, required for the synthesis of PABA, a candicidin precursor, was reduced by 72% in cells grown in medium supplemented with 7.5 mM phosphate. Hybridization studies showed that the DNA region of *S. acrimycini* carrying the *pab*AB gene (encoding PABA synthase) is very similar to the homologous region of *S. griseus* 3570. *S. acrimycini* was easily transformed with plasmids containing the *pab*AB gene of *S. griseus*. Four transformants were studied in detail; three of the transformants synthesized higher levels of PABA synthase and two transformants produced more candicidin than control cultures transformed with pIJ699. The fourth transformant was unable to synthesize the antibiotic. Formation of PABA synthase and candicidin production was equally sensitive to phosphate regulation in transformants with the *pab*AB than in the untransformed *S. acrimycini* strain.

INTRODUCTION

Candicidin (Cd) is a polyene (heptaene) macrolide antibiotic with antifungal activity produced by *Streptomyces griseus* IMRU 3570. The chemical structure of Cd entails in addition to the macrolide ring, a *p*-aminoacetophenone group (the aromatic moiety) derived from *p*-aminobenzoic acid (PABA) and the aminosugar mycosamine (3-amino-3,6-dideoxy-D-mannopyranose) attached glycosidically to the macrolide ring [18]. The biosynthesis and regulation by phosphate of Cd production in *S. griseus* has been studied [1,12,13]. However, genetic manipulation of this strain has been hampered by its poor sporulation on solid medium and difficulties in transformation or electroporation. Therefore it was important to find new Cd-producing strains that were more suitable for manipulation, in order to improve our molecular knowledge on candicidin biosynthetic genes and their regulation.

The *pab*S gene, renamed *pab*AB since it is equivalent to a fusion of *pab*A and *pab*B genes of *E. coli* [2] encodes PABA synthase, an enzyme involved in Cd biosynthesis [5]. Hybridization techniques using this gene have allowed the search for Cd-producing *Streptomyces* strains containing *pab*AB homologous genes; six of the 16 strains tested gave positive hybridization and produced Cd [6].

In the present report we study the biosynthesis and phosphate regulation of Cd production by *Streptomyces acrimycini* JI2236, a strain that is readily transformed and, therefore, would be suitable for recombinant DNA studies. This strain has been used to study the effect of increasing the gene dosage of the *pab*AB gene on candicidin biosynthesis.

MATERIALS AND METHODS

Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table 1.

Culture conditions

To measure Cd and PABA synthase activity, *S. acrimycini* JI2236 was grown in SPG antibiotic production medium containing in g L^{-1}: starch-free soya peptone (Staley 4S, USA), 25; glucose, 60; zinc sulfate, 0.143, pH 7.5 [14]. The cultures were incubated at 32 °C in 500-ml triple-baffled flasks containing 100 ml SPG medium, in an orbital shaking incubator at 225 r.p.m. The production medium was inoculated with 10 ml of a 24-h inoculum grown in YED medium (yeast extract, 10 g L^{-1}; glucose 10 g L^{-1}, pH 7.2). Thiostrepton (5 μg ml^{-1}) was added to the cultures of the transformed strains.

Determination of candicidin and PABA synthase activity

Cd was extracted and quantified from the culture broth at different times during fermentation in SPG medium as described by Martín and McDaniel [14]. PABA synthase activity was assayed in cell-free extracts from cultures grown for 24 h, and the formation of PABA was measured by the diazotization method [7]. A unit of PABA synthase produces 1 nmol of PABA in 30 min. All determinations were made in duplicate from two duplicate flasks.

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TABLE 1

Streptomyces strains and plasmids

Strain or plasmid	Characteristics	Reference
Strain		
S. acrimycini JI2236	Cloramphenicol resistant mutant from *S. acrimycini* JI1201. Cd producer	[17]
S. griseus IMRU3570	First reporter Cd producer	[11]
S. lividans JI1326	Wild type	[8]
Plasmid		
pIJ814	pBR322-derived, carrying the *pab*AB gene from *S. griseus* in a 4.5-kb *Bam*HI DNA fragment	[5]
pIJ819	pBR322-derived, carrying the *pab*AB gene from *S. griseus* in a 3.5-kb *Bam*HI DNA fragment	[5]
pIJ699	High-copy positive selection vector	[9]
pULJA61	pIJ699-derived, carrying the 4.5-kb *Bam*HI DNA fragment from pIJ814	This work
pULJA62	pIJ699-derived, carrying the 3.5-kb *Bam*HI DNA fragment from pIJ819	This work

DNA manipulations

Isolation of plasmid and chromosomal DNA from *S. acrimycini* was done as described by Hopwood et al. [8], except that the medium used for growth was YED-glycine medium (YED medium supplemented with 5 g L^{-1} glycine and 100 mM $MgCl_2$) in which *S. acrimycini* possesses good dispersed growth. Restriction enzymes, agarose gel electrophoresis, transfer of DNA to nitrocellulose, nick translation and hybridization conditions were carried out as described by Sambrook et al. [16]. Extraction of DNA from low melting point agarose (LMA) gels was done as described by Langridge et al. [10].

Transformation of protoplasts

S. acrimycini protoplasts were obtained as described by Hopwood et al. [8], after growth in YED-glycine medium as noted above. As this strain has a minimal inhibitory concentration for thiostrepton of 3 μg ml^{-1}, transformants carrying the *tsr* gene were selected by overlaying the regeneration plates (R2YE medium) with 1.5 ml of an aqueous solution of 100 μg of thiostrepton ml^{-1} (final concentration on plates of 7.5 μg ml^{-1}).

RESULTS

Production of Cd and PABA synthase activity are regulated by phosphate in S. acrimycini

Growth and Cd production by *S. acrimycini* and *S. griseus* were determined during incubation in SPG medium and in SPG medium supplemented with 7.5 mM sodium phosphate buffer, pH 7.0. The growth of both actinomycetes was similar; in the presence of phosphate (7.5 mM) the cell dry weight increases about three fold in relation to unsupplemented cultures. However, in cultures with 7.5 mM phosphate, *S. acrimycini* cell lysis started 24 h earlier than in *S. griseus* (Fig. 1(A)). The production of Cd by *S. acrimycini*, at 72 h (before lysis), is about half of that of *S. griseus* (78 versus 140 μg Cd mg^{-1} dry weight). It is also strongly regulated by phosphate although to a lesser degree than in *S. griseus* (10% of the control in the case of *S. griseus* and 20% for *S. acrimycini*) (Fig. 1(B)).

PABA synthase activity in cell-free extracts obtained from 24-h-old cells grown in SPG medium without phosphate supplementation, was similar in both strains. As described previously for the *S. griseus* PABA synthase [1,7], the homologous enzyme in *S. acrimycini* was regulated also by phosphate; activity was only 28% of that observed in cells grown without supplemented phosphate.

Similarity of the region containing the pab*AB gene in* S. acrimycini *and* S. griseus

Since *S. acrimycini* DNA hybridizes with the *pab*AB gene of *S. griseus* [6], we mapped the region of DNA that hybridizes. Chromosomal DNA from *S. acrimycini* was digested separately with *Sac*II, *Pvu*II, *Bam*HI and *Sal*I. Digested DNAs were electrophoresed, transferred to nitrocellulose and hybridized with the 3.5-kb *Bam*HI fragment from *S. griseus pab*AB gene (cloned in pIJ819). The pattern of hybridization of the digested DNAs (Fig. 2) matched quite well with the restriction map of the 4.5-kb *Bam*HI fragment of *S. griseus* [15] indicating that both genes probably have a marked similarity in their sequences. However the size of the *Sal*I bands in *S. acrimycini* DNA that hybridize with the *pab*AB probe is slightly different from those of *S. griseus*.

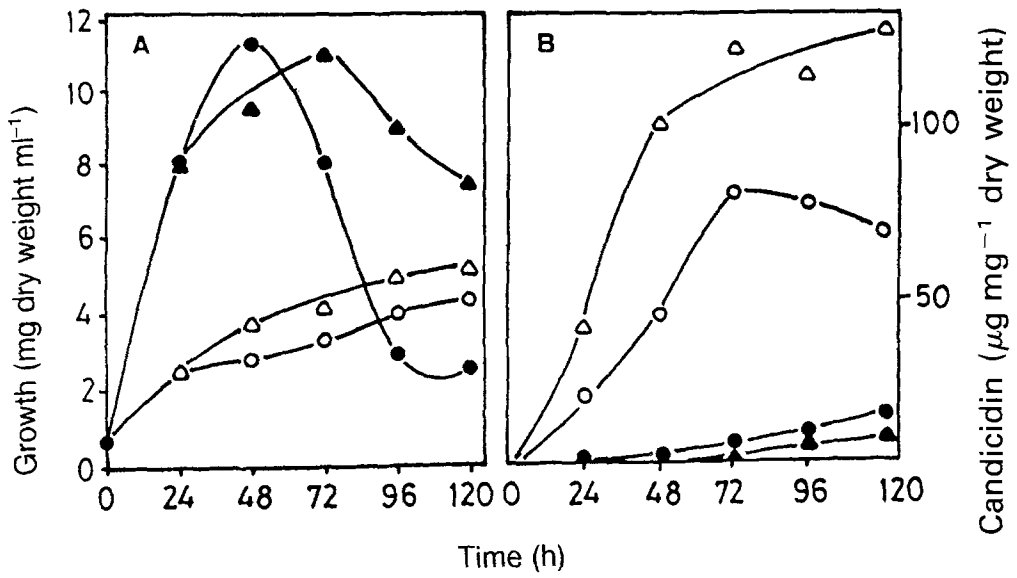

Fig. 1. Growth (A) and Cd production (B) of *S. acrimycini* JI2236 (○, ●) and *S. griseus* IMRU 3570 (△, ▲) grown in SPG medium with (●, ▲) or without (○, △) supplementation with 7.5 mM sodium phosphate.

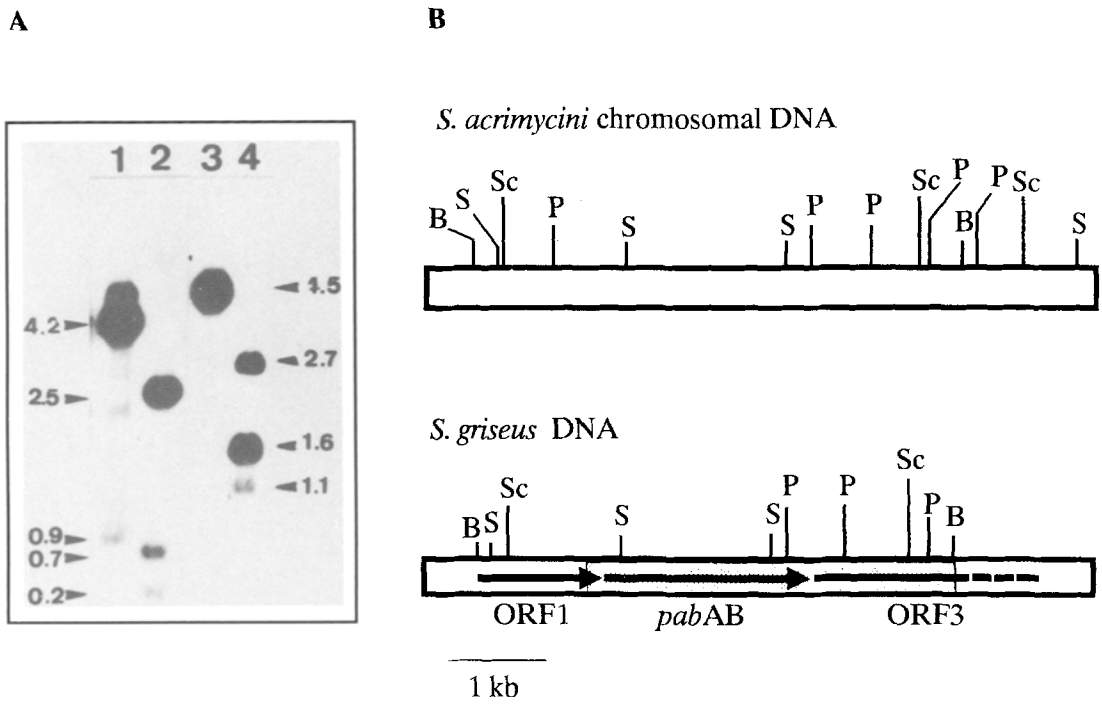

Fig. 2. (A) Southern hybridization of genomic DNA from *S. acrimycini* digested with *Sac*II (1), *Pvu*II (2), *Bam*HI (3) and *Sal*I (4) with the 3.5-kb *Bam*HI fragment from *S. griseus* containing the *pab*AB gene. (B) Map of the Cd-cluster region of *S. acrimycini* showing the restriction fragments. The map of the equivalent region of *S. griseus* DNA, including the ORFs present, is shown below for comparison. The DNA used as probe is shadowed. Symbols: *Sac*II (Sc), *Pvu*II (P), *Bam*HI (B), *Sal*I (S).

Construction of high-copy number plasmids containing the pab*AB gene*

In order to study whether an increase in the number of copies of *pab*AB has any effect on the production of candicidin, plasmid constructions were made to introduce the *pab*AB gene from *S. griseus* into *S. acrimycini*.

The plasmid pIJ699 [9] was digested with *Bgl*II (Fig. 3) and the 5-kb fragment containing the *tsr* gene was isolated from low melting agarose. In parallel, pIJ814 and pIJ819 [5] were digested with *Bam*HI, and the 4.5- and 3.5-kb fragments containing the *pab*AB gene were also isolated from low melting agarose. These fragments were separately ligated to the 5-kb fragment isolated from pIJ699 and the ligation mixture was used to transform *S. lividans* protoplasts.

Thiostrepton-resistant clones were screened for plasmid DNA and the plasmids, designated pULJA61 and pULJA62 respectively, were mapped to confirm their presence and integrity. These plasmids were then used to transform *S. acrimycini* protoplasts. Both constructions differ in the presence or absence of a 1-kb DNA fragment upstream from the *pab*AB gene that corresponds to a thioesterase activity present in the candicidin gene cluster.

Cd production by S. acrimycini *transformed with the* pab*AB gene*

A total of 30 thiostrepton-resistant clones of *S. acrimycini*-[pULJA61] and *S. acrimycini*[pULJA62] were tested for Cd production in solid medium. Three clones of *S. acrimycini*-[pULJA61] named R2, R4 and R9, showing different production levels of Cd on agar medium were chosen for further studies. All the clones of *S. acrimycini*[pULJA62] had similar production levels of Cd. *S. acrimycini*[pIJ699] produced considerably less Cd (32%) than the wild type *S. acrimycini* JI2236 strain and it also synthesized less PABA synthase. This phenomenon is related to the presence of thiostrepton in the culture [4; V. Kumar, personal communication, 1993]. When the production of Cd from clones carrying several copies of the *pab*AB gene was compared to that of control *S. acrimycini*[pIJ699] in liquid medium, two clones, *S. acrimycini*[pULJA61]R9 and *S. acrimycini* [pULJA62], produced more Cd (188% and 129%, respectively), although less than the untransformed wild type strain. The regulation by phosphate of candicidin biosynthesis was similar in all the transformants (Fig. 4), except transformant R2 which showed no production.

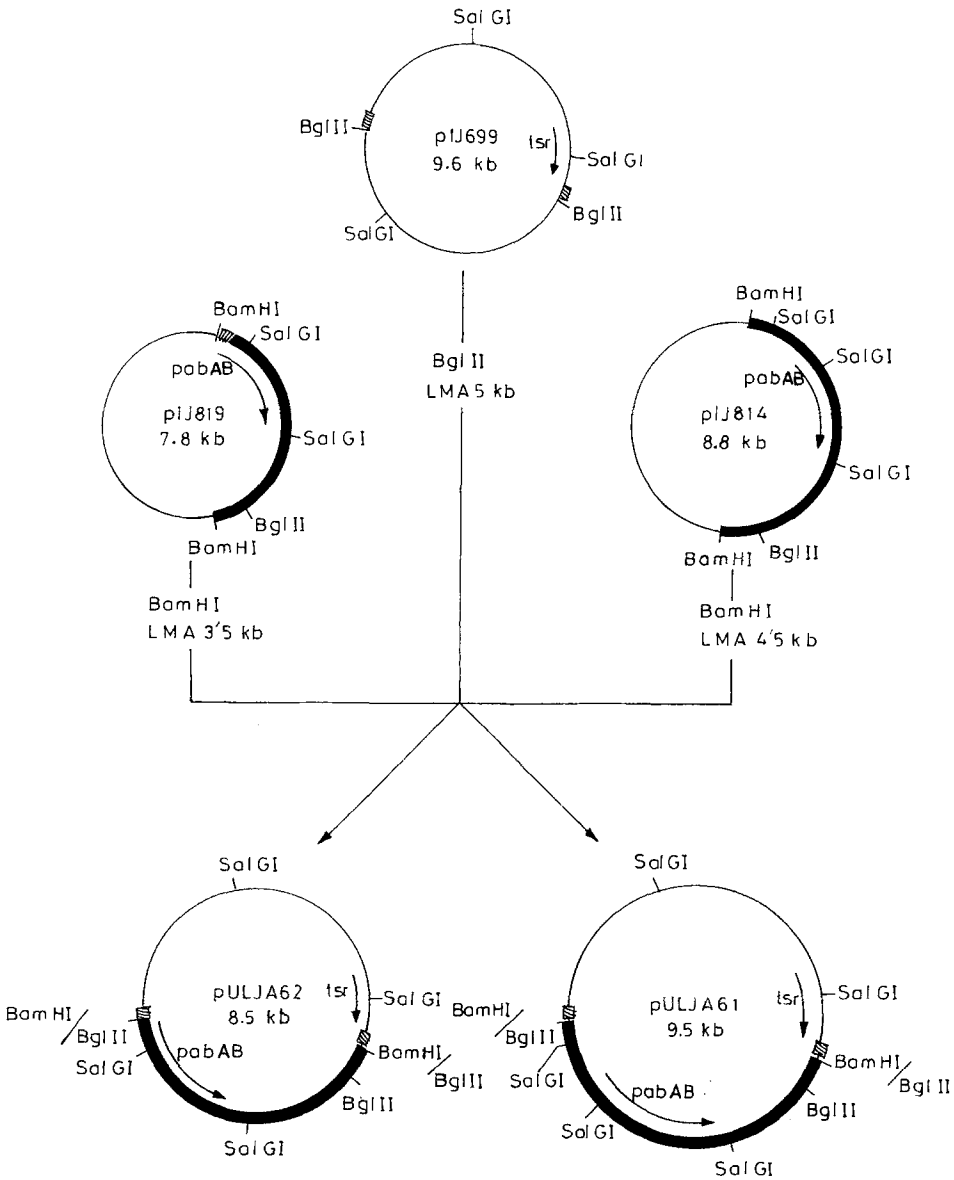

Fig. 3. Construction of plasmids pULJA61 and pULJA62, carrying the *pab*AB gene of *S. griseus*.

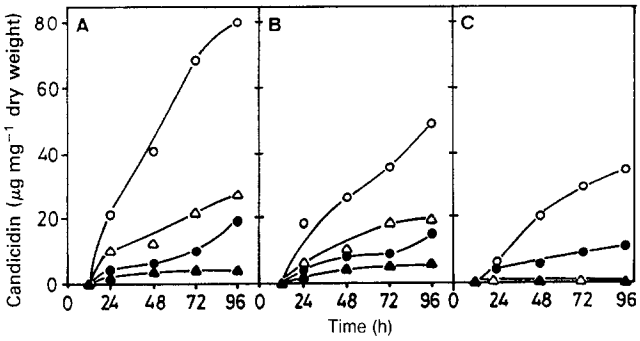

Fig. 4. Specific production of Cd in SPG medium with (●, ▲) or without (○, △) supplementation of 10 mM phosphate. (A) *S. acrimycini* JI2236 (○, ●) and *S. acrimycini*[pIJ699] (△, ▲); (B) *S. acrimycini*[pULJA61]R9 (○, ●) and *S. acrimycini*[pULJA61]R4 (△, ▲); and (C) *S. acrimycini*[pULJA62] (○, ●) and *S. acrimycini*-[pULJA61]R2 (△, ▲).

PABA synthase activity of S. acrimycini *transformed with the* pab*AB gene*

PABA synthase activity was measured in cultures of all the transformant clones. As shown in Table 2, there is a good correlation between PABA synthase activity and Cd production. As observed with candicidin production, PABA synthase activity is lower in *S. acrimycini*[pIJ699] or *S. acrimycini* transformed with pIJ699-derived plasmids as it was found also for Cd production. All the strains carrying a high number of copies of the *pab*AB gene (except for *S. acrimycini*[pULJA61]R2), have more PABA synthase activity than the control transformant *S. acrimycini*[pIJ699]. Phosphate regulation is similar or slightly lower in *S. acrimycini*-[pULJA61]R9 and *S. acrimycini*[pULJA62] (48 and 50%, respectively) than in *S. acrimycini*[pIJ699] (61%). PABA synthase activity of *S. acrimycini*[pULJA61]R2, the transformant that did not produce Cd, is very low and appears to be stimulated by phosphate.

TABLE 2

PABA synthase activity and regulation by phosphate of *S. acrimycini* transformants

Strain	PABA synthase activity[a]			
	(U mg^{-1} protein)	(%)	(%)	Regulation by phosphate[b] (%)
S. acrimycini JI2236	14.00	100[c]	–	72
S. acrimycini[pIJ699]	4.20	30	100[d]	61
S. acrimycini[pULJA61]R9	7.19	51	171	48
S. acrimycini[pULJA61]R4	5.80	41	138	67
S. acrimycini[pULJA61]R2	1.83	13	43	−14[e]
S. acrimycini[pULJA61]	5.20	37	124	50

[a] Data at 24 h in SPG.
[b] 10 mM phosphate.
[c] Percentage of PABA synthase activity with respect to the control *S. acrimycini* JI2236.
[d] Percentage of PABA synthase activity with respect to the control *S. acrimycini*[pIJ699].
[e] Phosphate stimulates PABA synthase activity in this transformant.

Pattern of the pab*AB region in the transformants*

The different expression of the *pab*AB gene could be due to recombination between the chromosomal *pab*AB gene and the plasmid gene. Total DNA of the different strains was isolated, digested with *Sal*I, and hybridized with the 3.5-kb *Bam*HI fragment containing the *pab*AB gene of *S. griseus*. As shown in Fig. 5(A), all the transformed strains have a pattern of hybridization consistent with the plasmid being introduced (either pULJA61 or pULJA62, Fig. 5(B)). The high intensity of the 2.5, 1.6 and 1.1 *Sal*I bands (belonging to pULJA61) as compared to the 2.7, 1.6 and 1.1 bands of the untransformed *S. acrimycini* indicate that several copies of the pULJA61 (or pULJA62) are present in the transformants. The pattern of hybridization of transformant [pULJA61]R2 showed no changes in the small (1.6- and 1.1-kb) chromosomal bands but lacked the 2.7-kb *Sal*I chromosomal fragment of *S. acrimycini* that was present in all other transformants (see discussion).

DISCUSSION

The slow advance of the genetics of Cd biosynthesis has been due to the unavailability of a producing strain suitable for genetic manipulation. *S. acrimycini* JI2236 has several advantages over the *S. griseus* strain presently used to study candicidin production: efficient sporulation and easy transformability, in addition to the presence of the *pab*AB gene and adjacent DNA regions that appears to be similar to the homologous region of *S. griseus* IMRU 3570 [2,15]. Furthermore, the production of Cd and *pab*AB gene expression are regulated by phosphate, as in *S. griseus* [1]. Attempts to introduce into *S. acrimycini* low copy number plasmids containing the *pab*AB gene, such as pIJ806, pIJ822 or pIJ811 [5] proved unsuccessful, probably due to the fact that these plasmids contain the SLP1.2 replicon and the host range of this replicon is very narrow. The introduction by transformation of a high number of copies of the *pab*AB gene into *S. acrimycini* increased the PABA synthase activity with respect to control transformants carrying pIJ699, in which PABA synthase activity is severely reduced. The observation that strains transformed with plasmids containing the *tsr* gene, when grown in the presence of thiostrepton, exhibited lower antibiotic production than strains without the plasmid, has been previously described with plasmid pIJ702 in relation to cephamycin C production [4].

The PABA synthase activity in the transformants was not proportional to the theoretical gene dose of *pab*AB, as cells generally carry about 50 copies of the plasmid pIJ699 per cell [3]. In both *S. acrimycini*[pUL61]R9 and *S. acrimycini*[pUL62] the increase in PABA synthase activity (171 and 124%) in relation to *S. acrimycini*[pIJ699] relates well to the increase in candicidin production (188 and 129%) but no correlation was found in *S. acrimycini*[pUL61]R4.

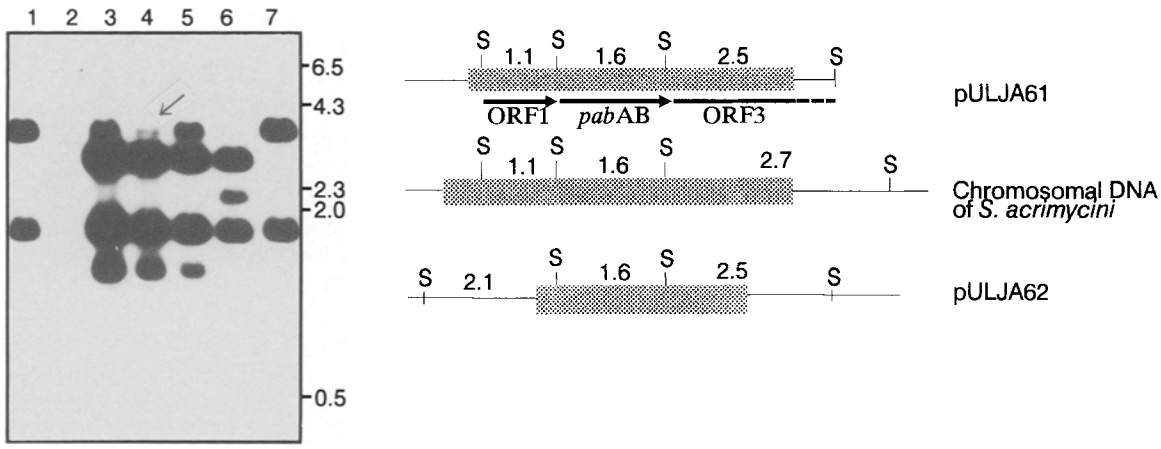

Fig. 5. (A) Southern hybridization of total DNA from different strains digested with *SalI* using the same probe as in Fig. 2(A): *S. acrimycini* JI2236 (1), Size markers (no hybridization) (2), *S. acrimycini*[pULJA61]R4 (3), *S. acrimycini*[pULJA61]R2 (4), *S. acrimycini*[pULJA61]R9 (5), *S. acrimycini*[pULJA62] (6) and *S. acrimycini*[pIJ699] (7). (B) Schematic representation of the results. The thin line corresponds to the vector (pIJ699) DNA. S = *SalI* restriction site.

No recombination between the chromosomal *pab*AB gene and the plasmid *pab*AB gene was observed in transformant [pULJA61]R2. This mutant seems to have suffered a deletion of the 2.7-kb *SalI* fragment (Fig. 5, arrow) that contains the *p*-aminobenzoyl-CoA ligase (ORF3). Therefore the inability to synthesize candicidin even if additional copies of PABA synthase are introduced, may be attributed to the cloned 4.5-kb fragment of *S. griseus* lacking the full ORF encoding *p*-aminobenzoyl-CoA ligase. The low level of PABA synthase activity in [pULJA61]R2, despite the presence of the 4.5-kb fragment of *S. griseus* DNA in the plasmid (Fig. 5, lane 4), suggests that expression of the *pab*AB gene is activated by a mechanism specified by a region of the DNA fragment that is missing in mutant R2.

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