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

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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.

Ablcicidin 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 (Albr) 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
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 (Fshi et al., 1993). The mechanism of albicidin transport across the outer membrane is unknown.

A dominant Alb$^\beta$ 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 antibiotic. 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$^\beta$ 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$^\beta$) 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 $^\circ$C, and other bacteria at 29 $^\circ$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 $\mu$L.

Alicicidin remaining in culture supernatants was determined after overnight growth at 29 $^\circ$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 $\mu$L 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 alb$\beta$ gene.** A genomic library of *A. denitrificans* SO-9 was prepared by cloning fragments from partial *SalIII* endonuclease digests of chromosomal DNA into the *BamHI* site of cosmid pH79, 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$^\beta$ 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$^\beta$ clones (pSB6, pSB9) and five Alb$^\alpha$ clones obtained after Exonuclease III digestion (pSB6-1, pSB7, pSB8, pSB10, pSB11) were used to obtain the complete sequence from both strands of the isolated alb$\beta$ 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

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**Table 1. Bacterial strains and plasmids**

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or feature</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><em>A. denitrificans</em> SO-9</td>
<td>Alb$^\beta$</td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em> DH5a</td>
<td>F$^+$*deoR end:A1 gyrA96 hisD17 (rpsL15,mcrA) Δ(lacZΔM15) recA1 relA1 supE44 thi-1 $\lambda^-$</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td><em>E. coli</em> LE 392</td>
<td>F$^+$galK2 galT22 hisD154 (rpsL15, mcrA) lacY1 metB1 supE44 supF58 trpR55 $\lambda^-$</td>
<td>Birch et al. (1990)</td>
</tr>
<tr>
<td><em>E. coli</em> UQM70</td>
<td>Protophytic, Val$^+$ wild type</td>
<td>Uhlin et al. (1983)</td>
</tr>
<tr>
<td><em>E. coli</em> 294 RecA$^-$</td>
<td>F$^+$hisD17 (rpsL15, mcrA) recA end:A1 thi-1 supE44 $\lambda^-$</td>
<td></td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBS SK +</td>
<td>Ap$^+$ sequencing vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pHC79</td>
<td>Ap$^+$ 'Tc' cosmid vector</td>
<td>Hohn &amp; Collins (1980)</td>
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manuscript'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* DH5α carrying plasmid pUC19 or pSB4 was grown overnight at 37 °C then mixed with albicidin at a final concentration of 1.5 μg 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 15,000 g for 10 min, washed in 10 mM sodium phosphate buffer (pH 7.8) or TEMED 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 15,000 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 15,000 g for 30 min. Approximately 100 μl of sonicated cell supernatant (containing 10–12 μg protein μl⁻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⁺ 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⁺ cells with albicidin.** To distinguish between binding and catalytic inactivation of albicidin in intact cells, overnight cultures of *E. coli* DH5α(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⁺ 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/HC1, 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 (662 kDa), ovalbumin (427 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 (35–27% 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 apoferitin (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* DH5α(pSB4) were excised and assayed for the ability to inactivate albicidin.

Electrophoretic transfer onto PVDF membrane was carried out in a Hoeffer electrophorat 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⁺ bacteria**

Of 19 Gram-negative Alb⁺ 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,
Cloning of the Alb\(^{+}\) gene

An Alb\(^{+}\) clone containing a 27.7 kb insert in cosmid pH79 was recovered from a cosmid genomic library of \(A.\) \textit{denitrificans} SO-9 in \(E.\) \textit{coli} 294 RecA\(^{+}\). From this cosmid, a 23 kb fragment from digestion with \(EcoRI\) and \(HindIII\) was inserted into pUC19, to obtain pSB4 which conferred albicidin resistance on \(E.\) \textit{coli} DH5\(\alpha\). A range of subclones of pSB4 was produced by digestion with \(EcoRI\), \(HindIII\), \(PvuII\) and Exonuclease III, in the cloning vector pBluescript SK\(+\) (Fig. 1). Each of these plasmids was transformed into \(E.\) \textit{coli} DH5\(\alpha\), and the sensitivity of each to albicidin was determined (Table 2). All of these clones remained sensitive to bacteriophage T6, indicating no interference with the Tsx pore involved with albicidin uptake. The \(albB\) gene was located on a 1.1 kb \(EcoRI-PvuII\) 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 \(E.\) \textit{coli} expressing the cloned \(albB\) gene**

Antibiotic activity up to 30 ng of albicidin was completely abolished when mixed with \(E.\) \textit{coli} DH5\(\alpha\)(pSB4) cell extract (Alb\(^{+}\)) containing 10 \(\mu\)g cellular protein prepared in TEMM buffer, but extracts prepared in sodium phosphate buffer failed to inactivate albicidin. At higher albicidin 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 albicidin was abolished within 10 min at temperatures above 55 \({}^\circ\)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 albicidin with cell extracts from \(E.\) \textit{coli} DH5\(\alpha\)(pBluescript) (Fig. 2).

Albicidin is fairly heat stable, but albicidin–protein mixtures generally show reduced activity upon boiling, probably due to loss of albicidin in the denatured protein pellet (e.g. pBluescript + boiling in Fig. 2). In contrast, mixtures of albicidin and cell extract from \(E.\) \textit{coli} DH5\(\alpha\)(pSB4) or DH5\(\alpha\)(pSB12), which showed no measurable antibiotic activity before heating, showed substantially restored albicidin activity upon boiling. This indicates release of albicidin upon denaturation of a binding protein encoded by the \(albB\) gene cloned from \(A.\) \textit{denitrificans}. Extended incubation of albicidin in \(E.\) \textit{coli} DH5\(\alpha\)(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 albicidin inactivation under these conditions.

**Reversible albicidin binding in intact cells**

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

**Isolation and characterization of the albicidin resistance gene product**

A single novel protein band was present in the cell extracts of \(E.\) \textit{coli} DH5\(\alpha\)(pSB4) but absent in cell extracts of DH5\(\alpha\)(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 DH5α 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 (µg ml⁻¹). Data are means of two replicates. All plasmids were contained in E. coli DH5α. NT, not tested.

<table>
<thead>
<tr>
<th>Clone or strain</th>
<th>IPTG</th>
<th>Sensitivity to albicidin doses</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>5.0</td>
<td>3.0</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>±</td>
<td>NT</td>
</tr>
<tr>
<td>pSB4</td>
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<tr>
<td>pSB6</td>
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<tr>
<td>pSB6-1</td>
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<td>NT</td>
</tr>
<tr>
<td>pSB7</td>
<td>±</td>
<td>NT</td>
</tr>
<tr>
<td>pSB8</td>
<td>±</td>
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<td>pSB12</td>
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</table>

**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 DH5α(pBluescript) control; squares, E. coli DH5α(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-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 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).

**Fig. 3.** LIDS-PAGE of protein extracts from cells of E. coli DH5α. Lanes: 1, molecular mass markers (kDa); 2-15, protein extracts of E. coli DH5α 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' protein is indicated by an arrowhead.

**Albicidin-induced accumulation of the resistance gene product**

Cell-free extracts from E. coli DH5α(pSB4) cultures exposed to 1.5 µg albicidin ml⁻¹ for 6 h during early stationary phase showed approximately fivefold greater...
albicidin inactivation than extracts with the same total protein content from parallel cultures of DH5α(pSB4) exposed to albicidin for only the final hour of incubation. Native PAGE revealed a corresponding increase in the cellular concentration of \( \text{albB} \) gene product with increasing time of exposure to albicidin (Fig. 3).

**Nucleotide sequence of the \( \text{albB} \) gene**

The \( \text{EcoRI/PvuII} \) fragment of pSB12 contained a 620 bp ORF that commenced about 0.1 kb from the \( \text{EcoRI} \) 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
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 kcal mol−1 (−10.46 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' 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' 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' protein and the K. oxytoca Alb' protein or any other protein (Lipman & Pearson, 1985). Also, five of the eight identical amino acids at the N terminus of the two Alb' 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 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-I) (Fig. 6, Table 2). Sequencing across the vector/insert junctions showed that plasmid pSB6 carries the complete structural gene for the Alb' 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' protein to confer resistance.

Plasmid pSB6-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' 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' protein. This is consistent with the indication from amino acid homology between A. denitrificans and K. oxytoca Alb' 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage inactivation of albicidin</th>
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<tbody>
<tr>
<td></td>
<td>400 ng albicidin ml⁻¹</td>
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<tr>
<td>H₂O control</td>
<td>0</td>
</tr>
<tr>
<td>pSB12 cell extract</td>
<td>(4.8 mg ml⁻¹)</td>
</tr>
<tr>
<td>Oligopeptide</td>
<td>(4.5 mg ml⁻¹)</td>
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<tr>
<td>Oligopeptide</td>
<td>(0.45 mg ml⁻¹)</td>
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</table>

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* DH5α(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 extracellular albicidin inactivation only in older cultures of *A. denitrificans*, where lysing cells would release some of the protein into the medium.

The Alb' 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' 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' 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* DH5α(pSB6) and *A. denitrificans* SO-9, confirmed the non-catalytic action of the *A. denitrificans* Alb' protein.

The regions of partial DNA sequence homology between the *A. denitrificans* and *K. oxytoca* Alb' 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' 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* DH5α(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' protein. We have not determined whether this induction involves regulation of transcription, translation, or protein stability.

558
After a 6 h exposure to albicidin, the Alb \(^{B}\) protein was estimated to comprise approximately 10% of the total protein in the \(E. \) coli DH5\(\alpha\)(pSB4) cell extract, based on band intensity after PAGE. On this basis, the observed inactivation of 30 ng of albicidin (842 Da) by 10 \(\mu\)g of cellular protein (10 \% Alb\(^{B}\) protein of 23 kDa) occurred at a molar ratio of 1 Alb\(^{B}\) protein.

We speculated that the amino acid homology in the N terminus of the Alb\(^{B}\) 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\(^{B}\) 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\(^{B}\) protein also bound albicidin, but with lower efficiency than the full protein.

The normal role of the \(A. \) denitrificans \(a b \) 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 \(a b \) 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 cytoplasm 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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REFERENCES


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