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Genetic Analysis of Albicidin Resistance in Bacteria

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ABSTRACT. *Albicidins produced by sugarcane leaf scald pathogen Xanthomonas albilineans selectively inhibit prokaryotic DNA synthesis without binding to or damaging DNA (Birch and Patil, 1985). Therefore, bacterial genes for albicidin resistance were sought by selecting for resistant mutants of Escherichia coli, and by screening soil microbial populations for resistance. Albicidin resistant mutants of E. coli. revealed that stable albicidin resistance occurred by loss of nucleoside transport mechanism involved in rapid, illicit, intracellular accumulation of albicidin. This mechanism is encoded by the tsx gene which is responsible for an outer membrane pore of E. coli. A gram-negative soil bacterium resistant to albicidin was isolated and identified as Alcaligenes denitrificans and determined to be a potential source of detoxifying ability. In vivo cloning experiments revealed that albicidin resistance was dominant, carried on a single genomic fragment, and expressed in E. coli. without any effect on nucleoside uptake associated with spontaneous albicidin resistance in E. coli. The albicidin resistance gene product was heat sensitive and inactivated albicidin rapidly by reversible binding to albicidin to form a complex without antibiotic activity. Southern hybridization experiments did not show cross hybridization of alb' gene from A. denitrificans with the other available alb' genes tested. When 'Gen Bank' and 'NBRF' data bases were used to detect any homology with other sequenced genes at nucleotide level or amino acid level, Klebsiella oxytoca gene for albicidin resistance (Walker et al., 1988) emerged homologous after realigning the regions of genes. A homology of 62.5% between the amino-terminal sequences of alb' proteins from K. oxytoca and A. denitrificans suggested that this may be a conserved region for albicidin binding.*

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INTRODUCTION

The xylem-invading sugarcane pathogen, *Xanthomonas albilineans*, produces a family of toxins, albicidins *in vitro*, which are bactericidal at low concentrations to a range of bacteria by inhibiting prokaryote DNA replication. In uninvaded chlorotic tissues of leaf scald diseased sugarcane plastids are present as proplastids, etioplasts and vesicular forms smaller than chloroplasts (Birch and Patil, 1983). Since chloroplasts appear to use prokaryotic mechanisms of DNA replication, transcription and translation, it was postulated that albicidin or a structural analogue produced in diseased plants may be responsible for blocked chloroplast differentiation (Birch and Patil, 1983 & 1985).

Many naturally occurring microorganisms are known to possess various mechanisms capable of converting herbicides, phytotoxins, or antibiotics into non-toxic compounds (Amy *et al.*, 1985; Ingvorsen *et al.*, 1991). Since the phytotoxin albicidin is an antibacterial compound, bacteria resistant to albicidin may have suitable mechanisms which can be utilized in transgenic sugarcane plants to provide leaf scald resistance. Previous studies based on linkage to known markers in conjugative transfer, a locus (*alb*) controlling albicidin resistance was mapped at 9 to 10 min on the *E. coli*. chromosome. P1 transduction experiments for precise mapping of the alb locus indicated that the alb gene was located at the *tsx* locus, and all the *alb*^{r} transductants were observed to be phage T6 resistant (Birch *et al.*, 1990). The *tsx* gene codes for the phage T6 receptor protein of *E. coli.*, which is an outer membrane protein involved in the transport of all nucleosides and deoxynucleosides, except cytidine and deoxycytidine (Krieger-Brauer and Braun, 1980).

The objective of this study was to detect and analyse bacterial genes for albicidin resistance which can provide leaf scald disease resistance to transgenic sugarcane plants.

MATERIALS AND METHODS

Bacterial and phage strains used in this study are given in Appendix I. Albicidins produced in culture by *Xanthomonas albilineans* strain LS155 were purified as described previously (Birch and Patil, 1985), and were used in these experiments. Resistance to phage T6 was tested by cross-streaking bacteria against phage T6 on Z agar plates (Gerhardt, 1981).

Albicidin resistance mechanism in *E. coli.*

Albicidin-resistant (Alb^r) mutants of *E. coli.* strains were selected by plating untreated cell suspensions onto Z agar containing 100ng albicidin ml^{-1}. All the Alb^r mutants were tested for phage T6 resistance as described above. *E. coli.* strains (Appendix I) grown in Luria broth at 37°C for approximately 4h were harvested and resuspended in 0.01 times the original culture volume of the minimal medium (MGM) containing 100ng albicidin ml^{-1} (Miller, 1972). At intervals after addition of albicidin, 0.2ml samples were removed and immediately centrifuged at 15000g for 1min. Albicidin activity remaining in the supernatant was determined for each sample as described previously (Birch and Patil, 1985) except that *E. coli.* UQM70 was used as the assay strain.

Albicidin resistance mechanism in soil bacteria

Albicidin resistant bacteria were isolated from a diluted suspension of soil from grassland at Samford, Queensland, Australia, by plating on to Luria medium containing 100ng ml^{-1} albicidin. The bacteria resistant to albicidin were tested for phage T6 resistance as described above, and only the phage T6 resistant bacteria were used for further experiments.

Uptake, inactivation and intracellular accumulation of albicidin

Selected organisms were grown overnight at 29°C in LM broth containing 100ng albicidin ml^{-1}. Albicidin remaining in the culture supernatants was assayed as described by Birch and Patil (1985), with the exception of UQM70 as the albicidin sensitive *E. coli.* strain. Isolates were tested for production of extracellular products able to inactivate albicidin. Intracellular effects on albicidin removed from the medium were tested by assaying for albicidin activity in the sonicated cell supernatants of the organisms grown as above in the presence of albicidin. Cells were sonicated in either TEMM buffer (10mM tris-(hydroxymethyl)-aminomethane pH 7.0, 10mM EDTA and 2mM 2-mercaptoethanol) or 10mM sodium phosphate buffer (pH 7.8). Cell extracts containing 200ng-25μg of total protein from the selected organism were mixed with 250ng of albicidin in a final volume of 15μl to determine the ability of the resistance gene product to inactive albicidin. Antibacterial activity was immediately measured on MGM plates overlaid with albicidin sensitive *E. coli.* strain UQM70 as described

previously (Birch *et al.*, 1990). Cell extracts containing 25μg of total protein were also tested for ability to protect *alb*^{r} *E. coli.* UQM70 in an overlayer on freshly prepared plates of LM containing 100ngml^{-1} albicidin.

Identification of the isolated bacterium

Isolate SO-9 was selected for further experiments and identified using key characteristics and API 20NE strips.

Response of albicidin resistance gene product to heat

Cell extracts were incubated for 10min at temperatures between 4°C and 100°C, then 25μg of incubated total protein was mixed with 100ng of albicidin and immediately assayed for albicidin activity to test the heat sensitivity of the albicidin inactivating material. Cell extract containing 100μg total protein was mixed with 400ng of albicidin in a final volume of 30μl to test the heat-reversible binding of albicidin to the albicidin resistance gene product. One half of this was treated at 100°C for 15min, then the boiled and unboiled samples were assayed for albicidin activity. Control treatments consisted of albicidin resistance protein only and BSA (a non-specific protein) mixed with albicidin and albicidin only, at concentrations equivalent to those in test samples (Basnayake *et al.*, 1993).

Nucleotide sequence of the *alb*^{r} gene

For dideoxy DNA sequencing, RNA-free, double-stranded, closed circular DNA templates were used with Universal and Reverse primers according to the manufacturer's instructions (Pharmacia: T7 sequencing^{TM} kit; Basnayake *et al.*, 1993). 'Gen Bank' data base (Release 63) was used to search for any homologous genes which can provide with clues for evolutionary relationships or the function of this gene in *A. denitrificans*.

Amino-terminal sequence of *alb*^{r} protein

Single dimensional Poly Acrylamide Gel Electrophoresis (PAGE) of cell extract was carried out to isolate the alb^r protein for sequencing using Edman degradation method (Basnayake *et al.*, 1993). A computer search of the

'NBRF' protein sequence database 'Release 40.0' was used to identify proteins homologous to the *alb*^{r} gene product from *A. denitrificans*.

Comparison of *alb*^{r} genes

The availability of the cloned alb^r gene from *A. denitrificans* (Basnayake *et al.*, 1993) provided an opportunity to test for homology with other *alb*^{r} genes by Southern hybridization. Homology was tested with (i) the gene for an albicidin binding protein from *Klebsiella oxytoca* (Walker *et al.*, 1988) and (ii) the genome of *X. albilineans*, as this organism must have a mechanism to protect its own DNA replication from inhibition by albicidin. Homology was also tested with the wild-type *E. coli*. genome, to test the hypothesis that the *alb*^{r} gene from *A. denitrificans* might encode a broadly conserved protein (*eg.* a DNA replication protein) which has become albicidin-resistant in *A. denitrificans* due to a minor sequence change. Genomic DNA was isolated from *X. albilineans* LS155 and *E. coli*. MG 1655, and 7μg of each was digested with *Eco*R1, and after gel electrophoresis the resulting DNA were transferred to nylon membranes (Hybond-N$^+$: Amersham International, U.K.) by capillary action (Southern, 1975). The *alb*^{r} gene from *A. denitrificans* was used as the probe, and the probe was labelled and hybridization and colour development steps were performed using a 'Digoxygenin Detection Kit' (Boeringer Mannheim, Australia) according to the manufacturer's instructions. The labelled probe was hybridized with DNA on the membranes at 55°C for 20h. Excess probe was washed twice with 15mM NaCl and 1.5mM sodium citrate at the same temperature. Washed membranes were incubated for 20hr in antibody-conjugate solution according to the manufacturer's instructions.

RESULTS

Albicidin resistance mechanism in *E. coli*.

The *alb*^{s} *E. coli*. strains rapidly accumulated albicidin from the surrounding medium whereas the Alb^r mutants were defective in uptake of albicidin at low extracellular concentrations (Figure 1). Stable *alb*^{r} mutants of *E. coli*. obtained from Z agar plates containing 100ng albicidin ml^{-1} showed an approximate 100-fold increase in albicidin resistance relative to their parent strains (Figure 1). All the Alb^r mutants of *E. coli*. were resistant to phage T6.

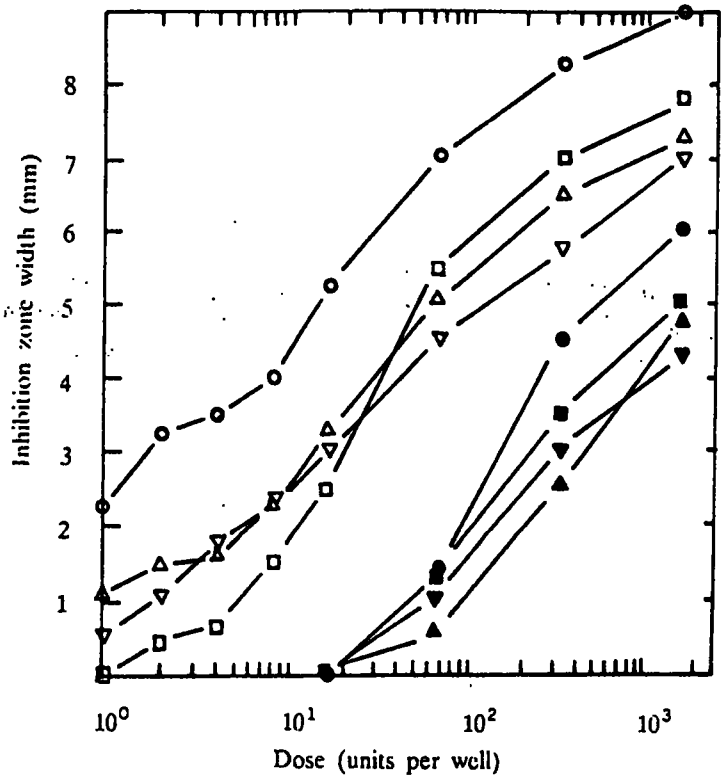


Figure 1. Dose-response relationships for inhibition of growth of *E. coli.* strains by albicidin. Parent strains 294 (○), KL251 (■), Q358 (△) and LE392 (△), and spontaneous mutants 294 Alb^r (◆), KL251 Alb^r (▬), Q358 Alb^r (▲) and LE392 Alb^r (▼). Plate-assay conditions have been described in Methods. The results shown are from a typical experiment.

Albicidin resistance and albicidin uptake

The albicidin-sensitive *E. coli.* strain Q358 rapidly removed albicidin from the surrounding medium, whereas the spontaneous Alb^r mutant of this strain did not (Figure 2a). To provide a more rigorous test of the hypothesis that albicidin resistance in *E. coli.* results from mutations at *tsx* which reduce albicidin uptake, an isogenic pair of *E. coli.* strains differing only in one thoroughly characterized mutation which eliminates production of the Tsx

identical to BRE2050 except for an insertion mutation that generated a *tsx*-lacZ protein fusion close to the amino-terminus of Tsx, so that strain GP4 did not produce any Tsx protein (Bremer *et al.*, 1988). Strain BRE2050 was Alb^s and rapidly removed albicidin from the surrounding medium; strain GP4 proved Alb^r and showed greatly reduced albicidin uptake (Figure 2b).

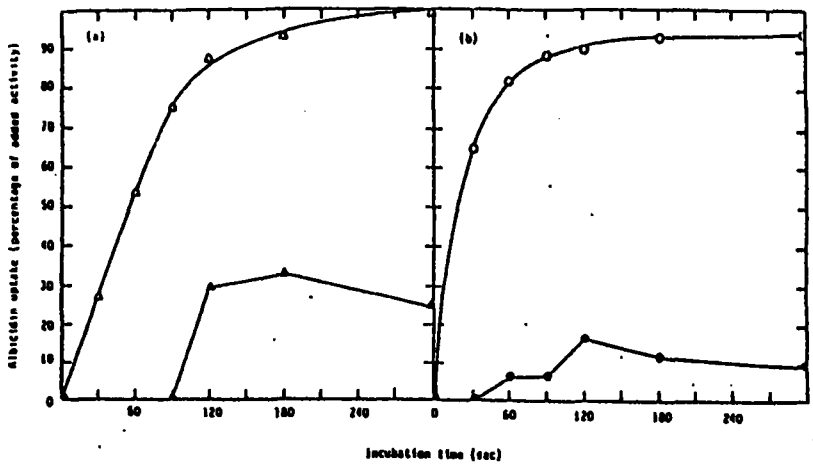


Figure 2. Uptake of albicidin by *E. coli.* strains. (a) Parent strain Q358 (△) and spontaneous mutant Q358 Alb^r (▲). (b) Parent strain BRE2050 (○) and isogenic φ (*tsx-lacZ*) 1 (Hyb) fusion derivative strain GP4 (◆). Albicidin uptake was estimated by following the removal of albicidin from the medium surrounding cells, as described in Methods. Data points are means of two replicates. The results shown are from a typical experiment.

Albicidin resistance mechanism in soil bacteria

Uptake, Inactivation and intracellular accumulation of albicidin

Six of the 19 isolates (SO-1, SO-4, SO-8, SO-9, SO-15 and SO-18) substantially reduced the albicidin activity in the culture medium during Cell free culture supernatants prepared from ageing cultures of the two isolates (SO-8 and SO-9) which removed all albicidin from their culture

medium during overnight growth were able to inactivate albicidin, and this capacity increased as cultures aged from 2 to 6 days. There was no albicidin-inactivation by cell free culture supernatants of the other tested isolates. When the sonicated cell supernatants were assayed for the presence of intracellularly accumulated albicidin, all tested isolates except SO-8 and SO-9 had intracellularly accumulated albicidin. Cell extracts of SO-8 and SO-9 prepared in phosphate buffer also failed to inactivate albicidin, but albicidin activity was completely abolished when mixed with cell extracts of SO-9 prepared in TEMM buffer (Basnayake *et al.*, 1993). Therefore, TEMM buffer was used in all subsequent experiments with cell extracts.

Capacity of cell extracts to inactivate albicidin was assayed by mixing with a known quantity of albicidin and immediately assaying the remaining albicidin activity in the mixture. In the alternative assay where samples were added to wells in overlaid plates containing albicidin, growth of albicidin sensitive *E. coli.*, was completely inhibited, except in a zone of 10-17mm diameter around all the wells containing cell extracts of SO-9, where dense growth occurred (data not shown).

Identification of the isolated albicidin resistant soil bacterium

Isolate SO-9 was selected for further experiments. Bacterial motility, cell morphology, flagella arrangement, Gram reaction, biochemical tests for organism identification and computer analysis of API 20NE data suggested this organism being either *Pseudomonas acidovorans* or *Alcaligenes denitrificans*. Based on high mol % of G+C of its DNA, peritrichous flagella, negative chitin metabolism, negative pigmentation, failure to oxidise or ferment glucose or fructose, presence of PHB inclusions under nitrogen starvation, metabolism of organic acids as the sole carbon source, and anaerobic nitrate reduction to N_2, soil isolate SO-9 was identified as *Alcaligenes denitrificans* subspecies denitrificans, (Kreig, 1984).

Response of albicidin resistance gene product to heat

Ability of cell extracts to inactivate albicidin was abolished within 10min at temperatures above 55°C. Antibiotic activity was rapidly abolished from solutions containing albicidin-protein mixture. However, most albicidin activity was restored by boiling such mixtures (data not shown). BSA did not inactivate albicidin, but boiling BSA-albicidin mixtures also slightly

reduced the activity possibly due to loss of albicidin in the denatured protein pellet (Basnayake *et al.*, 1993).

Nucleotide sequence of the *alb'* gene

Computer analysis of the bacterial, plant and organelle genes in the 'Gen Bank' data base revealed no genes with 'significant' homology to the *A. denitrificans* gene. The search revealed three genes with 'possible' homology (*Klebsiella oxytoca* gene for albicidin resistance, *Pseudomonas aeruginosa amiR* gene for aliphatic amidase regulator, and barley alpha-amylase type B (*Amy46*) gene). Homology at the nucleotide level may indicate some evolutionary relationship between these genes. When the computer analysis was conducted to allow regions to be realigned, the highest overall homology was between the *alb'* genes from *A. denitrificans* and *K. oxytoca.*

Amino-terminal sequence of *alb'* protein

The computer search revealed a 62.5% homology in the amino-terminal sequences of albicidin binding proteins of *A. denitrificans* and *K. oxytoca.* This was due to the presence of identical amino acids or amino acids grouped according to properties of their side chains, in a 16 amino acid region at the NH_2-terminus of both albicidin binding proteins (Figure 3). However, comparison of the entire sequence did not show homology to the *K. oxytoca* Alb' protein. In addition, no other significantly homologous (Lipman and Pearson, 1985) proteins were revealed even with distant relationships.

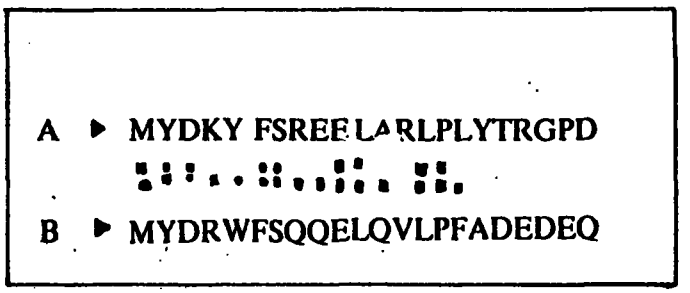
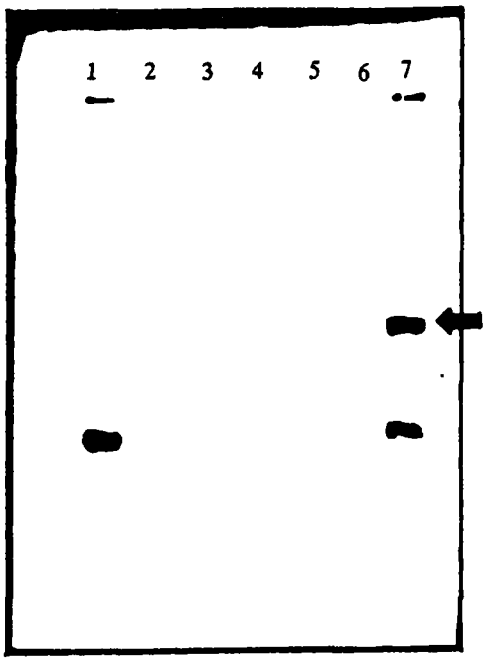


Figure 3. Comparison of 22 amino acids in the NH_2-terminal sequences of A, Alcaligenes denitrificans *alb'* gene and B, *Klebsiella oxytoca alb'* gene. Symbols -: identical amino acids; . amino acids with similar properties in their side chains.

Comparison of *alb*^{r} genes

No cross hybridization was detected, even at low stringency, between the labelled *alb*^{r} gene from *A. denitrificans* and the *alb*^{r} gene from *K. oxytoca*, or genomic DNA from *X. albilineans* LS155 or *E. coli.* MG1655 (Figure 4).



Lane 1 - *alb*^{r} gene from *A. denitrificans* (the probe),
Lanes 2-3 - genomic DNA of *X. albilineans* digested with *Eco*RI,
Lane 4 - lambda DNA digested with *Hind*III (as the Standard),
Lane 5 - genomic DNA of *E. coli.* MG1655 digested with *Eco*RI,
Lane 6 - albicidin resistance gene from *K. oxytoca*,
Lane 7 - albicidin resistance gene carrying plasmid digested with *Hind*III/*Eco*RI (Basnayake *et al.*, 1993)

Figure 4. Southern blot analysis of genomic DNA from *X. albilineans* LS155, *E. coli.* MG1655 and albicidin resistance gene from *K. oxytoca*, using *alb*^{r} gene from *A. denitrificans* as the probe.

DISCUSSION

Several mechanisms of resistance to albicidin in bacteria were investigated. Characterization of albicidin resistant mutants of *E. coli.* revealed an occurrence of stable albicidin resistance by loss of a nucleoside transport mechanism involved in rapid, illicit intracellular accumulation of the antibiotic. Since this involved an outer membrane protein of a Gram-negative bacterium, it seemed unlikely to be a suitable gene for transfer into plants.

No soil organisms were detected with the capability for extracellular detoxification of albicidin in young cultures. Two organisms, SO-8 and SO-9, were able to inactivate albicidin extracellularly in stationary phase cultures. In addition, cell extracts of these two were able to abolish albicidin activity rapidly. SO-8 was resistant to a range of tested antibiotics, whereas SO-9 was resistant only to albicidin. Thus, SO-9 was chosen for further characterization, and was identified as *Alcaligenes denitrificans*. *In vivo* cloning with RP4::Mini mu revealed that the *alb*^{r} gene can be transferred on a single genomic fragment and expressed in *E. coli*. Cosmid cloning was carried out to isolate the fragment containing albicidin resistance (Basnayake *et al.*, 1993). The albicidin resistance gene product in cell extracts was heat sensitive, and needed a buffer containing a reducing agent to inactivate albicidin *in vitro*, and was also able to inactivate albicidin by reversible binding. The antibiotic activity of the bound albicidin could be recovered by heating the complex to inactivate the albicidin resistance gene product.

Southern analysis at low stringency conditions showed no homology between the *alb*^{r} gene from *A. denitrificans* and any of the genes tested. Computer analysis of nucleotide homology in the 'Gen Bank' data base showed 'possible' homology between the alb^r gene from *A. denitrificans* and the *alb*^{r} gene from *Klebsiella oxytoca*. When regions of genes were allowed to be realigned, the highest overall homology was between the two alb^r genes. However, there is a 62.5% homology in a region of 16 amino acids in the amino-terminus of the *alb*^{r} proteins of *A. denitrificans* and *K. oxytoca* which might indicate a conserved albicidin binding domain suggesting at least a distant relationship between the two albicidin binding proteins, which were non-homologous at the amino acid level except at the functional amino-terminus due to insertions or deletions resulting in frame shifts with time.

CONCLUSIONS

There was a variety of resistance mechanisms available in bacteria for albicidin resistance. The stable albicidin resistance mechanism in *E. coli.* occurred by loss of a nucleotide uptake through *tsx* outer membrane channel. Since an outer membrane pore of *E. coli.* was responsible for this, it seemed unlikely to be a suitable mechanism to confer resistance to the effects of albicidin in sugarcane. Characterization of albicidin resistance in *A. denitrificans* and *K. oxytoca* indicated that both organisms have albicidin binding proteins which can inactivate/detoxify albicidin rapidly, but not progressively. The presence of *alb*^{r} genes from *A. denitrificans* and *K. oxytoca* did not inhibit the production or function of the *tsx* gene product responsible for albicidin uptake in *E. coli.* (Basnayake *et al.*, 1993). The computer search at the nucleotide level and the results of Southern hybridization experiments have revealed that there was no biologically significant homology between the *alb*^{r} genes. However, constitutive expression of the gene, and production of the corresponding protein in the cytosol suggest the suitability of the alb^r genes from *A. denitrificans* and *K. oxytoca* to be transferred to obtain albicidin resistance in sugarcane plants.

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APPENDIX I

Bacterial strains used in this study

Strain Designation	Genotype	Reference or Source
BRE 2050	*F metB ilv rpsL cytR9 deoR8 Δ(argF-lac) U169*	Bremer *et al.*(1988)
CR34 Thy^{s}m^{r}	*thr-1 leuB6 tonA21 lacY1 supE44 lambda thyA6 rpsL67 thi-1 deoC1*	B. Bachmann*
DH5α	*supF44V lacU169 (φlacZVM 15)hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Maniatis *et al.* (1982)*
GP4	BRE 2050 Φ(*tsx-lacZ*) 1(Hyh)	Bremer *et al.* (1988)
KL251	*F recA ara leuB azi tonA lacZ proC tsx-67 purE trpE rpsL xyl mtl metE thi supE lambda-*	B. Bachmann*
KL251 Alb^{r}		This study
KMBL 1789	*F argA pheA bio thyA deoB endA polA lambda-*	B. Bachmann*
KMBL 1789 Rif^{r}		This study
LE 392	*F hsdR (r^{m+}) lacY galK galT metB trpR supE supF lambda*	Maniatis *et al.* (1982)
LS 155	wild type	This study
MG 1655	wild type	B. Bachmann*
Q358	*hsdR (r^{m+}) supE Q 80^{R}*	Maniatis *et al.* (1982)
Q358 Alb^{r}		This study
UQM70	Prototrophic, Val^{R} wild type	L.Sly, Dept. of Microbiology, Univ. of Queensland, Australia.

All strains are *E. coli.* K12, and UQM70 which is an Australian isolate used for albicidin assays.

* Strains denoted B. Bachmann were kindly provided by Dr. Barbara Bachmann, *E. coli.* Genetic Stock Centre. Yale University. CT. USA.