A gene from *Alcaligenes denitrificans* that confers albicidin resistance by reversible antibiotic binding

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Albicidin antibiotics specifically block prokaryote DNA replication. The albicidin resistance gene (*albB*) cloned from a soil isolate of *Alcaligenes denitrificans* encodes a 23 kDa protein capable of detoxifying albicidin by reversible binding. This mechanism operates intracellularly to protect DNA replication in albicidin-sensitive *Escherichia coli* expressing the cloned resistance gene, which can be induced fivefold in the presence of 1.5 µg albicidin ml⁻¹ in the surrounding medium. The coding region of 621 bp has regions with partial DNA sequence homology to an albicidin resistance gene (*albA*) from *Klebsiella oxytoca*, but with rearrangements and frame-shifts resulting in loss of protein homology. There is a short region of N-terminal homology between the albicidin resistance (Alb^r) proteins from *A. denitrificans* and *K. oxytoca*, although the two genes use different codons for shared amino acids. The N-terminal homology suggested a common functional domain; this was confirmed by deletion analysis, translational fusions and albicidin binding by a synthetic oligopeptide. Antibiotic binding provides a high level of albicidin resistance in *E. coli*. The gene appears to be a useful candidate for transfer to plants to protect plastid DNA replication from inhibition by albicidin phytotoxins involved in sugarcane leaf scald disease.

Keywords: *Alcaligenes denitrificans*, antibiotic binding, albicidin resistance, *alb* gene

INTRODUCTION

The phytopathogen *Xanthomonas albilineans*, which causes leaf scald disease of sugarcane, produces a family of toxins that selectively block DNA replication in bacteria and chloroplasts (Birch & Patil, 1985b, 1987b). The major antibiotic produced by *X. albilineans* in culture is a low molecular mass aromatic compound called albicidin, which is rapidly bactericidal at concentrations between 1 and 100 ng ml⁻¹ to a range of Gram-positive and Gram-negative bacteria. Near the minimum inhibitory concentration against *Escherichia coli*, albicidin causes a rapid and complete block to DNA replication without binding to or damaging DNA, indicating likely interaction with a DNA replication protein (Birch & Patil, 1985b; Birch *et al.*, 1990). There is no cross-resistance between albicidin

and other antibiotics affecting DNA replication. At 8 µg ml⁻¹, the compound shows no cytotoxicity to mammalian cells in tissue culture (Birch & Patil, 1985a). Albicidins are therefore of interest as potential clinical antibiotics and as tools to study prokaryotic DNA replication.

Albicidins produced by *X. albilineans* cause the characteristic chlorotic symptoms in emerging leaves of sugarcane with leaf scald disease, by preferentially inhibiting DNA replication in differentiating plastids (Birch & Patil, 1983, 1987a, b). This mode of action is unique among phytotoxins, and provides a rare example of a known mechanism of development of a plant disease from the molecular level through to visible symptom expression. Mutants of *X. albilineans* which do not produce albicidins have failed to produce any systemic disease symptoms in inoculated sugarcane (Birch & Patil, 1987a). Albicidin resistance may therefore convey leaf scald disease resistance in sugarcane. The recent development of techniques for gene transfer into sugarcane cells (Franks & Birch, 1991; Rathus & Birch, 1992), and production of transgenic sugarcane plants (Bower & Birch, 1992),

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Abbreviation: Alb^r, albicidin-resistant, albicidin resistance.

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creates an opportunity to test novel genes for disease resistance in this crop.

Albicidin is effective at very low concentrations against *E. coli* because it is rapidly concentrated within cells by illicit transport through the *tsx*-encoded outer-membrane channel normally involved in nucleoside uptake (Birch *et al.*, 1990). Although albicidin has a molecular mass three to four times that of common nucleosides, and partial characterization by proton and ^{13}C NMR spectroscopy reveals no apparent nucleoside moiety (Birch & Patil, 1985a), the compound apparently uses the nucleoside binding site of the *Tsx* channel for efficient flux across the *E. coli* outer membrane. Albicidin resistance results from loss of the mechanism of albicidin transport through the outer membrane (Birch *et al.*, 1990), and is accompanied by diminished capacity for *Tsx*-mediated deoxynucleoside uptake (Fsihi *et al.*, 1993). The mechanism of albicidin transport across the inner membrane is unknown.

A dominant *Alb^r* gene cloned from *Klebsiella oxytoca* encodes a 25 kDa protein which binds to albicidin *in vitro* to form a complex without antibiotic activity, but without catalysing further chemical modification of the albicidin. Binding of the antibiotic is transiently blocked by an excess of ATP, but not by other nucleosides or nucleotides (Walker *et al.*, 1988). This was the first example of antibiotic resistance due to a non-catalytic antibiotic binding protein. The normal function of the *Alb^r* protein in *K. oxytoca* remains unknown.

We report here that *Alcaligenes denitrificans* also has a gene for an albicidin binding protein which confers albicidin resistance when expressed in *E. coli*. The *A. denitrificans* gene is distinct from the one previously demonstrated in *K. oxytoca*, but shows a short region of amino acid homology at the N terminus, which appears to play a role in albicidin binding.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are given in Table 1. Albicidin-resistant (*Alb^r*) bacteria were isolated from diluted suspensions of soil from grassland at Samford, Queensland, by plating onto

Luria medium (LM; Miller, 1972) containing 100 ng albicidin ml^{-1} . Determinative tests were performed using routine procedures (Krieg & Holt, 1984) and API 20NE test strips.

Media and growth conditions. The nutrient medium used for *E. coli*, *A. denitrificans* and other isolated soil bacteria was LM. Where necessary this was solidified with 1.5% agar. Cultures of *E. coli* were incubated at 35 °C, and other bacteria at 29 °C. Broth cultures were aerated by shaking at 200 r.p.m. on an orbital shaker.

Inactivation of albicidin by isolated soil bacteria. Albicidins were produced and assayed as described previously (Birch *et al.*, 1990). The mixture of albicidins obtained after HW-40(S) chromatography was used in these experiments. Dose-response relationships to albicidin were determined as described previously (Birch & Patil, 1985b), except that basal layers were of LM agar, and the sample volume per well was 15 μl .

Albicidin remaining in culture supernatants was determined after overnight growth at 29 °C in LM broth containing 100 ng albicidin ml^{-1} . To test for production of extracellular products able to inactivate albicidin, isolates were grown in LM broth containing 100 ng albicidin ml^{-1} . Each day for 7 d, 15 μl of cell-free culture supernatant was removed, mixed with 50 ng albicidin and assayed for antibiotic activity. To measure intracellular accumulation of albicidin, cells grown as described above in the presence of albicidin were disrupted by sonication as described below, and assayed for antibiotic activity in the cell extract.

Cloning and sequencing of the *albB* gene. A genomic library of *A. denitrificans* SO-9 was prepared by cloning fragments from partial *Sau3A*I endonuclease digests of chromosomal DNA into the *Bam*HI site of cosmid pHCT9, followed by transfection into *E. coli* 294, using published protocols (Priefer *et al.*, 1984) and biochemicals from Promega. Recombinant clones were selected on LM agar containing carbenicillin. *Alb^r* clones were identified by patching on LM agar supplemented with albicidin, and T6 sensitivity was confirmed by cross streaking. Routine techniques were used for subcloning into pUC19 and pBluescriptII SK+ vectors (Sambrook *et al.*, 1989).

Two *Alb^r* clones (pSB6, pSB9) and five *Alb^s* clones obtained after Exonuclease III digestion (pSB6-I, pSB7, pSB8, pSB10, pSB11) were used to obtain the complete sequence from both strands of the isolated *albB* gene. For dideoxy sequencing (Chen & Seeburg, 1985), RNA-free, double-stranded, closed circular DNA templates were denatured in alkali and annealed to either T7 universal primer or reverse primer according to the

Table 1. Bacterial strains and plasmids

Plasmids constructed in this work are shown in Fig. 1.

Strain or plasmid	Genotype or feature	Reference or source
<i>A. denitrificans</i> SO-9	<i>Alb^r</i>	This work
<i>E. coli</i> DH5 α	$\text{F}^{-}\text{deoR endA1 gyrA96 hsdR17 (r}_k^-\text{m}_k^+) \Delta(\text{lacZYA-argF}) \text{U169 } (\phi 80\text{d lac}\Delta\text{M15})$	Sambrook <i>et al.</i> (1989)
<i>E. coli</i> LE 392	$\text{F}^{-}\text{galK2 galT22 hsdR514 (r}_k^-\text{m}_k^+) \text{lacY1 metB1 supE44 supF58 trpR55 } \lambda^{-}$	
<i>E. coli</i> QQM70	Prototrophic, <i>Val^r</i> wild type	Birch <i>et al.</i> (1990)
<i>E. coli</i> 294 RecA $^{-}$	$\text{F}^{-}\text{hsdR17 (r}_k^-\text{m}_k^+) \text{recA endA1 thi-1 supE44 } \lambda^{-}$	Uhlin <i>et al.</i> (1983)
Plasmid		
pBS SK+	<i>Ap^r</i> sequencing vector	Stratagene
pHC79	<i>Ap^r</i> <i>Tc^r</i> cosmid vector	Hohn & Collins (1980)
pUC19	<i>Ap^r</i> cloning vector	Vieira & Messing (1982)

manufacturer's instructions (Pharmacia: T7 sequencing kit), except that primer annealing was carried out for 10 min at 70 °C and gradually cooled to room temperature over 20 min to reduce secondary structures.

Preparation of cell extracts containing the *albB* gene product.

E. coli DH5 α carrying plasmid pUC19 or pSB4 was grown overnight at 37 °C then mixed with albicidin at a final concentration of 1.5 $\mu\text{g ml}^{-1}$ and incubated for a further 5 h. To examine the inducibility of albicidin resistance, cell extracts were collected at intervals after addition of albicidin. Cells were harvested from 25 ml of culture by centrifuging at 15000 *g* for 10 min, washed in 10 mM sodium phosphate buffer (pH 7.8) or TEMM extraction buffer [10 mM tris(hydroxymethyl)amino-methane pH 7.0, 10 mM EDTA, 10 mM MgCl₂ and 2 mM β -mercaptoethanol] and recentrifuged at 15000 *g* for 5 min. The cell pellet was resuspended in the same buffer to a final volume of 125 μl , and cells were disrupted by sonication on ice, with a microprobe at 50% duty cycle, applying 8 s sonication periods with an output of 25–45 W, for 6 min (Branson model 250 sonifier). Cell disruption was confirmed by phase contrast microscopy, and cell debris was removed by centrifugation at 15000 *g* for 30 min. Approximately 100 μl of sonicated cell supernatant (containing 10–12 $\mu\text{g protein } \mu\text{l}^{-1}$) was obtained from a 50 ml stationary phase culture, and stored at –20 °C in small samples for use as cell extract. Protein concentrations in cell extracts were measured by dye-binding (Bradford, 1976), using bovine serum albumin (BSA) for calibration.

Interaction of Alb^r cell extracts with albicidin. To test for ability to inactivate albicidin, cell extracts containing 10 μg total protein were mixed with 15–75 ng of albicidin in a final volume of 15 μl and antibacterial activity was assayed immediately. To test for heat-reversible binding of albicidin to the *albB* gene product, cell extract containing 100 μg total protein was mixed with 12 ng albicidin in a final volume of 30 μl . One half of the mixture was treated at 100 °C for 15 min, then both the boiled and unboiled samples were assayed for antibacterial activity. Mixtures were incubated for intervals up to 1 h before boiling to detect progressive inactivation, indicative of catalytic activity. Materials tested as cofactors for catalytic inactivation of albicidin by cell extracts were 1 mM ATP, 1 mM NAD⁺, 1 mM Mg²⁺ and 100 μM each of reducing agents dithiothreitol or β -mercaptoethanol.

Interaction of intact Alb^r cells with albicidin. To distinguish between binding and catalytic inactivation of albicidin in intact cells, overnight cultures of *E. coli* DH5 α (pSB6) and *A. denitrificans* SO-9 were mixed with an equal volume of LM containing albicidin. Samples were removed at intervals and placed on ice, or boiled for 15 min to release bound albicidin then placed on ice. At the end of the sampling period, all samples were assayed for remaining albicidin activity.

Isolation and characterization of the Alb^r protein. Denaturing one-dimensional (1D) PAGE was performed on 100 μg of cell extract, using linear acrylamide gradients [15–20% of 39% (w/v) acrylamide/1% (w/v) *N,N*-methylenebisacrylamide, 100 mM sucrose, 50 mM Tris/HCl, pH 8.3, 0.1% (w/v) LiDS, 0.05% (w/v) ammonium persulphate and 0.001% (v/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED)] with 8% acrylamide stacking gels on a slab gel apparatus (Bio-Rad Protean II) at a constant current of 12 mA for 16 h per gel at 4 °C (Ryrie, 1983). Molecular mass markers were bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (26 kDa), trypsinogen (24 kDa) and lysozyme (14.4 kDa). 2D-PAGE was performed as described by Holloway & Arundel (1988).

1D-gel electrophoresis of cell extracts under non-denaturing conditions was carried out on linear acrylamide gradients (3.5–27% of 39% acrylamide/1% *N,N*-methylenebisacrylamide) with 3.5% acrylamide stacking gels, without LiDS in the gel mix or electrophoresis buffers. The molecular mass markers were apoferritin (dimer 880 kDa; monomer 440 kDa) and BSA (dimer 132 kDa; monomer 66 kDa). Electrophoresis was carried out at a constant current of 12 mA for 16 h per gel at 4 °C. Novel bands from cell extracts from *E. coli* DH5 α (pSB4) were excised and assayed for the ability to inactivate albicidin.

Electrophoretic transfer onto PVDF membrane was carried out in a Hoefer electroblotting apparatus at a constant current of 60 V for 4 h at 15 °C. The desired protein band was excised from the PVDF membrane and the N-terminal protein sequence was determined by Edman degradation, using a gas-phase protein sequencer (Applied Biosystems model 407A) at the Brisbane Centre for Protein and Nucleic Acid Research.

Albicidin binding by a synthetic oligopeptide. An oligopeptide corresponding to the first 20 amino acids at the N terminus of the *albB* gene product was synthesized at the Brisbane Centre for Protein and Nucleic Acid Research, using 'tea bag' solid phase synthesis (Houghten, 1985). The product was 60–70% pure as judged by analytical HPLC, with the remainder consisting of various single amino acid deletions. Ability to inactivate albicidin was tested as described above for extracts.

RESULTS

Selection and identification of Alb^r bacteria

Of 19 Gram-negative Alb^r bacterial isolates from soil samples, six isolates substantially reduced albicidin activity in the culture medium during overnight incubation. Two isolates (SO-8 and SO-9) removed all albicidin from their culture medium during overnight growth and accumulated no intracellular albicidin, whereas all other isolates partially depleted albicidin in the growth medium and showed some albicidin within cells. No isolates were capable of extracellular detoxification of albicidin in young cultures. Two isolates (SO-8 and SO-9) were able to inactivate albicidin extracellularly in stationary phase cultures, and this capacity increased as cultures aged from 2 to 6 d. These two isolates were also most resistant to albicidin, with minimum inhibitory concentrations 10³-fold higher than *E. coli* Q358. Isolate SO-8 was resistant to kanamycin, ampicillin, tetracycline and rifampicin. Isolate SO-9 was sensitive to these antibiotics, and was chosen for further characterization.

The cells of isolate SO-9 from broth culture were Gram-negative, non-spore-forming rods, 0.5–0.7 μm in diameter and 1.8–2.5 μm in length, with 4–8 peritrichous flagella of 20–22 nm diameter. An inert reaction resulted in the test for oxidation or fermentation of glucose in Hugh and Leifson's medium. The organism was positive in tests for oxidase, reduction of nitrates to nitrites and N₂, metabolism of mannitol, gluconate, adipate, malate, citrate and phenyl acetate, and accumulation of intracellular polyhydroxybutyrate (PHB) under conditions of nitrogen starvation. The organism was negative in tests for indole formation, arginine dihydrolase, urease, β -galactosidase, protease, chitinase, esculin hydrolysis, metabolism of fructose, arabinose, mannose, *N*-acetyl glucosamine,

Fig. 1. Restriction endonuclease map of pSB4 and its derivatives. The cloning vector pBluescriptII SK+ (pUC19 for pSB4) is indicated by a heavy line. In all cases, except pSB4, the *lac* promoter would direct transcription into the insert from right to left. Albicidin resistance or sensitivity of *E. coli* DH5 α containing each plasmid and grown on medium without IPTG is indicated.

maltose, and caprate, and pigment production on King B medium. The G+C content of the DNA was 69.5–70 mol %. These features are sufficient for identification of isolate SO-9 as *A. denitrificans* subsp. *denitrificans* (Krieg & Holt, 1984). Ability to metabolize mannitol was the only feature in which isolate SO-9 differed from the species description.

Cloning of the Alb^r gene

An Alb^r clone containing a 27.7 kb insert in cosmid pH79 was recovered from a cosmid genomic library of *A. denitrificans* SO-9 in *E. coli* 294 RecA⁻. From this cosmid, a 2.3 kb fragment from digestion with *Eco*RI and *Hind*III was inserted into pUC19, to obtain pSB4 which conferred albidin resistance on *E. coli* DH5 α . A range of subclones of pSB4 was produced by digestion with *Eco*RI, *Hind*III, *Pvu*II and Exonuclease III, in the cloning vector pBluescript SK+ (Fig. 1). Each of these plasmids was transformed into *E. coli* DH5 α , and the sensitivity of each to albidin was determined (Table 2). All of these

clones remained sensitive to bacteriophage T6, indicating no interference with the Tsx pore involved with albidin uptake. The *albB* gene was located on a 1.1 kb *Eco*RI–*Pvu*II fragment from pSB4. Clones with deletions into this region allowed complete sequencing of both strands of the *albB* gene.

Albicidin inactivation by cell-free extracts from *E. coli* expressing the cloned *albB* gene

Antibiotic activity up to 30 ng of albidin was completely abolished when mixed with *E. coli* DH5 α (pSB4) cell extract (Alb^r) containing 10 μ g cellular protein prepared in TEMM buffer, but extracts prepared in sodium phosphate buffer failed to inactivate albidin. At higher albidin concentrations, there was no progressive antibiotic inactivation by cell extracts during extended incubation in the presence of the tested cofactors. Ability of cell extracts to inactivate albidin was abolished within 10 min at temperatures above 55 °C. Albicidin inactivation was not due to non-specific adsorption of the antibiotic to proteins, as only slight loss of antibiotic activity resulted in mixtures of albidin with cell extracts from *E. coli* DH5 α (pBluescript) (Fig. 2).

Albicidin is fairly heat stable, but albidin–protein mixtures generally show reduced activity upon boiling, probably due to loss of albidin in the denatured protein pellet (e.g. pBluescript + boiling in Fig. 2). In contrast, mixtures of albidin and cell extract from *E. coli* DH5 α (pSB4) or DH5 α (pSB12), which showed no measurable antibiotic activity before heating, showed substantially restored albidin activity upon boiling. This indicates release of albidin upon denaturation of a binding protein encoded by the *albB* gene cloned from *A. denitrificans*. Extended incubation of albidin in *E. coli* DH5 α (pSB12) cell extract before boiling did not result in any progressive decrease in recovered antibiotic activity upon boiling (Fig. 2), indicating no catalytic activity of the binding protein in albidin inactivation under these conditions.

Reversible albidin binding in intact cells

There was no evidence of catalytic inactivation of albidin in intact cells expressing the *albB* gene. Albicidin was rapidly removed from the culture medium by *E. coli* DH5 α (pSB6), but antibiotic activity was recovered by boiling the cells, 5 h after removal of all activity from the culture medium. *A. denitrificans* SO-9 removed albidin from the culture medium more slowly, but boiling also released albidin from these cells after 5 h incubation.

Isolation and characterization of the albidin resistance gene product

A single novel protein band was present in the cell extracts of *E. coli* DH5 α (pSB4) but absent in cell extracts of DH5 α (pUC19) after 1D-LiDS-PAGE. This novel protein appeared as a single spot at 23 kDa after 2D-PAGE, and as a 23 kDa band after native PAGE (Fig. 3). When protein bands were excised from the native PAGE gel, only the gel fragment containing the 23 kDa protein

Table 2. Sensitivity to albicidin of *A. denitrificans* SO-9 and of *E. coli* DH5 α containing subclones of the SO-9 *albB* gene

Sensitivity of bacterial strains to albicidin was measured as zone width (mm) surrounding a 4 mm diameter well containing albicidin at the concentrations given ($\mu\text{g ml}^{-1}$). Data are means of two replicates. All plasmids were contained in *E. coli* DH5 α . NT, not tested.

Clone or strain	IPTG	Sensitivity to albicidin doses					
		5.0	3.0	1.0	0.5	0.1	0.01
<i>E. coli</i> DH5 α	$\pm$	NT	NT	8.7	7.6	4.4	2.8
pSB4	—	2.0	0	0	0	0	0
pSB6	—	2.2	0	0	0	0	0
pSB6-I	+	4.0	3.5	1.0	0.2	0	0
pSB6-II	—	NT	NT	8.5	7.2	4.5	2.5
pSB7	$\pm$	NT	NT	8.4	5.9	3.7	2.1
pSB8	$\pm$	NT	NT	8.6	7.1	4.0	2.5
pSB9	—	2.0	0	0	0	0	0
pSB10	$\pm$	NT	NT	8.4	7.2	3.6	2.2
pSB11	$\pm$	NT	NT	8.7	7.4	4.3	2.8
pSB12	—	2.0	0	0	0	0	0
<i>A. denitrificans</i> SO-9	—	2.2	1.3	0	0	0	0

Fig. 2. Effect of extended incubation on reversible inactivation of albicidin by extracts of cells expressing the *albB* gene. Open symbols indicate samples assayed without boiling, closed symbols indicate samples boiled then assayed. Circles, TEMM buffer control; triangles, *E. coli* DH5 α (pBluescript) control; squares, *E. coli* DH5 α (pSB12) (expressing *albB* gene).

was able to inactivate albicidin. The similar protein band obtained under denaturing and native conditions indicates that the albicidin resistance protein is a monomer consisting of a single subunit. The first 26 amino acid residues from the N terminus of the isolated 23 kDa protein, revealed by Edman degradation, were Met-Tyr-Asp-Lys-Tyr-Phe-Ser-Arg-Glu-Glu-Leu-Ala-Arg-Leu-Pro-Leu-Tyr-Thr-Arg-Gly-Pro-Asp-Gly-Asp-Pro-Ser. This N-terminal sequence does not have the general features of a signal peptide. The full amino acid composition deduced

Fig. 3. LiDS-PAGE of protein extracts from cells of *E. coli* DH5 α . Lanes: 1, molecular mass markers (kDa); 2–15, protein extracts of *E. coli* DH5 α containing pSB4 (2–8) or pUC19 (9–15) prepared at intervals of 0, 1, 2, 3, 4, 5 and 6 h after exposure to albicidin. Lanes 9 and 10 were loaded with 80 μg total protein. All other lanes were loaded with 100 μg total protein. The position of the Alb^r protein is indicated by an arrowhead.

from the nucleotide sequence of the cloned gene (Fig. 4) contained 16.85% hydrophobic residues (Kyte & Doolittle, 1982) which were mostly scattered from the middle to the end of the sequence. The predominantly hydrophilic character and the absence of a signal peptide indicate an intracellular protein (Lewin, 1990).

Albicidin-induced accumulation of the resistance gene product

Cell-free extracts from *E. coli* DH5 α (pSB4) cultures exposed to 1.5 $\mu\text{g ml}^{-1}$ albicidin for 6 h during early stationary phase showed approximately fivefold greater

	-60	-50	-40	-30	-20	-10	
	*	*	*	*	*	*	
	CTA	CCTCAGGCCT	GGATGCTCCA	TCCGCGAACC	GTTTCGATGCA	GGCTGCTGGA	GATGATGACT
						SD	
	10	20	30	40	50	60	
	*	*	*	*	*	*	
ATG	TAT	GAT	AAA	TAT	TTT	TCC	CGA
Met	Tyr	Asp	Lys	Tyr	Phe	Ser	Arg
	70	80	90	100	110	120	
	*	*	*	*	*	*	
CCG	GAC	GGG	GAC	CCG	AGT	GGC	TTG
Pro	Asp	Gly	Asp	Pro	Ser	Gly	Leu
	130	140	150	160	170	180	
	*	*	*	*	*	*	
GGC	CAG	GGA	GGC	TAT	ACC	GCG	CCA
Gly	Gln	Gly	Gly	Tyr	Thr	Ala	Pro
	190	200	210	220	230	240	
	*	*	*	*	*	*	
CAA	CGG	CGA	TCC	GCG	CTT	GTT	GGT
Gln	Arg	Arg	Ser	Ala	Leu	Val	Gly
	250	260	270	280	290	300	
	*	*	*	*	*	*	
GCA	GAC	GCA	CGT	GCA	TTT	CCA	AAG
Ala	Asp	Ala	Arg	Ala	Phe	Pro	Lys
	310	320	330	340	350	360	
	*	*	*	*	*	*	
CAA	GCT	GCT	TAT	CTA	CGA	AAA	ATA
Gln	Ala	Ala	Tyr	Leu	Arg	Lys	Ile
	370	380	390	400	410	420	
	*	*	*	*	*	*	
CGC	AGC	ATT	ACT	GCG	ACA	GAT	CCT
Arg	Ser	Ile	Thr	Ala	Thr	Asp	Pro
	430	440	450	460	470	480	
	*	*	*	*	*	*	
TTG	TTC	CGC	GCC	CAC	TCG	AAA	CCG
Leu	Phe	Ala	Ala	His	Ser	Lys	Pro
	490	500	510	520	530	540	
	*	*	*	*	*	*	
TCG	TAT	GCG	GGC	AAG	AAC	CCG	GCC
Ser	Tyr	Ala	Gly	Lys	Asn	Pro	Ala
	550	560	570	580	590	600	
	*	*	*	*	*	*	
CCC	GAC	TTG	TCC	GCT	GGC	ACA	TGG
Pro	Asp	Leu	Ser	Ala	Gly	Thr	Trp
	610	620	630	640	650	660	670
	*	*	*	*	*	*	*
GAT	GGG	ACC	CTA	GCG	AAG	TAG	CGAAGTAGC
Asp	Gly	Thr	Leu	Ala	Lys	---	---
	680	690	700	710	720	730	740
	*	*	*	*	*	*	*
CAAGTGGCGA	AACTCATCGC	CGCCGCAATA	CGACGACGTT	CATGCCAGAG	CACCCTTGTG	GCATCGGTCA	
	750	760					
	*	*					
TGTAAGATTG	CGTTCGTTGC	TGGGCGGT					

Fig. 4. Nucleotide sequence of the *albB* gene and the predicted amino acid sequence of its gene product. The sequence is numbered commencing at the first base of the translation initiation codon; bases 5' to the initiation codon have negative numbers. The core regions of the putative promoter are underlined in the -20 to -60 region, as is a SD sequence from -10 to -13. A region of dyad symmetry is indicated at nucleotides 641-661 (underlined).

albicidin inactivation than extracts with the same total protein content from parallel cultures of DH5 α (pSB4) exposed to albicidin for only the final hour of incubation. Native PAGE revealed a corresponding increase in the cellular concentration of *albB* gene product with increasing time of exposure to albicidin (Fig. 3).

Nucleotide sequence of the *albB* gene

The *EcoRI*/*PvuII* fragment of pSB12 contained a 620 bp ORF that commenced about 0.1 kb from the *EcoRI* site. A potential ribosome-binding site with a GGAG Shine-

Dalgarno (SD) sequence (Shine & Dalgarno, 1974; Stormo *et al.*, 1982) was found 10 bp upstream from an ATG assumed to act as the initiation codon (Fig. 4). The N-terminal amino acid sequence of the 23 kDa albicidin resistance protein produced by pSB4 corresponds to that predicted from this ORF. However, there are two additional in-frame ATG codons immediately downstream of the SD sequence, and another ORF commences at the ATG at base 5 and continues to base 592 in Fig. 4. Expression of the cloned gene in pSB4 indicates that there may be an effective promoter within the 100 bp of cloned DNA upstream of the SD sequence, as there is no

Fig. 5. Comparison of 22 amino acids in the N-terminal sequences of the *A. denitrificans* *albB* gene (B), and the *K. oxytoca* *albA* gene (A). Symbols: double dot, identical amino acids; single dot, amino acids with similar properties in their side chains.

recorded promoter activity in this direction in the polylinker of pUC19. The putative *A. denitrificans* promoter region did not conform well to the -35 and -10 consensus sequences for *E. coli* promoters, the best match being CTGGAT and TTCGAT (underlined in Fig. 4), centred approximately 50 and 27 bp, respectively, upstream of the start codon of the *A. denitrificans* gene.

A region of GC-rich dyad symmetry (Platt, 1986) occurred 20 bp downstream from the termination codon TAG. This region would yield a potential hairpin loop structure of low stability [$-2.5 \text{ kcal mol}^{-1}$ ($-10.46 \text{ kJ mol}^{-1}$)] in the mRNA, but is not followed by consecutive thymidine residues or the TCTG consensus sequence characteristic of many factor-independent termination sites (Brendel & Trifinov, 1984).

Computer analysis of the bacterial, plant and organelle genes in the GenBank database (Release 63) revealed no genes with close homology to the *albB* gene. When the computer analysis was conducted to allow regions to be realigned, there were regions of partial DNA homology between the Alb^r genes from *A. denitrificans* and *K. oxytoca*. However, these regions are separated by deletions, insertions and frame-shifts so that translation results in dissimilar proteins.

Computer analysis of the NBRF protein sequence database (Release 40.0) revealed 62.5% homology at the N terminus of Alb^r proteins of *A. denitrificans* and *K. oxytoca*. Fifteen of the first 16 amino acids of these albicidin binding proteins are either identical or have side chains with similar properties (Fig. 5). However, comparison of the entire sequence did not show even distant homology between the *A. denitrificans* Alb^r protein and the *K. oxytoca* Alb^r protein or any other protein (Lipman & Pearson, 1985). Also, five of the eight identical amino acids at the N terminus of the two Alb^r proteins used different codons.

Deletion analysis of the promoter and N terminus regions

To test the role of the possible promoter region between -24 and -52 bp, deletions were made in this region of a subclone in plasmid vector pBluescriptII SK+. Two surprising results emerged. Firstly, deletion to -14 bp (which removes the putative *A. denitrificans* promoter) did not affect albicidin resistance (pSB6). Secondly, deletion

Fig. 6. Deletions introduced into the promoter region of the *albB* gene, and their effects on sensitivity to albicidin. The nucleotide sequence of the promoter region is shown at the top. Nucleotides are numbered from the translation-initiation site. Underlined upstream sequences indicate a putative SD sequence and regions of homology to prokaryote promoter consensus sequences. Lengths of the deleted clones after Exonuclease III digestions are shown by the lengths of the bars. *E. coli* DH5 α (pSB6-I) was resistant to albicidin only in the presence of IPTG, indicating transcription from the *lac* promoter.

to - 8 bp (which also removes the putative promoter and SD sequence) abolished albicidin resistance, but resistance was partially restored when IPTG was added to induce the adjacent *lac* promoter (pSB6-1) (Fig. 6, Table 2). Sequencing across the vector/insert junctions showed that plasmid pSB6 carries the complete structural gene for the Alb^r protein and its SD sequence as an out-of-frame transcriptional fusion behind the *lac* promoter. Thus the low level of constitutive expression from the *lac* promoter results in sufficient production of the Alb^r protein to confer resistance.

Plasmid pSB0-I carries an in-frame translational fusion between the N terminus of the *lac* gene and the *albB* gene, driven by the *lac* promoter. Since it is in-frame with the *lac* gene, an additional 20 amino acids have been added to the N terminus of the normal Alb^r gene product. The presence of these additional amino acids apparently interferes with the albicidin binding property of the gene product, so that albicidin resistance does not result from the low level of constitutive expression from the *lac* promoter. However, when the *lac* promoter is induced by IPTG, overproduction of the fusion protein partially restores albicidin resistance, possibly because the fusion protein has some residual albicidin binding activity, or due to removal of N-terminal amino acids by intracellular protease activity, releasing a small amount of the effective Alb^r protein. This is consistent with the indication from amino acid homology between *A. denitrificans* and *K. oxytoca* Alb^r genes that the conserved N terminus may be important for albicidin binding.

The deletion in plasmid pSB7 extended to nucleotide 174 in the *albB* gene and an out-of-frame fusion, accounting for the loss of albicidin resistance. With pSB8, the gene product is an in-frame translational fusion with 17 amino acids from the *lacZ* gene product fused to amino acid 86 of the *albB* gene product. This fusion protein lacked the conserved N-terminal amino acids, and conveyed no albicidin resistance even when the *lac* promoter was induced by IPTG. This result is again consistent with the importance of the N-terminal region for albicidin binding.

Table 3. Binding of albicidin by the *albB* gene product and a synthetic N terminus oligopeptide

Results are means of two replicates at each albicidin concentration.

Treatment		Percentage inactivation of albicidin		
		400 ng albicidin ml ⁻¹	200 ng albicidin ml ⁻¹	100 ng albicidin ml ⁻¹
H ₂ O control		0	0	0
pSB12 cell extract	(4.8 mg ml ⁻¹)	100	100	100
Oligopeptide	(4.5 mg ml ⁻¹)	93	94	95
Oligopeptide	(0.45 mg ml ⁻¹)	50	56	50

Albicidin binding by a synthetic oligopeptide

As a further test of the role of the conserved N terminus of the albicidin binding proteins from *A. denitrificans* and *K. oxytoca*, we tested the ability of a synthetic 20 amino acid oligopeptide MYDKYFSREELARLPLYTRG (identical to the N terminus of the *A. denitrificans* protein) to bind albicidin. At equivalent albicidin-protein ratios, albicidin binding by this oligopeptide was almost as effective as cell extracts from *E. coli* DH5 α (pSB12) (Table 3). However, based on visual estimates of band intensity on polyacrylamide gels (Fig. 3), the *albB* gene product constituted approximately 10% of total protein in these cell extracts. Therefore on a molar basis, binding by the synthetic oligopeptide was less efficient than for the full protein in cell extracts (Table 3).

DISCUSSION

Albicidin-resistant *A. denitrificans* isolated from soil carried a dominant gene which conferred albicidin resistance when expressed in *E. coli*. The isolated product of the resistance gene was a 23 kDa protein which corresponded in molecular mass and N-terminal sequence to the predicted 206 amino acid polypeptide translated from the nucleotide sequence of the cloned gene. This protein was able to inactivate albicidin *in vitro* when recovered from non-denaturing gels. The protein lacked a signal sequence for transport across the cytoplasmic membrane (von Heijne, 1983). This may explain the presence of extra-cellular albicidin inactivation only in older cultures of *A. denitrificans*, where lysing cells would release some of the protein into the medium.

The Alb^r protein from *A. denitrificans* appeared to inactivate albicidin by binding rather than covalently modifying the antibiotic, as previously observed with *K. oxytoca* (Walker *et al.*, 1988). In both cases, a reducing agent was required for binding and albicidin inactivation was reversible following heat treatment of the albicidin-protein mixture. This indicates reversible binding of the resistance gene product to albicidin, to form a complex without antibiotic activity. Since albicidin is relatively heat stable, when the bound Alb^r protein is denatured by heat treatment, most of the antibiotic activity is restored.

The possibility that binding is a pre-requisite to catalytic inactivation of albicidin has not previously been tested. Albicidin binding by the *A. denitrificans* Alb^r protein in cell extracts was reversible by heat even after extended incubation (Fig. 2), and there was no progressive inactivation at albicidin-protein ratios resulting in some remaining antibiotic activity from unbound albicidin. This indicates no catalytic inactivation of albicidin under these assay conditions, but does not exclude the possibility that the gene product is an enzyme with a required cofactor lost during preparation of the cell extracts. To test this possibility, we measured albicidin recovery upon heating intact cells which had removed albicidin from the culture medium. Reversible albicidin binding, even after extended incubation in intact cells of *E. coli* DH5 α (pSB6) and *A. denitrificans* SO-9, confirmed the non-catalytic action of the *A. denitrificans* Alb^r protein.

The regions of partial DNA sequence homology between the *A. denitrificans* and *K. oxytoca* Alb^r genes indicate that there is an evolutionary relationship between the genes. However, sequence rearrangements have resulted in loss of homology at the amino acid level, except for a region of 16 amino acids at the N terminus, and codon usage is different even for identical amino acids of the two proteins in this region. There are also differences in the organization of the two Alb^r genes. The *K. oxytoca albA* gene occurs in an operon, downstream of a gene for a 36 kDa protein of unknown function (Walker *et al.*, 1988), whereas in *A. denitrificans* the *albB* gene occurs immediately downstream of the promoter and overlaps a second ORF of unknown function for most of its length.

The sequence of the *A. denitrificans* promoter region deviates considerably from the canonical *E. coli* promoter. Exposure of *E. coli* DH5 α (pSB4) cells to albicidin resulted in a fivefold increase in albicidin inactivating ability within 6 h, paralleled by an increase in the concentration of the 23 kDa protein in cell extracts (Fig. 3). Because other plasmid-encoded proteins did not increase during this treatment, the effect was not due to plasmid amplification but rather to specific albicidin-induced production of the Alb^r protein. We have not determined whether this induction involves regulation of transcription, translation, or protein stability.

After a 6 h exposure to albicidin, the Alb^r protein was estimated to comprise approximately 10% of the total protein in the *E. coli* DH5 α (pSB4) cell extract, based on band intensity after PAGE. On this basis, the observed inactivation of 30 ng of albicidin (842 Da) by 10 μ g of cellular protein (10% Alb^r protein of 23 kDa) occurred at a molar ratio of 1 albicidin:1 Alb^r protein.

We speculated that the amino acid homology in the N terminus of the Alb^r proteins of *A. denitrificans* and *K. oxytoca* (Fig. 5) could indicate a conserved albicidin binding domain. Experiments with translational fusions and deletions confirmed the importance of the N terminus of the *A. denitrificans* Alb^r protein for albicidin resistance (pSB6 to pSB8, Fig. 6, Table 2). A synthetic oligopeptide of 20 amino acids from the N terminus of the *A. denitrificans* Alb^r protein also bound albicidin, but with lower efficiency than the full protein.

The normal role of the *A. denitrificans* *albB* gene is unknown, as we do not expect this organism to be naturally exposed to albicidin. The fate of bound albicidin in cells is also unknown. However, it is clear that binding can provide a high level of resistance to the antibiotic. Although albicidins are extremely potent antibacterial agents, effective at concentrations around 10 ng ml⁻¹ (Birch & Patil, 1985b), this depends on active accumulation by sensitive cells, resulting in much higher intracellular antibiotic concentrations (Birch *et al.*, 1990). Expression of a binding protein with a high affinity for the antibiotic presumably keeps the free albicidin concentration in the cytoplasm below the level for lethal interaction with its target. Antibiotic-induced accumulation of the resistance gene product is clearly advantageous for this purpose, although the natural inducer and target of this intriguing mechanism in *A. denitrificans* is presumably a substance other than albicidin.

This detoxifying mechanism is encoded by a single gene, does not require complex cofactors for activity, operates rapidly and efficiently within cells, and results in a product of little or no toxicity. Thus, the *albB* gene seems suitable to be cloned into a suitable plant expression vector and transformed into sugarcane. Constitutive expression of the gene, and production of the corresponding protein in the cytosol should result in binding and inactivation of albicidin before it reaches its target in the plastids. This should allow a test of the hypothesis that albicidin sensitivity is important for leaf scald disease sensitivity in sugarcane. In the longer term, the gene might be expressed from an albicidin-inducible promoter to convey efficient resistance in sugarcane against leaf scald disease caused by albicidin-producing strains of *X. albilineans*.

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