Genetic Analysis of Agrocin 84 Production and Immunity in Agrobacterium spp.

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Mutations affecting agrocin production on the 48-kilobase (kb) plasmid, pAgK84, can be complemented in trans with cloned portions of the plasmid. Five complementation groups ranging in minimum size from 1.2 to 5.6 kb were identified within a 14-kb segment. Plasmid pAgK84-encoded immunity to agrocin 84 was located to two separate regions of the plasmid. Either region alone was sufficient to protect sensitive strains, and both loci mapped to the agrocin 84 biosynthesis region. One region is located within complementation group I, while the other forms a part of complementation group IV. Production of agrocin 84 was unaffected by nopaline, agrocinopine A, acetylsyringone, or low or high levels of ferric iron. Agrocin 84 production was greatly suppressed when the strain also contained a Ti plasmid nutritionally or mutagenically derepressed for agrocinopine A catabolism. RNA dot-blot analysis indicated that decreased agrocin 84 production by such strains was not due to transcriptional repression of agrocin 84 biosynthetic loci. In strains also harboring pAtK84b, the opine catabolite plasmid of Agrobacterium radiobacter K84, induction of the agrocinopine A catabolic locus of this plasmid had no such effect on agrocin 84 production.

Crown gall disease, which affects a wide range of dicotyledonous plants, is caused by the soil-borne bacterium Agrobacterium tumefaciens. The disease causes economic losses worldwide, particularly in plant nurseries, stone fruit orchards, and vineyards (1, 13, 19). A biological control to combat crown gall has been developed (14) which utilizes a nonpathogenic Agrobacterium radiobacter strain, K84. This agent is commercially available and is used in countries around the world (19).

Production of agrocin 84 by strain K84 is a major determinant in the biological control of crown gall (3, 15). This agent exhibits specific toxicity toward Agrobacterium strains able to utilize the agrocinopine-type opines (4, 6, 29). These sugar phosphate opines are found in tumors incited by such strains (23), and their catabolism is encoded by the Ti plasmids resident in the pathogens (4). Sensitivity to agrocin 84 is related to agrocinopine uptake, and there is evidence that both molecules are transported by the same Ti plasmid-encoded permease (4, 20).

Agrocin 84 represents a novel antibiotic class; it is a disubstituted, fradulent adenine nucleoside analog (29). 3'-Deoxy-D-arabino-sugar substitutes for deoxyribose, and the molecule therefore lacks a 3'-OH. The glucosyluridylyl substituent at the N9 position is required for specific uptake (21) but not for toxicity. However, because this derivative is poorly transported, its specific toxicity is greatly decreased. The role of the methyl pentanamide is unknown, although chemical removal of this substituent in a fashion converting the phosphate ester to a hydroxyl group at the C-5' position abolishes toxicity without affecting transport (21).

Agrocin 84 is not unique. The agrocin produced by A. tumefaciens K108 has been identified as a cytidine analog (16), while a third agrocin, produced by A. tumefaciens D286, has a UV absorption spectrum characteristic of an adenine nucleoside (11). However, agrocin D286 shows a different specificity and is most probably not identical to agrocin 84. The pathways for the biosynthesis of these novel antiagrobacterial antibiotics have not yet been determined.

Production of agrocin 84 is encoded by a 48-kilobase (kb) plasmid, pAgK84 (3, 25), which has been physically and genetically characterized (3, 8, 25). Tn5 mutagenesis mapped the agrocin 84 biosynthetic determinants to a 20-kb region divisible into three segments on the basis of two insertions not affecting antibiotic synthesis. We report here further genetic analysis indicating that at least five transcriptional groups are involved in agrocin 84 biosynthesis. The plasmid also encodes immunity to agrocin 84, and we show that there are two distinct loci involved in this trait. Both have been characterized by transposon mutagenesis, and each maps to a different transcriptional unit in the biosynthetic region of the plasmid.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1. The published locations of Tn5 insertions (8) in six of the tested pAgK84 derivatives (B.15, B.59, C.10, B.19, D.26, and C.24) were found to be in error. Correct positions are given in Table 1.

Media and buffers. L broth, nutrient agar, Storier's medium (27), and AB minimal medium were prepared as described by Farrand et al. (8). Nopaline (Calbiochem-Behring, La Jolla, Calif.) and agrocinopine A were added to 0.2 mg/ml to Storier's medium solidified with 1.5% agar. Sugars were added to a final concentration of 10 mM. Acetylsyringone (3',5'-dimethoxy-4'-hydroxyacetophenone; Aldrich Chemical Co., Inc., Milwaukee, Wis.) was included in Storier's plates at final concentrations of 1 and 10 mM. Antibiotics were added to media at the following concentrations (micrograms per milliliter): kanamycin, 25; neomycin, 100; and spectinomycin, 150.

Isolation of agrocinopine A. Agrocinopine A was isolated by 70% ethanol extraction of tomato tumors induced by strain T37 and was partially purified by high-voltage paper
TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant plasmid</th>
<th>Relevant traits</th>
<th>Reference</th>
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<tr>
<td>Agrobacterium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>radiobacter K84</td>
<td>pAgK84</td>
<td>Agrocin 84 production</td>
<td>5, 15</td>
</tr>
<tr>
<td></td>
<td>pAtK84b</td>
<td>Nopaline and agrocinopine A catabolism</td>
<td></td>
</tr>
<tr>
<td>Agrobacterium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumefaciens C58</td>
<td>pTiC58</td>
<td>Tumorigenicity, nopaline and agrocinopine A catabolism</td>
<td>30</td>
</tr>
<tr>
<td>NT-1</td>
<td>pW11000</td>
<td>Ti plasmid-cured C58, agrocin 84 resistant</td>
<td>30</td>
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<tr>
<td></td>
<td></td>
<td>Tra-constitutive pTiC58, agrocin 84 supersensitive</td>
<td>5</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>HB101</td>
<td>leu-6 pro thi-2 recA lacY hsdR rpsL</td>
<td>18</td>
</tr>
<tr>
<td>1231</td>
<td></td>
<td>serB leu-6 thi-2 hsdR lacY</td>
<td>22</td>
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<tr>
<td>1830</td>
<td>pJB4JI</td>
<td>Tn3-donating suicide plasmid; met-63 pro-22 nal Kan'</td>
<td>8</td>
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<tr>
<td>Plasmids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSa152</td>
<td>IncW, Km'-Nm' Sp' Cm'</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>pAgK84::Tn5b</td>
<td>Km'</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>pSc304</td>
<td>pSc101::Tn3 temperature sensitive for replication</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

* Abbreviations: Cm', chloramphenicol resistance; hsd, host specificity determinant; IncW, incompatibility group W; Kan', Km', kanamycin resistance; lac, lactose; leu, leucine; met, methionine; nal, nalidixic acid resistance; Nm', neomycin resistance; pro, proline; rec recombination; rps, ribosome small subunit protein; ser, serine; Sp', spectomycin resistance; thi, thiamine; Tn, transposon transfer.

** DNA enzymology.** DNA ligation reactions were performed at 12°C in a buffer containing 66 mM Tris hydrochloride (pH 7.5), 5 mM MgCl₂, 5 mM dithiothreitol, 1 mM ATP, and 2 units of T4 DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). pAgK84 fragments completely or partially digested with XmaI were treated with calf intestine alkaline phosphatase (Boehringer Mannheim) and ligated into the chloramphenicol locus of pSa152 (28).

** RNA isolation.** Bacterial RNA was isolated essentially as described by Glivin et al. (10) and modified by Deretic et al. (2). Cultures (100 ml) were grown to the early to mid-exponential phase in either L broth or Stonier's medium. Cells were harvested by centrifugation at 0°C and lysed at 56°C in the presence of 3.7% (w/v) sodium dodecyl sulfate. RNA was collected by centrifugation through a 5.7 M CsCl cushion at 35,000 rpm in an SW41 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 12 h at 20°C. The RNA pellet was dissolved in sterile distilled water and extracted once with chloroform-isoamyl alcohol (24:1, vol/vol). The RNA was ethanol precipitated at −20°C, collected by centrifugation, dissolved in sterile distilled water, and stored at −70°C.

** DNA dot blots.** RNA was denatured for 1 h at 56°C in the presence of glyoxal (1 M) and 12.5 mM sodium phosphate (pH 6.5 before the addition of glyoxal). Twofold serial dilutions were made with sterile distilled water, and samples were applied to Gene Screen Plus membranes (New England Nuclear Corp., Boston, Mass.) as outlined by the manufacturer.

** Nick translations and hybridizations.** Probe DNA was labeled by nick translation with a kit from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and [32P]dCTP (650 Ci/mmol; ICN Radiochemicals). DNA dot blots were hybridized in 1% sodium dodecyl sulfate–1 M NaCl–10% dextran sulfate for 30 min and
hybridized at 60°C in the presence of 180 μg of denatured calf thymus DNA per ml and 9 ng of probe per ml. Blots were autoradiographed for 2 to 4 days at −70°C with Kodak XAR-5 X-ray film and intensifying screens.

RESULTS

Genetic complementation of mutations on pAgK84 abolishing agrocin 84 production. The 20-kb region of pAgK84 encoding agrocin 84 biosynthesis was defined by complementing a number of selected Tn5 insertion mutations with XmaI-generated fragments of pAgK84 cloned in the broad-host-range vector pSal52 (28). XmaI is a complementary end-generating isoschizomer of SmaI, and since a map of the plasmid has been determined (8) for the latter enzyme, we will refer to these clones as containing SmaI fragments. Strain NT-1, which is recombination proficient, was used for all complementations. However, plasmid analyses at the beginning and ending of each experiment showed that recombination was not taking place at a detectable level. Also, positive complementations resulted in wild-type levels of agrocin 84 production (data not shown), indicating that we were observing trans-acting complementation events. Agrocin production due to low-level recombination would have resulted in considerably reduced antibiotic levels. Strains C58 and K439 were used as the indicator strains. The latter strain is supersensitive to agrocin 84 (5), making it useful for detecting small amounts of the antibiotic (8). Strain NT-1(pAgK84::Tn5A.1) carrying a Tn5 insertion outside the agrocin 84 biosynthetic region (8) was used to evaluate wild-type levels of agrocin production.

Figure 1 shows the results of bioassays in which merodiploids were tested for agrocin 84 production. Table 2 presents a summary of the results. Single cloned fragments were able to complement insertions mapping only to the SmaI-B and SmaI-F2 regions of the plasmid. This suggested to us that some transcriptional groups extend through one or more SmaI fragments. To test this, we assayed partial XmaI digests of pAgK84 cloned into pSal52 for complementation of the same set of insertion mutants. With these clones all the Tn5 insertion mutations tested were complementable except A.46. This mutation, mapping to coordinate 39.7 (8), was not complemented with its corresponding SmaI F2 fragment or by a larger clone containing SmaI fragments F2, B, and G. However, insertions flanking A.46 could be complemented with fragments F2 or B (Fig. 1).

The Tn5 insertions affecting agrocin production were divided into five complementation groups (Fig. 1). Table 2 lists the sizes and locations of the five groups and the SmaI fragments required for their complementation. Two groups, II and III, were defined by complementation with the single SmaI fragments F2 and B, respectively. The remaining groups were delineated with clones containing multiple SmaI fragments.

All mutations in group I mapped to the SmaI-D region of the plasmid but required both fragments D and H for complementation (Fig. 1). The minimum size of group III was defined by Tn5 insertions A.60 (coordinate 40.8) and D.11 (coordinate 46.4). These insertions cause a slight decrease in agrocin production (8), but wild-type levels were produced when they were complemented with SmaI fragment B.

Mutations in group IV are of two types. Those at the left (anticlockwise) end produce no detectable agrocin 84. Mutations to the right, mapping between coordinates 1.2 and 2.8, allow production of low amounts of agrocin 84 detectable only with strain K439 as the indicator. Both types are complemented only with a clone containing fragments B and G even though all insertions of the latter class map to the SmaI-G region of the plasmid.

Group V mutations, constituting the right end of the biosynthetic locus, require SmaI fragments G and E for complementation. The left end of this group is defined by the two insertions mapping to the right end of the SmaI-G region which are not complemented by the clone containing fragments G and B (Fig. 1).

Immunity function of pAgK84. No single Tn5 insertion mutation affected immunity conferred by pAgK84 on a sensitive strain. We therefore tested the individual SmaI clones for immunity functions. Two recombinant clones, one

<table>
<thead>
<tr>
<th>Group</th>
<th>Location (coordinates in kb)</th>
<th>Size (kb)</th>
<th>Complementing SmaI fragments(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>32.7–34.4</td>
<td>4.5</td>
<td>D + H</td>
</tr>
<tr>
<td>II</td>
<td>36.7–38.8</td>
<td>3.5</td>
<td>F2</td>
</tr>
<tr>
<td>III</td>
<td>40.8–46.4</td>
<td>6.9</td>
<td>B</td>
</tr>
<tr>
<td>IV</td>
<td>47.2–2.8</td>
<td>4.7</td>
<td>B + G</td>
</tr>
<tr>
<td>V</td>
<td>3.5–4.7</td>
<td>2.2</td>
<td>G + E</td>
</tr>
</tbody>
</table>
with SmaI fragment D and the other with SmaI fragment G, conferred complete immunity on strain C58 (Fig. 2). Strains harboring recombinant clones with the other SmaI inserts remained sensitive to the nucleotide analog. Electrophoretic analysis showed that all the test strains harbored both the recombinant plasmids and pTiC58, the resident Ti plasmid encoding agrocin 84 sensitivity (data not shown). As expected, none of these constructs produced any detectable antibiotic (data not shown).

The immunity regions were more precisely defined by transposon mutagenesis. A group of 10 Tn5 insertions were identified on the SmaI-G clone which abolished the immunity phenotype (Fig. 3B). Insertions to the left and right of the 1.8-kb region defined by these insertions had no effect on agrocin 84 immunity. No Tn5 insertions in the SmaI-D clone affected the immunity function encoded by this fragment (Fig. 3A). However, the central portion of this fragment is evidently refractory to this transposon. When Tn3 was used, three insertions mapping between pAgK84 coordinates 32.3 and 33 abolished immunity in strain C58.

**Control of agrocin 84 production.** Virtually nothing is known about the control of agrocin 84 biosynthesis. The opines nopaline and agrocinopine A, the plant vir gene inducer, acetosyringone, and ferric iron were tested as candidate regulators along with glucose, arabinose, and mannitol and adenosine and deoxyadenosine. None of these compounds, when incorporated into Stonier's medium, had any demonstrable effect on agrocin 84 production as compared with standard controls (data not shown).

![Image](http://jb.asm.org/) on February 23, 2021 by guest

**FIG. 2.** Location of the agrocin immunity functions on pAgK84. Individual SmaI fragments (A through H) cloned in pSa152 were transformed into strain C58. Transformants were tested for sensitivity to agrocin 84 produced by strain K84 as described in Materials and Methods.

**FIG. 3.** Transposon insertion maps of the agrocin 84 immunity regions encoded on SmaI fragments D (A) and G (B). The cloned fragments were mutagenized with Tn5 or Tn3 and transformed into strain C58, and the progeny were tested for sensitivity to agrocin 84. Mutations in the immunity loci rendered transformants sensitive to agrocin 84 (immunity -), while insertions outside the determinants had no effect (immunity +). The lower portion of each map shows alignment with the biosynthetic complementation groups defined as in Fig. 1 and the text.
TABLE 3. Effect of agrocinopine A on agrocin 84 production

<table>
<thead>
<tr>
<th>Producer strain</th>
<th>Relevant plasmid(s)</th>
<th>Diam of growth inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Agrocinopine A</td>
<td>− Agrocinopine A</td>
</tr>
<tr>
<td>K439</td>
<td>pAgK84, pW110000</td>
<td>0</td>
</tr>
<tr>
<td>NT-1</td>
<td>pAgK84</td>
<td>30</td>
</tr>
<tr>
<td>NT-1</td>
<td>pAgK84, pAtK84b</td>
<td>32</td>
</tr>
<tr>
<td>C58</td>
<td>pAgK84, pTIC58</td>
<td>9</td>
</tr>
<tr>
<td>K84</td>
<td>pAgK84, pAtK84b</td>
<td>36</td>
</tr>
</tbody>
</table>

* In each case the agrocinogenic plasmid was pAgK84::Tn5A.1 (8), except for strain K439, which contained the wild-type type.
* Strains were grown on Stonier's medium with or without agrocinopines A and B (10 μM) for 48 h. Producer colonies were approximately 7 mm in diameter. After chloroform treatment, the plates were overlaid with strain K439 as the indicator.

Effect of Ti plasmid agrocinopine-constitutive mutation on agrocin 84 production. Ellis et al. (5) noted that agrocin 84 production was almost completely inhibited in a strain also containing pW11000, a mutant of pTIC58 constitutive for conjugal transfer and agrocinopine catabolism. From their experiments it was not clear whether this was a genetic or physiological effect. To approach this, we first determined whether the addition of agrocinopine A to strain C58 harboring pAgK84 and pTIC58 had any effect on agrocin 84 production. When this strain was cultured with 10 μM agrocinopine A, production of agrocin 84 was almost completely blocked (Table 3). As predicted, strain K439 containing the agrocinopine plasmid produced no detectable agrocin 84 when cultured with or without agrocinopine A. No such effect was seen with strain K84 or with strain NT-1 harboring pAgK84 and pAtK84b. The latter plasmid is the opine catabolic element of strain K84 (24) and encodes utilization of nopaline and uptake of agrocinopine A (5; G. T. Hayman and S. K. Farrand, unpublished observations).

We analyzed for transcriptional control by RNA dot-blot hybridization. Hybridization with a probe containing Smal fragments F2, B, and G was intense to RNA samples from strains containing pAgK84, but there was no detectable difference in the levels observed between the C58 and K439 backgrounds (data not shown). However, analyses of culture supernatants of the cells grown for RNA isolation clearly showed that while C58(pAgK84::Tn5A.1) yielded a high amount of antibiotic activity, the K439(pAgK84::Tn5A.1) culture produced little if any detectable agrocin 84 (data not shown). No hybridization was detected to RNA from strain C58 lacking the agrocin plasmid.

DISCUSSION

In this paper we present results indicating a complex arrangement of determinants involved in the biosynthesis of agrocin 84. All but one of the previously mapped (8) Tn5 insertions abolishing agrocin production could be complemented in trans. The single exception, insertion A-46, may be either a cis-acting mutation or an insertion in a plasmid carrying a second, undefined mutation elsewhere in the biosynthetic region. Such occasional double mutants have been observed in studies of A. tumefaciens virulence functions (9).

The agrocin biosynthetic region can be divided into at least five complementation groups. The smaller regions, such as group V (1.2 kb) may correspond to individual transcriptional units or even single genes, while the largest, group III (5.6 to 6.9 kb), may contain one or more transcriptional units. Taken as a whole, the region, which occupies at least 14 kb of pAgK84, may encode 12 to 14 proteins. The results suggest that the pathway for agrocin 84 production consists of a relatively large number of steps. Mutagenesis of the clones and transcript analysis will be required for further characterization of these regions.

Transcription polarity for groups I and IV can be inferred from the complementation data and the locations of the agrocin immunity functions. One immunity function is expressible from fragment D and maps to the group I region (Fig. 1 and 3A). Thus, the promoter for this function and therefore for the group I region must lie in Smal fragment D. However, Tn5 insertions in the group I region cannot be complemented with fragment D alone but also require Smal fragment H. This suggests that transcription is initiated in fragment D and proceeds clockwise into fragment H. Presumably the requirement for fragment H results from Tn5 insertions in the Smal-D region of the plasmid exerting polar effects on genes of this complementation group extending into the Smal-H region. Results for mutations in group IV parallel those obtained for group I, and by analogous reasoning, the direction of transcription is likely to be anticlockwise from the Smal-G region into the Smal-B region (Fig. 1 and 3B). The direction of transcription for groups II, III, and V cannot be inferred from the present data.

Immunity functions occupy two separate loci on the plasmid (Fig. 2 and 3). That either Smal fragment D or G is sufficient to confer immunity suggests that the two loci function independently rather than in concert. This is supported by the observation that no single insertion mapping to regions of the agrocin plasmid defined by fragments D or G abolishes immunity. However, insertions mapping to the immunity regions do block agrocin production, suggesting that immunity functions are associated with agrocin biosynthesis. The immunity function encoded by Smal fragment G corresponds closely in size and position to the clockwise end of complementation group IV, while that encoded by Smal fragment D is contained within complementation group I (Fig. 3).

Insertions in the 1.8-kb Smal-G immunity region result in production of small amounts of agrocin 84 (data not shown). This determinant might encode an antibiotic export system. Mutants in this function could well secrete small amounts of agrocin 84 by passive means. The second immunity function might be associated with attachment of an inactivating side group to the toxic molecule. The methyl pentanamide residue at the C-5' position could fulfill such a role since this group would have to be removed before agrocin 84 could be incorporated into replicating DNA as suggested by Murphy and Roberts (20).

In defined medium agrocin 84 is synthesized during the exponential phase of culture growth (P. J. Murphy, Ph.D. thesis, University of Adelaide, Adelaide, South Australia, 1981), but it is not known whether this represents the maximal production rate. It has been suggested that strain K84 parasitizes preexisting crown gall tumors, so one might expect synthesis to be stimulated in the vicinity of such plant hyperplasias. However, nopaline and agrocinopine A, the two opines utilized by strain K84 and associated with sensitive A. tumefaciens strains, had no detectable effect on agrocin production. Similarly, agrocinopine-associated exudate-associated vir gene induction signal (26), neither increased nor decreased the production of agrocin 84. Glucose and mannitol had no effect on agrocin 84 production, indicating that biosynthesis of the antibiotic is not catabolite repressible by either of these sugars.
Results (Table 3) showing that growth with agrocinopine A greatly reduces the amount of agrocin 84 produced by a strain also harboring wild-type pTiC58 confirm and extend the findings of Ellis et al. (5). While they demonstrated a similar effect in the absence of agrocinopine using a pTiC58 mutant constitutive for catabolism of this opine, our work shows that the phenomenon is associated with normal induction of the catabolic pathway. Levels of RNA homologous to the agrocin 84 biosynthetic region are as high in strain K439(pAgK84) as they are in strain C86(pAgK84) (data not shown), suggesting that this regulation does not function at the transcriptional level. The probe we used does not rule out the possibility that decreased transcription is occurring in only one of the transcriptional groups. However, the results are consistent with a physiological mechanism for decreased agrocin 84 production as proposed by Ellis et al. (5).

We did observe differences in the agrocinogenic response to agrocinopine A between strains harboring pTiC58 or pAtK84b. Whereas strains containing pTiC58 showed little or no detectable agrocin 84 production when induced with agrocinopine A, the opine had no such effect on strain K84 (Table 3). Strain NT-1 harboring both pAgK84 and pAtK84b responded in an identical manner, ruling out influence of the K84 chromosome and suggesting that there is some fundamental difference between the agrocinopine A catabolic systems encoded on pTiC58 and pAtK84b. This would be expected considering that under natural conditions strain K84 most likely continues to produce agrocin 84 in the near vicinity of tumors secreting agrocinopine A. Physiological suppression of agrocin 84 production by the opine would compromise the ability of strain K84 to displace the sensitive A. tumefaciens strains colonizing the tumor.

ACKNOWLEDGMENT

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LITERATURE CITED


