Biocontrol of the Sugarcane Borer *Eldana saccharina* by Expression of the *Bacillus thuringiensis cry1Ac7* and *Serratia marcescens chiA* Genes in Sugarcane-Associated Bacteria

KATRINA J. DOWNING,† GRAEME LESLIE, and JENNIFER A. THOMSON

**Department of Microbiology, University of Cape Town, Rondebosch 7701, 1 and South African Sugar Association Experiment Station, Mount Edgecombe 4300, 2 South Africa**

Received 12 October 1999/Accepted 28 April 2000

The *cry1Ac7* gene of *Bacillus thuringiensis* strain 234, showing activity against the sugarcane borer *Eldana saccharina*, was cloned under the control of the *tac* promoter. The fusion was introduced into the broad-host-range plasmid pKT240 and the integration vector pJFF350 and without the *tac* promoter into the broad-host-range plasmids pML122 and pKmM0. These plasmids were introduced into a *Pseudomonas fluorescens* strain isolated from the phylloplane of sugarcane and the endophytic bacterium *Herbaspirillum seropedicae* found in sugarcane. The *p tat-cry1Ac7* construct was introduced into the chromosome of *P. fluorescens* using the integration vector pJFF350 carrying the artificial interposon Omegon-Km. Western blot analysis showed that the expression levels of the integrated *cry1Ac7* gene were much higher under the control of the *tac* promoter than under the control of its endogenous promoter. It was also determined that multiplicity expression in *P. fluorescens* and *H. seropedicae* of *p tat-cry1Ac7* carried on pKT240 caused plasmid instability with no detectable protein expression. In *H. seropedicae*, more *Cry1Ac7* toxin was produced when the gene was cloned under the control of the *NmR* promoter on pML122 than in the opposite orientation and bioassays showed that the former resulted in higher mortality of *E. saccharina* larvae than the latter. *P. fluorescens* 14:*p tat-tox* resulted in higher mortality of larvae than did *P. fluorescens* 14:*tox*. An increased toxic effect was observed when *P. fluorescens* 14:*p tat-tox* was combined with *P. fluorescens* carrying the *Serratia marcescens* chitinase gene *chiA*, under the control of the *tac* promoter, integrated into the chromosome.

The gram-positive, aerobic, spore-forming bacterium *Bacillus thuringiensis* has been used as a safe alternative and supplement to chemical pesticides for over 2 decades. It is a pathogen of insect larvae which produces highly specific crystal inclusions during sporulation. These parasporal crystals consist predominantly of protoxin molecules known as δ-endotoxins, Cry toxins, or Cry proteins. The crystal inclusions dissolve in the larval midgut, where one or more protoxins are released and proteolytically converted into smaller toxic polypeptides. The activated toxins are highly specific to the insect and very predominant of protoxin molecules known as δ-endotoxins, Cry toxins, or Cry proteins. The crystal inclusions dissolve in the larval midgut, where one or more protoxins are released and proteolytically converted into smaller toxic polypeptides. The activated toxins are highly specific to the insect and very specific in their activity (14). Despite the success of conventional *B. thuringiensis*-based products, they have several disadvantages as bioinsecticides. In the case of the sugarcane borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae), a widespread sugarcane pest which causes considerable crop loss in the cane-growing areas of South Africa and Swaziland, these include instability in the environment and on the surface of sugarcane, as well as difficulty in reaching the internal regions where the larvae feed. The use of recombinant DNA technology has provided solutions to the problems through the development of two approaches, namely, genetically modified microorganisms and transgenic plants (18, 21, 22, 25, 26).

As part of an integrated pest management approach to the control of *E. saccharina* in South Africa, the *cry1Ac7* gene from *B. thuringiensis* strain 234 was previously introduced into *P. fluorescens* isolate 14 (13, 33). This organism was isolated from the surface of sugarcane leaves, stems, and borings and shown to be a good colonizer of the phylloplane of sugarcane. Toxicity bioassays indicated that *P. fluorescens* 14 clones that expressed the gene were toxic to *E. saccharina* larvae, and greenhouse trials showed that sugarcane plants inoculated with the strain carrying the integrated gene were more resistant to *E. saccharina* damage than were untreated controls.

Although these results were encouraging, it was felt that there was room for further improvement in the use of recombinant bacteria for the control of this sugarcane pest. The aim of the work presented in this paper was to increase δ-endotoxin expression by cloning the *cry1Ac7* gene under the control of the *tac* promoter with subsequent integration of the cassette into the chromosome of *P. fluorescens* 14. In addition, since recombinant *P. fluorescens* 14 populations are not stably maintained on sugarcane over long periods (33), the potential of endophytic bacteria present in the interior regions of healthy sugarcane plants that express the gene as a biocontrol agent was investigated. Of particular interest is the gram-negative, obligately endophytic, nitrogen-fixing bacterium *Herbaspirillum seropedicae*, which has been isolated only from monocotyledonous plants such as sugarcane, rice, sorghum, maize, 13 different graminaceous weeds, and the roots of a pigeonpea plant (3, 6, 7). The use of an endophytic bacterium was also seen as a possible solution to the problem of inaccessibility of conventional *B. thuringiensis*-based products to the interior regions of the plant. The advantages of using these recombinant endophytes are their high stability in sugarcane and the ability to be transferred to subsequent generations via sugarcane sets (4, 6, 7).

A further strategy to improve the biocontrol of *E. saccharina* involved combining *P. fluorescens* strains producing the Cry1Ac7 protein and a *Serratia marcescens* chitinase, ChiA.
Nal1, was isolated. These strains were grown in JNFb medium, which was provided by J. Do ¨bereiner of the Empresa Brasiliera de Pesquisa m

of MgSO4 at 4°C, and of NH4Cl was added per liter in addition to yeast extract. The micronutrients added per liter with the yeast extract omitted; and for liquid JNFb medium, 1 g KOH (pH 5.8) and was supplemented with the appropriate antibiotic. For solid medium, 0.2 g of Na 2MoO4 5H2O. Phenotypic expression was carried out at 30°C for 24 h in 1 ml of LB or JNFb medium (for P. fluorescens and H. seropedicae, respectively). The cells were plated undiluted onto LB medium with agar or JNFb solid medium supplemented with kanamycin (100 µg/ml) and grown at 30°C. To increase the electroporation efficiency of H. seropedicae, the method described by Wirth et al. (37) was attempted.

Bacterial transformation by electroporation and conjugation. Broad-host range plasmids and the integration vector carrying the puc-cry1Ac7 cassette were electroporated into P. fluorescens 14 and H. seropedicae Nal1 using a modification of the method of Waalwijk et al. (36). Cells harvested at mid-exponential phase and washed three times in 100 mM sucrose were electroporated in 40-µl volumes with 1 to 3 µg of DNA using a Bio-Rad Gene Pulser and controller set at 25 w i t h1to3

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Rifampin-resistant P. fluorescens 14 was grown on Luria-Bertani medium (LB) or LB medium with agar supplemented with rifampin (100 µg/ml). The sugarcane endophyte H. seropedicae HRC54 was provided by J. Dohbereiner of the Empresa Brasileira de Pesquisa Agropecuaria, Brasilia, Brazil. A spontaneous nalidixic acid-resistant mutant, H. seropedicae Nal1, was isolated. These strains were grown in JNFb medium, which contained, per liter, 5 g of malic acid, 0.6 ml of K2HPO4, 1.8 ml of KH2PO4, 0.2 g of MgSO4·7H2O, 0.1 g of NaCl, 0.2 g of CaCl2·H2O, 0.066 g of FeEDTA, 2 ml of bromothymol blue, 2 ml of micronutrients, 0.02 g of yeast extract, and 4.5 g of KOH (pH 5.8) and was supplemented with the appropriate antibiotic. For solid JNFb, 17 g of agar was added per liter; for semi-solid JNFb, 1.9 g of agar was added per liter with the yeast extract omitted; and for liquid JNFb medium, 1 g of NH4Cl was added in per liter in addition to yeast extract. The micronutrients consisted of 0.2 g of Na2MoO4·2H2O, 0.235 g of MnSO4·H2O, 0.28 g of H3BO3, 0.008 g of CuSO4·5H2O, and 0.024 g of ZnSO4·7H2O per 200 ml of H2O.

All bacteria were grown at 30°C. P. fluorescens was maintained in 0.1 M MgSO4 at 4°C, and H. seropedicae strains were maintained in JNFb medium supplemented with 10% glycerol at −70°C.

Molecular techniques. Molecular techniques were performed as described by Sambrook et al. (29).

Western blot analysis. Determination of the expression of the cry1Ac7 gene in P. fluorescens 14 and H. seropedicae Nal1 was carried out by Western blot analysis. Cell extracts were prepared from 1 ml of stationary-phase cultures by resuspending cell pellets in 100 µl of denaturing loading buffer (20). Samples (20 µl) were loaded onto a denaturing gradient (10 to 5%) acrylamide gel, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (20). For the quantitative analysis of Cry1Ac7 production, Escherichia coli cultures were grown overnight at 30°C in LB medium supplemented with the appropriate antibiotic, diluted 100-fold, grown to mid-exponential phase at 37°C, and induced with 0.3 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) as described by Ausubel et al. (1). Uninduced controls were prepared by dividing the mid-exponential-phase (optical density at 600 nm, 0.4) culture in two before adding IPTG. Samples from both uninduced and induced cultures were removed at various time intervals after induction. Samples (1 ml) of cultures induced for 24 h were sonicated, and the protein concentration was determined by the method of Bradford (5). Volumes of denatured cell extracts containing 50 µg of protein were separated by SDS-PAGE.

Proteins were transferred from SDS-polyacrylamide gels onto nitrocellulose membranes by the method of Towbin et al. (34). Western blot analysis was carried out using the primary antibody raised against the Cry1Ac7 protein (supplied by SASEX) and goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma) as the secondary antibody.
onto JNfb plates after each cycle of growth. One hundred of the resulting CFU were patched onto JNfb plates supplemented with kanamycin (100 μg/ml). The percentage of patched colonies which grew on these plates was recorded. This experiment was performed three times.

**Southern blot analysis.** Southern blot analysis was used to demonstrate integration of the Omegon-Km-tac-cry1Ac7 cassette into the chromosome of *P. fluorescens* 14 clones. Total bacterial DNA was isolated as described by Ausabel et al. (1). Probes were labeled with digoxigenin-11-dUTP (Boehringer Mannheim) by the random primed DNA labeling method in accordance with the manufacturer’s instructions. Southern blot analysis was carried out by the method described in the digoxigenin-11-dUTP system user’s guide.

**Toxicity bioassays.** Weighed quantities of the various freeze-dried bacterial species were mixed with weighed quantities of an artificial insect diet (11) such that a known amount of bacterial preparation per gram of diet was obtained. Aliquots (0.2 g) were added to Eppendorf tubes, and five 2-week-old *E. saccharina* neonate larvae were placed in each tube. Each treatment comprised five tubes. Mortality of the larvae was recorded every 24 h for 5 days. An analysis of variance was done with one factor, bacterial species, the Omegon-Km–p tac-cry1Ac7 cassette was inserted into the chromosome and integration was confirmed by Southern blot analysis. Total DNA of *P. fluorescens* 14::p tac-cry1Ac7 was cut with EcoRI and probed with the 4-kb BamHI fragment of p tac-tox carrying the p tac-cry1Ac7 cassette (Fig. 1). Four fragments of 3.5, 3.0, 0.7, and 0.2 kb hybridized to EcoRI-restricted pJTT. In all of the clones analyzed, two EcoRI fragments of 0.7 and 0.2 kb, corresponding to the fragments internal to the Omegon-Km–p tac-cry1Ac7 cassette, and two of different sizes greater than 3.5 and 3.3 kb, hybridized to the probe. Random, single integration of the cassette was indicated by the fact that the two larger EcoRI fragments were of different sizes and only two of the larger EcoRI fragments were detected in these clones.

The stability of the integrated cassette was not definitively established, but there was no evidence of decreased Cry1Ac7 expression in SDS-PAGE after 48 h (results not shown) and the growth rate of the recombinant strain did not appear to be different from that of *P. fluorescens* 14.

The plasmids pKTT240 and pJFF350, their recombinant p tac-cry1Ac7 derivatives, and the pML122- and pKmM0-derived plasmids carrying the cry1Ac7 gene were electroporated into *H. seropedicae* Nal1. pKTT240 and the pKmM0- and pML122-based plasmids carrying the cry1Ac7 gene were successfully introduced with an efficiency of ca. 8 × 10^3 transforms/μg of DNA. Electroporation of pKTT240-derived pKTT carrying the p tac-cry1Ac7 cassette resulted in only a few Km r colonies, possibly due to the instability of this construct in *H. seropedicae*. Plasmid stability studies showed that pMT7 carrying the cry1Ac7 gene inserted downstream of the strong Nm r promoter on pML122 was extremely unstable after overnight growth, whereas pMT11, with the gene in the opposite orientation with respect to this promoter, was stable over the 60 generations tested (results not shown). This is likely to be due to the intolerance of high levels of constitutively expressed cry1Ac7 in *H. seropedicae* cells. pKmM0-tox was stable over the 40 generations tested (results not shown). Efforts to introduce the p tac-cry1Ac7 cassette on the integrative construct pJTT into *H. seropedicae* by electroporation and conjugative transfer resulted in very low numbers of Km r colonies, indicative of low transposition frequencies, believed to be due to inefficient transformation.

**Expression of the 0-endotoxin gene.** Expression of the cry1Ac7 gene in *P. fluorescens* 14 and *H. seropedicae* Nal1 was determined by quantitative Western blot analysis. The 134-kDa Cry1Ac7 protein was not detected in *P. fluorescens* 14 (pKTT) clones carrying the p tac-cry1Ac7 cassette on pKTT240 (Fig. 2A, lane 4). However, this gene, under the control of its endogenous promoter on pKTT240 and pDER405, was expressed in *P. fluorescens* 14 at two toxin protein levels of 3.5 and 2.2%, respectively, of the total proteins (12). This implied that constitutive expression of cry1Ac7 at high levels in *P. fluorescens* 14(pKTT) must have resulted in the accumulation of mutants defective in cry1Ac7 expression after overnight growth.
All of the analyzed \textit{P. fluorescens} 14::\textit{ptac-cry1Ac7} clones, carrying the integrated Omegon-Km–\textit{ptac-cry1Ac7} cassette, produced the 134-kDa protein at levels considerably greater than that of the previously constructed \textit{P. fluorescens} 14::Omegon–Km–\textit{cry1Ac7} strain, referred to in this report as \textit{P. fluorescens} 14::\textit{tox} (Fig. 2A, compare lanes 6 to 10 with lane 5).

The Cry1Ac7 protein was not detected by Western blot assay of \textit{H. seropedicae} (pKTT) carrying the \textit{ptac-cry1Ac7} cassette on pKT240 (results not shown). As in \textit{P. fluorescens} 14(pKTT) clones, this is possibly due to the accumulation of Cry1Ac7 mutants resulting from high levels of the constitutively expressed \textit{cry1Ac7} gene in \textit{H. seropedicae} (pKTT). In contrast, \textit{H. seropedicae} (pMT11) clones with the \textit{cry1Ac7} gene downstream of the Nmr promoter on pML122 produced higher levels of the Cry1Ac7 protein than did \textit{H. seropedicae} (pMT11) clones with the gene in the opposite orientation with respect to this promoter (Fig. 2B). This indicated that expression of the gene in the former clones was under the control of the Nmr promoter, which could explain the high levels of instability of this plasmid. Labes et al. (19) reported that the Nmr promoter was an efficient and more effective promoter than the \textit{tac} promoter for overexpression of foreign genes in soil bacteria, including \textit{Pseudomonas} spp. The Cry1Ac7 protein was also detected in strains carrying pKmM0–\textit{tox} (results not shown).

The effect of Cry1Ac7 on \textit{P. fluorescens} and \textit{H. seropedicae} strains on \textit{E. saccharina} larvae. The biological activity of Cry1Ac7 on \textit{P. fluorescens} and \textit{H. seropedicae} strains was determined in toxicity bioassays using 3 mg of freeze-dried bacteria per g of diet (Table 2). The results, at the 5\% significance level, showed that \textit{P. fluorescens} 14::\textit{ptac-cry1Ac7} was significantly different from \textit{P. fluorescens} 14::\textit{cry1Ac7}. Both \textit{P. fluorescens} 14::\textit{cry1Ac7} and 14::\textit{ptac-cry1Ac7} were significantly different from the parental strain, which was not significantly different from the untreated control. \textit{H. seropedicae} Nal1(pMT7) was significantly differ-
ent from *H. seropedicae* Nal1, which was not significantly different from the control. *H. seropedicae* Nal1(pMT11) was different from *H. seropedicae* Nal1, but the sample size was not large enough to declare significance at the 5% level.

**Effect of *P. fluorescens* strains expressing the cry1Ac7 and chiA genes on *E. saccharina* larvae.** *P. fluorescens* 14::tac-cry1Ac7 and *P. fluorescens* Rif1::tac-chiA were combined at different concentrations and used in toxicity bioassays (Table 3). Mortality was determined after 2 and 5 days. The results, at the 5% level of significance, show that when the chitinase-expressing strain was added at either 0.3 or 30 mg/g of diet, there was a significantly increased toxic effect. The reason for the lack of increased toxicity when the chitinase-expressing strain was added at 0.3 mg/g of diet, it did not do so at 0.3 or 3 mg/g of diet. However, there was a significant increase in toxicity when it was mixed with the Cry1Ac7-expressing strain at 0.3 mg/g of diet.

**DISCUSSION**

*B. thuringiensis* cry genes have been introduced into bacteria other than *B. thuringiensis*, such as the root colonizers *P. fluorescens* and *Agrobacterium radiobacter* and *Anylcobacter aquaticus*, a bacterium isolated from aquatic habitats. These strains were toxic against the larvae of the tobacco hornworm (*Manduca sexta*), the malaria mosquito *Anopheles stephensi*, and the leatherjacket (*Tipula oleracea*) (16, 23, 24, 35). The introduction of the cry1A(c) gene from *B. thuringiensis* subsp. *kurstaki* into the chromosome of *Clavibacter xylii* subsp. *cyanodontis*, which naturally colonizes the xylem of Bermuda grass, is the only report of the use of a genetically modified endo-

**TABLE 2.** Toxicity to *E. saccharina* neonate larvae of *P. fluorescens* 14 and *H. seropedicae* Nal1 strains expressing the cry1Ac7 gene

<table>
<thead>
<tr>
<th>Day</th>
<th>Controla</th>
<th>14b</th>
<th>14::cry1Ac7</th>
<th>14::tac-cry1Ac7</th>
<th>Nal1c</th>
<th>Nal1(pMT7)</th>
<th>Nal1(pMT11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4 (4.0)</td>
<td>16 (7.5)</td>
<td>0 (0)</td>
<td>4 (4)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>4 (4.0)</td>
<td>48 (8.0)</td>
<td>0 (0)</td>
<td>8 (4.9)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>8</td>
<td>8 (4.9)</td>
<td>68 (4.9)</td>
<td>0 (0)</td>
<td>16 (7.5)</td>
<td>16 (7.5)</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>16</td>
<td>11.7)</td>
<td>84 (7.5)</td>
<td>12 (8)</td>
<td>48 (12)</td>
<td>28 (10.2)</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>28</td>
<td>8 (6.5)</td>
<td>92 (4.9)</td>
<td>20 (8.9)</td>
<td>52 (10.2)</td>
<td>36 (14.7)</td>
</tr>
</tbody>
</table>

a Averages of five experiments are shown. The statistical analysis was done on transformed data (see Materials and Methods; 22).
b No treatment.
c *P. fluorescens* 14.

**TABLE 3.** Toxicity to *E. saccharina* neonate larvae of *P. fluorescens* 14::tac-cry1Ac7 and *P. fluorescens* Rif1::tac-chiA

<table>
<thead>
<tr>
<th>Concentration (mg/g of diet)</th>
<th>Avg % mortality (SE)a Day 2</th>
<th>Avg % mortality (SE)a Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em> 14::tac-cry1Ac7</td>
<td><em>P. fluorescens</em> Rif1::tac-chiA</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>5.5 (2.2)</td>
</tr>
<tr>
<td>0.3</td>
<td>0.3</td>
<td>12.5 (3.7)</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>30.8 (8.1)</td>
</tr>
<tr>
<td>0.3</td>
<td>0.3</td>
<td>8.2 (3.9)</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>7.7 (2.8)</td>
</tr>
<tr>
<td>0.3</td>
<td>0.3</td>
<td>21.8 (9.1)</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>39.3 (7.7)</td>
</tr>
<tr>
<td>0.3</td>
<td>0.3</td>
<td>37.5 (6.0)</td>
</tr>
</tbody>
</table>

a Averages of 11 to 16 experiments are shown. The statistical analysis was done on transformed data (see Materials and Methods; 22).

Our results proved that the tac promoter is capable of operating efficiently in *Pseudomonas* and is responsible for the increased levels of expression of the gene. We are unaware of any reports of a cry1A(c) gene under the control of the tac promoter having been integrated into the chromosome of a *Pseudomonas* sp. Quantitative analysis of the δ-endotoxin by enzyme-linked immunosorbent assay in *P. fluorescens* 14::tac-cry1Ac7 clones was not determined, but Herrera et al. (13) showed that *P. fluorescens* 14::cry1Ac7 clones produced high levels of Cry1Ac7 protein similar to those produced by pKT240-cry1Ac7 clones, representing 3.7 and 3.5% of the total protein, respectively. These levels were comparable to those of 0.5 to 1% reported by Obuckowicz (23) for a similar cry gene
in root-colonizing pseudomonads. Ge et al. (10) reported that expression of the cry1Ac7 gene in both P. fluorescens and H. seropedicae improves the control of E. saccharina larvae. However, it is important to consider that although increased expression leads to increased toxicity, it can also be a burden on bacterial cells, resulting in the accumulation of nonexpressing mutants or in lethality. All of these factors need to be taken into account when planning strategies for biological control of E. saccharina in sugarcane.

Synergistic insecticidal effects with combined B. thuringiensis suspensions and chitinase or chitinase-producing bacteria, as well as the combined effects of a Cry1C protein and S. marcescens ChiA, have been demonstrated previously (28, 31). The addition of both B. thuringiensis and chitinase increased the insecticidal effect on Choristoneura funefera larvae significantly. Perforation of the peritrophic membrane by ChiA caused an increase in the toxicity of Cry1C, possibly due to an increase in the numbers of Cry1C toxin molecules binding to the membrane receptors present in the epithelium of the insect larvae. A Cry1C concentration of 20 μg/ml was required for a maximum toxic effect on larvae in the absence of chitinase, whereas only 3 μg of Cry1C per ml was needed for the same toxic effect in the presence of ChiA.

Our results demonstrate that by co-introduction of cry1Ac7 and chitinase genes into strains of P. fluorescens, increased biocontrol of insect pests could be achieved, requiring lower levels of Cry1Ac7 protein expression. This is advantageous, since lower expression may enable the bacteria to compete better in the environment with a diminished risk of generation of resistant larval populations resulting from exposure to high levels of Cry protein. The optimum, effective concentrations of the recombinant strains, as well as the synergistic toxic effect of H. seropedicae strains producing the Cry1Ac7 protein and chitinase, need to be investigated.

ACKNOWLEDGMENTS

We thank Imke Hansen-Wester for construction of pKmMII-tox and its expression in H. seropedicae, Gillian Mimmack for statistical analysis of the data, Barbara Hukett for assistance with bioassays, and Helena Boschoff for helpful discussions.

REFERENCES


