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Chemical Nature of Agrocin 84 and Its Effect on a Virulent Strain of *Agrobacterium tumefaciens*

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Agrocin 84, produced by *Agrobacterium radiobacter* K84, inhibited ribonucleic acid, deoxyribonucleic acid, and protein synthesis and amino acid transport in a susceptible, virulent strain of *A. tumefaciens* H-38-9. Cell motility was immediately stopped by action of the agrocin, 50% of the cells were killed within 15 min of contact, and the remainder were inhibited. Agrocin 84 is trypsin and pepsin resistant, but chemical analysis indicated a small peptide with a molecular weight of 2,500 containing six different amino acids, including nine molecules of glutamine or glutamic acid and seven molecules of serine.

Crown-gall is a neoplastic disease in plants produced by the bacterium *Agrobacterium tumefaciens*. Usually, two conditions must be met before the disease can be initiated: the plant must be wounded and the living virulent bacterial cells must come in direct contact with the dividing plant cells (for review see reference 10).

Htay and Kerr have shown that crown gall disease in plants can be controlled by the inhibitory action of an agrocin produced by *A. radiobacter* K84 upon the virulent *A. tumefaciens* strain (5). Some strains of *A. tumefaciens* are able to initiate tumors, and other closely related strains are unable to cause tumor production in plants. Recent work has shown this ability to be correlated with the presence of a large plasmid in the virulent strain. This plasmid in some strains not only codes for virulence but also codes for sensitivity to the agrocin produced by *A. radiobacter* K84 (15).

In this paper the action of agrocin 84 upon a susceptible, virulent strain of *A. tumefaciens* in regard to deoxyribonucleic acid, ribonucleic acid, and protein synthesis, amino acid transport, viability, and motility is described as well as the biochemical nature of agrocin 84.

MATERIALS AND METHODS

Bacterial strains. *A. radiobacter* K84, the agrocin 84-producing strain isolated by A. Kerr, was obtained from R. Hamilton, The Pennsylvania State University. The agrocin-susceptible, virulent strain of *A. tumefaciens* H-38-9 and the agrocin-resistant, avirulent strain of *A. tumefaciens* H-38-7 were derived from *A. tumefaciens* C-58 by R. Hamilton (4).

Media. Stock cultures of *A. radiobacter* K84 were maintained on AB agar, which contains K_2HPO_4, 3.0 g; NaH_2PO_4, 1.0 g; NH_4Cl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; KCl, 0.15 g; $CaCl_2$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 2.5 mg; glucose, 5.0 g (sterilized separately); agar, 15 g; and 1 liter of distilled water (2). *A. tumefaciens* H-38-9 and H-38-7 were grown on tryptic soy agar slants. All experiments were carried out in AB medium. For production of agrocin, AB broth was supplemented with 0.5% sodium glutamate. The cells were incubated for 3 days on a rotary shaker at 27°C. Upon centrifugation the agrocin was found in the supernatant fraction.

Agrocin 84 assay. Agrocin 84 activity was measured by the paper disk assay. A 30-ml portion of AB agar in a 15-cm petri dish was overlayed with 5 ml of AB 0.9% agar inoculated with a 0.5-ml suspension of *A. tumefaciens* H-38-9 from an overnight culture, which had been centrifuged and resuspended in AB broth to 3.0×10^9 cells/ml (30 Klett units, red filter).

A 60-μl portion of agrocin 84 was added to sterile 12.7-mm paper assay disks (Schleicher & Schuell Co.). The disks were dried and then placed on the surface of the agar overlay. After 24 h of incubation at 27°C, the zone of inhibition was measured from the edge of the disk to the edge of the zone. One arbitrary unit (AU) was defined as 1 mm of inhibition. The dose response was linear in the range of 16 to 133 AU/ml when tested at the appropriate dilution.

Isotopic labeling and radioactivity determination. Two 125-ml side-arm flasks containing 20 ml of AB broth were inoculated with 10^7 to 2×10^7 colony-forming units of of *A. tumefaciens* H-38-9 per ml and incubated on a rotary shaker at 27°C for 2 h. Agrocin at a final concentration of 8 AU/ml or an equal volume of AB broth was added, and immediately afterward ^{14}C-labeled mixed L-amino acids (NEC-445) or [^{14}C]uracil, to a final concentration of 0.5 μCi/ml, were added to both flasks (isotopes from New England Nuclear). Samples (50 μl) were pipetted onto 1-inch (ca. 2.5 cm) filter paper squares, dried, washed two times with cold 5% trichloroacetic acid and once with cold ethanol, dried, and counted in a Packard scintillation counter (8).

Transport of amino acids was measured in a similar manner, except that chloramphenicol (100 μg/

ml) was added to inhibit protein synthesis 30 min prior to the addition of ^{14}C-labeled mixed amino acids (0.5 μCi/ml). Cells were collected from 1.0-ml samples on a 0.22-μm membrane filter (Millipore Corp.), washed two times with 0.1 M phosphate buffer, pH 7.0, dried, and counted (8).

Motility was observed directly using a Zeiss phase contrast microscope. A drop of agrocin 84 was added to a wet mount of an overnight culture of *A. tumefaciens* H-38-9.

Cell killing was measured by the addition of agrocin 84 to 20 ml of a 2-h AB broth culture of *A. tumefaciens* and removing 0.1-ml samples at stated intervals for plating on AB agar.

Specificity of adsorption. Approximately 6×10^{10} *A. tumefaciens* H-38-9 or H-38-7 cells were mixed with 3 ml of agrocin 84, and at 0, 10, and 20 min 1-ml samples were filtered through a 0.22-μm membrane filter (Millipore Corp.) and assayed for biological activity.

Enzyme susceptibility. A 1-ml portion of agrocin 84 was incubated at 37°C for 2 h with 1 ml of Pronase (2 mg/ml) self-predigested for 2 h at 37°C (Sigma Chemical Co.) or trypsin (Sigma Chemical Co.) in 10 mM tris(hydroxymethyl)aminomethane buffer (pH 7.4) and then assayed for agrocin 84 activity by the paper disk assay. Pepsin was tested at pH 4.2.

Purification and molecular weight determination. The AB broth supernate was concentrated using an Amicon ultrafilter. The supernatant fluid was first passed through a XM300 membrane, which retains particles larger than 300,000 daltons. The filtrate was then passed through a UM10 membrane, which retains molecules larger than 10,000 daltons, and finally concentrated using a UM2 membrane, which retains molecules larger than 1,000 daltons and which, consequently, retained the agrocin. A 1-ml sample of this concentrated agrocin was then layered onto a G-25 Sephadex column (Pharmacia Fine Chemicals; bed size, 1.6 by 50 cm), equilibrated with 10 mM tris(hydroxymethyl)aminomethane buffer, pH 7.5. Fractions (0.5 ml) were collected with a Gilson automatic fraction collector, the adsorbance of each fraction was read at 260 nm, and the fractions were tested for biological activity by the paper disk assay. The molecular weight was determined from a standard curve prepared using oxytocin (Sigma Chemical Co.), glucagon (Sigma Chemical Co.), and bacitracin (Sigma Chemical Co.) as reference proteins (1). The amino acid analysis and molecular weight determination were made using the fraction from the G-25 column showing maximum biological activity, and the fractions collected before and after were used as controls.

RESULTS

Effect on *A. tumefaciens* H-38-9 cells. Protein synthesis, as measured by incorporation of ^{14}C-labeled amino acids, was almost completely inhibited within 10 min by the addition of agrocin 84. A similar pattern of inhibition occurred in DNA synthesis as measured by incorporation of [^{3}H]thymidine. The inhibitory effect on RNA synthesis was not apparent until 20 min after the addition of agrocin 84 and resulted in a 50% reduction of incorporation of [^{14}C]uracil when compared to the control cells (Fig. 1).

The transport of amino acids across the cell membrane was immediately stopped by the ad-

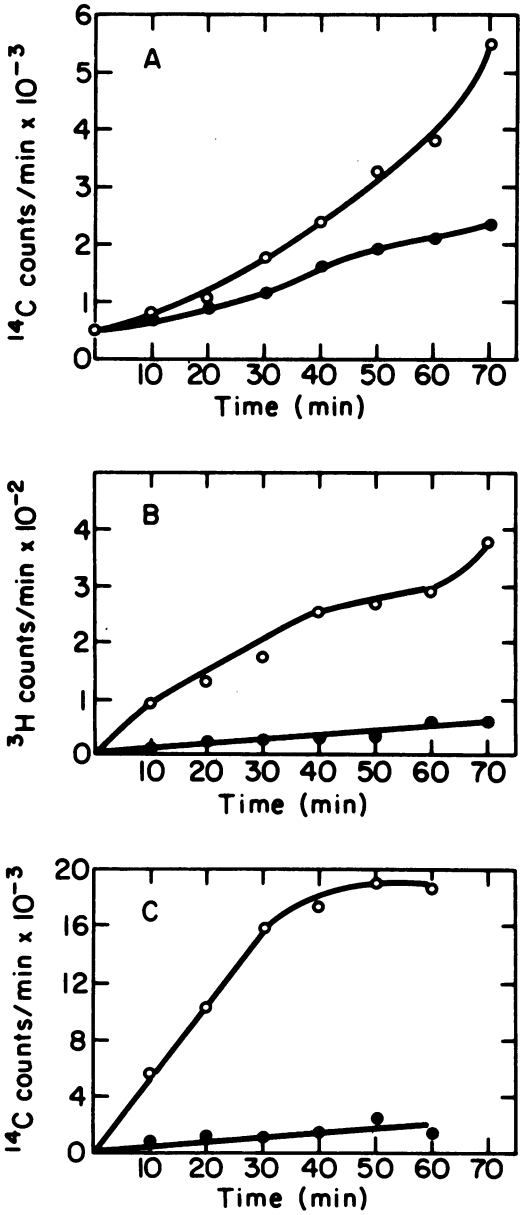

FIG. 1. *Effect of agrocin 84 on synthesis in A. tumefaciens H-38-9 agrocin added at zero time. (A) Ribonucleic acid synthesis at zero time. [^{14}C]uracil was added. (B) Deoxyribonucleic synthesis at zero time. [^{3}H]thymidine was added. (C) Protein synthesis at zero time. ^{14}C-labeled mixed amino acids were added. Symbols: ●, agrocin 84; ○, control.*

dition of agrocin to the cells, which correlates with the lack of incorporation of the ^{14}C-labeled amino acids into cell protein (Fig. 2).

The energy relationship of the effects of agrocin 84 on the susceptible cells was observed by the almost immediate cessation of motion, which occurred upon addition of agrocin 84 to a highly motile culture. The cells spun in place and then stopped entirely within 90 s.

Addition of agrocin 84 to an early-log-phase culture of *A. tumefaciens* H-38-9 resulted in the killing of between 40 and 50% of the cells within 15 min after addition of agrocin 84. No further reduction of colony-forming units occurred, but the remainder of the cells were inhibited, as the turbidity of the culture remained constant for 24 h.

Agrocin 84 did not adsorb to *A. tumefaciens* H-38-7, which is avirulent to plants but which does adsorb to the virulent, susceptible strain *A. tumefaciens* H-38-9 (Table 1). After 10 min of contact with the cells, 50% of the agrocin 84 was adsorbed.

Synthesis and characterization of agrocin 84. Maximum production of agrocin 84 occurred in AB medium supplemented with 0.5% sodium glutamate after 3 days of incubation at 27°C, when the *A. radiobacter* K84 culture was in stationary phase. No agrocin 84 was produced when the cells were grown in a complex medium containing peptone. Yields of agrocin 84 were less if the temperature of growth was 25 or 30°C rather than 27°C. The agrocin 84 was stable at room temperature, and no activity was lost after storage at 4°C for 6 months; however, boiling for 10 min caused complete inactivation. Treatment of the agrocin 84 with pepsin, trypsin, or Pronase did not reduce the biological activity.

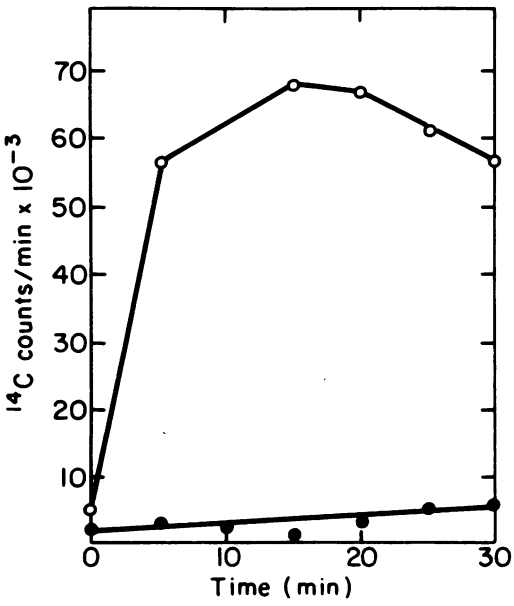

FIG. 2. *Effect of agrocin 84 on amino acid transport in A. tumefaciens H-38-9. Symbols: ●, agrocin 84; ○, control.*

TABLE 1. *Adsorption of agrocin 84*

Time (min)	Residual activity in filtrate (AU/ml)	
	H-38-9 (virulent)[a]	H-38-7 (avirulent)[a]
0	109	118
10	51	109
20	58	118

[a] *A. tumefaciens* strain used.

An estimate of the molecular weight of agrocin 84 was made by gel filtration on a Sephadex G-25 column. Agrocin 84 readily passes through a cellulose dialysis membrane, so the crude material was filtered through an Amicon ultra filter. The agrocin 84 passed through a UM10 membrane, which retains molecules larger than 10,000 daltons, but was retained by a UM2 membrane, which retains molecules larger than 1,000 daltons. When the filtrate was passed through a Sephadex G-25 column, the biological activity was located in a single peak, which also showed a slight absorbance at 260 nm (Fig. 3A). From a standard curve the molecular weight was estimated to be 2,450 (Fig. 3B).

Amino acid analysis of the fraction giving maximum biological activity demonstrated six different amino acids in the following proportions: arginine, 1; aspartic or asparagine, 1.7; serine, 6.9; glutamic or glutamine, 9; glycine, 3.1; and alanine residues, 3. On the basis of the amino acid analysis, the molecular weight estimate is 2,558 (Table 2).

DISCUSSION

The agrocin 84 produced by *A. radiobacter* K84 interacts specifically with the cell wall of the virulent strain of *A. tumefaciens* H-38-9 and does not adsorb onto the surface of the avirulent strain that was originally derived from the same parent strain, indicating that the receptor site on one strain is missing on the other strain. This reflects an alteration of a surface component coded for by the virulence plasmid. The bacterial cell surface is an important factor in virulence, as has been suggested by Lippincott and Lippincott (9). They postulate a specific receptor on the bacterial surface that interacts with a specific site on the plant

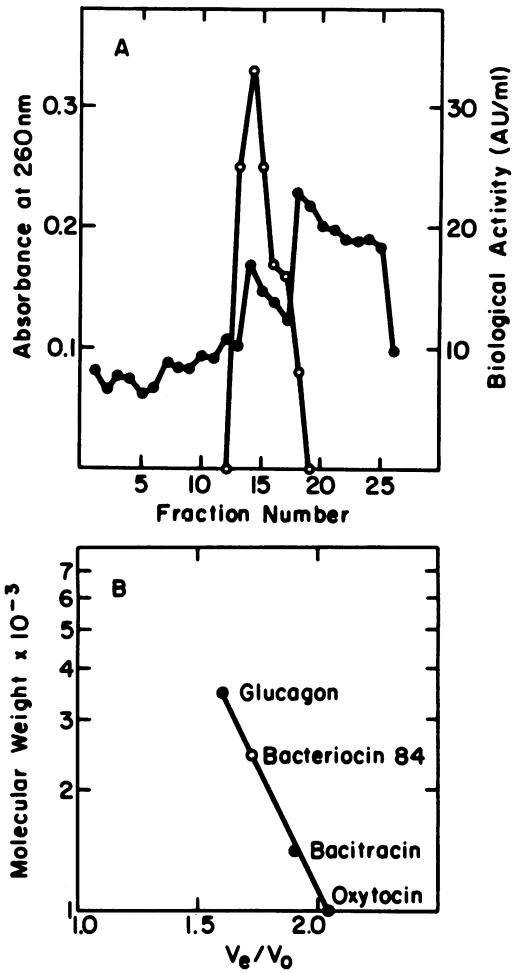

FIG. 3. *(A) Elution profile of agrocin 84 from a Sephadex G-25 column eluted with 10 mM tris(hydroxymethyl)aminomethane buffer, pH 7.5; 0.5-ml samples were collected. Symbols: ○, biological activity (AU/ml); ●, adsorbance at 260 nm. (B) Apparent molecular weight of agrocin 84 based upon elution from a Sephadex G-25 column. The void volume (V_0) was determined using blue dextran (2 mg/ml). To determine molecular weight, elution volume (V_e) divided by V_0 was plotted versus $\log_{10}$ of the molecular weight (1), using indicated marker proteins (●) and agrocin (○).*

cell wall to initiate crown gall disease. In competition experiments they demonstrate that virulent and avirulent strains compete for these sites. Kerr and Htay, however, demonstrated that an apparent competition between the virulent and avirulent strains could be the result of agrocin 84 produced by an avirulent strain that inhibited or killed the virulent strains (6). Both these mechanisms may be operative depending upon the strains involved in the competition.

The action of agrocin 84 is more than just the masking of a receptor site on the surface of the cell. The results show extensive inhibition of the susceptible strain by agrocin 84. The most immediate effect is loss of energy, as demonstrated by the abrupt loss of motility after contact with the agrocin. This reflects an influence upon the cell membrane, where the energy-generating reactions occur. Also, the transport of amino acids across the cell membrane was inhibited by the action of agrocin 84, again demonstrating an effect on the integrity of the membrane and associated transport functions.

A secondary effect on the cell was shown by the inhibition of deoxyribonucleic acid, ribonucleic acid, and protein synthesis, which ultimately led to the killing of half the cells, as measured by the ability to form colonies. The remainder of the cells, although still able to produce colonies, were inhibited from dividing in the presence of agrocin 84. On the susceptible cells, agrocin 84 has a very pronounced effect, similar to that shown by colicins K and E_1 on sensitive *Escherichia coli* cells (11, 12).

Agrocin 84 is, however, much smaller in size than the colicins. Most of the colicins have a

TABLE 2. *Amino acid analysis of agrocin fractions from a Sephadex G-25 column*[a]

Amino Acid	Preceding fraction (25 AU/ml)	Peak fraction (83 AU/ml)		Post-peak fraction (0 AU/ml)	Mol wt of amino acids
		Total	Ratio		
Lys		T			
Arg		73	1 (1)		156
Asp		127	1.7 (2)	113	230
Thr					
Ser		506	6.9 (7)	35	609
Glu	163	659	9.02 (9)	215	1161
Gly	54	230	3.1 (3)	250	171
Ala		220	3.0 (3)	91	213
Val					
Met					
Ile					
Leu					
Tyr					
Phe	X	X		X	
Water					18
Total mol wt					2558

[a] Values are expressed as picomoles per sample. Tryptophan, histidine, and proline are not detectable by the *O*-phthaladehyde method used (2). X indicates massive peak coming off in position of phenylalanine. The ratio represents the residues present calculated from the values obtained for the total amount. The numbers in parentheses indicate the theoretical content for each amino acid. Total number of residues was 25. T, Trace.

molecular weight of 50,000 or larger (7). Even the smaller bacteriocins such as streptocin A have a molecular weight of about 8,000 (14). Agrocin 84 has a molecular weight of 2,500, which is closer in size to the protein antibiotics such as gramicidin. The chemical analysis indicates agrocin 84 is a low-molecular-weight peptide composed of at least six different amino acids, predominated by serine and glutamine. The insensitivity of the molecule to trypsin indicates that the molecule lacks a peptide bond, in which the carbonyl function is donated by either a lysine or arginine residue, and resistance to pepsin shows lack of peptide bonds, in which the amino function is contributed by phenylalanine, tryosine, and tryptophan, as well as leucine and acidic amino acids (9). The amino acid analysis shows a lack of some of these amino acids, but it does contain the acidic amino acids aspartic and glutamic acid. Its resistance to Pronase suggests that it may have an unusual structure and be a circular polypeptide such as gramicidin or that it contains D- rather than L-amino acids.

Agrocin 84 is similar in some aspects to the agrocin produced by *A. tumefaciens* H100 and T37, first reported by Stonier (13). This agrocin was found to pass through a dialysis membrane and to be inhibited by the presence of peptone in the medium. Agrocin 84 is also not produced in a peptone-containing medium and does not show activity against cells growing in a complex medium. Different metabolic pathways are operative in a complex medium than in a defined medium, and either the agrocin 84 is not produced, or after production it is inactivated by combination with the ingredients in the complex medium.

The exact chemical nature of agrocin 84 and the receptor site on the bacterial cell surface are still unknown and remain for further study. This report shows the effect of agrocin 84 upon susceptible cells, separation of agrocin from the medium, and the chemical nature of this substance. Agrocin 84 should be a useful tool to identify exactly how the chemical structure of the cell surface of virulent cells differs from that of avirulent cells and provide a key to the interaction of the bacterial cell with the plant cell.

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