A Basis for Agrocin 84 Sensitivity in *Agrobacterium radiobacter*

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Agrocin 84 inhibits a virulent strain of *Agrobacterium radiobacter* containing a nopaline Ti plasmid, but not the same strain lacking the Ti plasmid; this specificity was investigated. Sensitivity to agrocin 84 was correlated with its active uptake by a high-affinity transport system with a K_m of 5.88×10^{-8} M. Unmetabolized agrocin 84 was transported inside the sensitive strain and was associated with the soluble fraction of ruptured bacteria and not with the outer walls or cytoplasmic membrane. Agrocin 84 bound to a protein fraction isolated from the periplasmic space of the sensitive strain; there was no equivalent binding activity in the insensitive strain. It is proposed that sensitivity to agrocin 84 is due to the presence of one or more plasmid-coded binding proteins which are associated with transport of agrocin 84 into sensitive strains.

INTRODUCTION

Crown gall is a neoplastic disease of plants induced by virulent strains of *Agrobacterium radiobacter*. In stone fruit, the disease is controlled biologically by dipping planting material in a cell suspension of an avirulent strain of *A. radiobacter* K84 (New & Kerr, 1972) which produces agrocin 84, a low molecular weight bacteriocin characterized as a fraudulent adenine nucleotide (Roberts *et al.*, 1977). The primary site of action of agrocin 84 has not yet been determined although McCardell & Pootjes (1976) demonstrated a major effect on DNA and protein synthesis.

The genes coding for sensitivity to agrocin 84 are located on the Ti plasmid, which also codes for virulence and nopaline metabolism (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975). Avirulent strains lacking a Ti plasmid are not sensitive to agrocin 84 (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975). The Ti plasmid is a conjugative plasmid and can be readily transmitted from virulent donor to avirulent recipient strains of *A. radiobacter*. The coded characters of virulence, nopaline metabolism and agrocin 84 sensitivity are also transmitted and expressed (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975).

The extraordinary selectivity of agrocin 84 for virulent strains of *A. radiobacter* carrying a nopaline Ti plasmid forms the subject of this paper.

METHODS

Bacterial strains. Four strains of *A. radiobacter* were used: K57, an avirulent strain insensitive to agrocin 84; K57A, the same strain after acquisition of a Ti plasmid following a Kerr cross (Kerr, 1971), sensitive to agrocin 84; K24, a virulent wild-type strain sensitive to agrocin 84 (our standard indicator strain for agrocin 84 production); K229, a rough colony variant of K84 which produces agrocin 84.

Media. The medium used for production of agrocin 84 contained (per litre): $(\text{NH}_4)_2\text{SO}_4$, 1.0 g; trisodium

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citrate, 0.5 g; sodium glutamate, 1.0 g; K_2HPO_4 , 1.05 g; KH_2PO_4 , 0.45 g; $Fe(NO_3)_3$, 5 mg; biotin, 200 μ g; trace elements (Gamborg & Eveleigh, 1968), 1.0 ml; and (added as separately sterilized solutions) glucose, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; thiamin, 5 mg; the final pH was 7.0. Strain K229 was inoculated and incubated on an orbital shaker for 48 h at 25 °C. For the preparation of [^{32}P]agrocin 84, the phosphate concentration was reduced to 1 μ g ml⁻¹ and 5 to 10 μ Ci ^{32}P ml⁻¹ was added after 24 h incubation. Agrocin 84 used for viability studies was prepared by incubating strain K229 as described above, removing the bacteria by centrifugation and sterilizing the supernatant by shaking with 0.5 vol. chloroform.

To determine the numbers of viable bacteria, samples were plated on Yeast Mannitol Agar (YMA) and incubated at 27 °C.

Chemicals. Carbenicillin was obtained from Beecham Research Laboratories; egg white lysozyme from Sigma. ^{32}P as orthophosphate was obtained from the Australian Atomic Energy Commission. The charcoal used was Nuchar Granular C (ash content, 2.95%; Fe, 0.11%). All other chemicals were analytical grade.

Protein determination. Protein concentrations were determined by Lowry's method, using bovine serum albumin as a standard. Where necessary, initial precipitation with 5% (w/v) trichloroacetic acid preceded protein determination.

Purification of [^{32}P]agrocin 84. Batches of 100 ml ^{32}P -labelled culture medium were stirred with 0.5 g charcoal at 4 °C for 24 h. The charcoal was removed by centrifugation (17000 g, 10 min) and stirred at 4 °C with two changes of 100 ml 70% (v/v) ethanol over 24 h. The pooled ethanol fractions were rotary evaporated at 35 °C, lyophilized, made up in 1 ml water and applied as a band to 3MM Whatman paper and subjected to electrophoresis (Tate, 1968); the buffer was triethylamine acetate, pH 5.0 [containing triethylamine (9.71 ml l⁻¹) and glacial acetic acid (5.72 ml l⁻¹)]. A thin strip of the electrophoretogram was bioassayed for agrocin 84 activity. Agrocin 84 was eluted with water and subjected to further electrophoresis in triethylamine borate buffer, pH 9.4 [containing triethylamine (14.0 ml l⁻¹) and boric acid (12.36 g l⁻¹)]. A thin strip of the electrophoretogram was scanned for ^{32}P activity on a Packard Radiochromatogram Scanner as well as bioassayed. The band corresponding to a single peak of ^{32}P activity, and also to biological activity, was eluted in water and stored at 4 °C. Agrocin 84 used to quantify kinetic studies was purified as outlined by Roberts *et al.* (1977).

Uptake studies. Cultures were grown overnight in Stonier's medium (Stonier, 1956) and then resuspended in fresh medium to give an A_{640} of 0.6 in a 1 cm cuvette. Portions (1 ml) of this bacterial suspension were maintained at 25 °C for 10 min, then [^{32}P]agrocin 84 was added and samples (2 $\times$ 50 μ l) were taken at the times indicated. These samples were immediately filtered through Sartorius membranes (0.45 μ m pore size) and the membrane and residue were washed with Stonier's medium (2 $\times$ 5 ml), placed in a scintillation vial and dried at 110 °C for 10 min. Scintillant [2 ml; containing 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (dimethyl-POPOP, 0.3 g l⁻¹) and 2,5-diphenyloxazole (PPO, 8 g l⁻¹)] was added to each vial and the radioactivity was counted in a Packard Tricarb scintillation counter. When inhibitors were added to cultures, the bacteria were preincubated with the inhibitor for 15 min before [^{32}P]agrocin 84 was added.

For kinetic studies, strain K57A was grown overnight in Stonier's medium and then resuspended to give an A_{640} of 0.15 in a 1 cm cuvette. Portions (1 ml) were taken and incubated at 25 °C for 5 min before [^{32}P]agrocin 84 was added. Samples (50 μ l) were removed at 30 and 60 s intervals, filtered and counted as above. The amount of agrocin 84 present was calculated by comparison with a standard solution of known absorbance at 264 nm and a molar extinction coefficient of 19860 (Roberts *et al.*, 1977).

Preparation of protein fractions. Protein extracts were prepared from 500 ml cultures grown in Stonier's medium for 48 h at 25 °C on an orbital shaker. The cultures were centrifuged at 6000 g for 10 min and then resuspended in 0.1 M-phosphate buffer pH 7.0 [containing $NaH_2PO_4 \cdot 2H_2O$ (7.8 g l⁻¹) and $Na_2HPO_4 \cdot 2H_2O$ (8.9 g l⁻¹)] to form a thick slurry. The bacteria were disrupted by three passages through a French pressure cell at 17.5 MPa. Whole bacteria were removed by centrifugation at 7500 g for 10 min at 4 °C. The supernatant was then filtered through Sartorius membrane filters (0.45 μ m pore size) to remove all bacteria and tested for sterility by plating on to YMA plates.

To produce periplasmic protein fractions, spheroplasts of *A. radiobacter* were made. This was done by a modification of the method of Klapwijk *et al.* (1975) used to lyse bacteria. Cultures were grown overnight in Stonier's medium and then resuspended in fresh medium to an A_{640} of 0.6. Sucrose (0.171 g ml⁻¹), carbenicillin (500 μ g ml⁻¹) and lysozyme (100 μ g ml⁻¹) were added and the cultures were incubated for a further 4 h. The bacteria so produced were susceptible to osmotic lysis, and the formation of spheroplasts with intact cytoplasm but modified outer cell walls and periplasmic space was verified by electron microscopy. In some preparations, up to 25% ghost bacteria were present. After centrifugation (5000 g, 15 min, 4 °C) the supernatant was removed and concentrated approximately 25-fold by filtering through an Amicon PM-10 filter. This fraction was designated the periplasmic fraction. The pellet was resuspended in 0.1 M-phosphate buffer pH 7.0 and the bacteria were disrupted by three passages through a French pressure cell at 17.5 MPa. After centrifugation (12000 g, 10 min, 4 °C) the supernatant was collected. This fraction was designated the cytoplasmic fraction.

Fig. 1. Effect of agrocin 84 on the viability of avirulent and virulent *A. radiobacter* strains. Avirulent strain K57: ●, control; □, with agrocin 84. Virulent strain K57A: ○, control; ■, with agrocin 84. (Agrocin 84: 7×10^{-7} M)

Binding assays. Agrocin 84 binding assays were performed using dialysis chambers made of 5 ml glass tubes in which 8/32 Visking dialysis tubing was placed. Stirring was by small magnetic stirrers. The protein sample (0.5 ml) was placed inside the dialysis bag; 3 ml 0.1 M-phosphate buffer pH 7.0, to which [32 P]-agrocin 84 (about 30000 c.p.m.) was added, was in the outside chamber. Dialysis equilibrium was attained after 24 h at 4 °C. Samples from inside and outside the bag were then taken and dried on glass fibre before counting in scintillant as previously described.

Bioassay. The bioassay technique was a modification of the method originally developed by Stonier (1960). Plates of Stonier's medium containing 1.3% (w/v) agar were poured to give 3 mm thickness; a well (5 mm diam.) was cut in the agar and 20 μ l agrocin 84 was added. This was left to diffuse for 3 h, during which time the plate was sterilized with chloroform, before the indicator strain was overlaid in buffered agar. Plates were incubated at 27 °C and the diameters of inhibition zones were measured after 48 h.

RESULTS

Effect of agrocin 84 on virulent and avirulent strains

The addition of non-purified agrocin 84 to pre-exponential phase cultures of the virulent strain K57A and the avirulent strain K57 resulted in the selective reduction of viability of K57A but had no measurable effect on K57 (Fig. 1).

Uptake of agrocin 84

When strain K57A was incubated in the presence of [32 P]agrocin 84, there was a rapid uptake of the bacteriocin which continued until a maximum level was reached at 2 h. Strain K57 showed negligible uptake of agrocin 84 (Fig. 2). The uptake of agrocin 84 into cells of the sensitive strain followed Michaelis-Menten kinetics; the uptake was by a high-affinity system with a K_m of 5.88×10^{-8} M. To determine whether the absence of uptake in strain K57 was due to a breakdown of agrocin 84, strain K57 was incubated with the bacteriocin for 2 h and then the incubation medium was examined by electrophoresis. No significant degradation of bacteriocin was observed.

Effect of temperature and metabolic inhibitors on the uptake of agrocin 84

When strain K57A was incubated at 0 °C there was no uptake of agrocin 84 (Fig. 3). On re-incubation at 25 °C, there was an immediate uptake at the same rate as with bacteria continuously incubated at 25 °C, indicating that the bacteria were not damaged by this treatment. Figure 4 shows the effect of some metabolic inhibitors on uptake of agrocin 84 by sensitive bacteria. Both sodium azide and dinitrophenol at 10 mM greatly reduced the

Fig. 2. Uptake of [³²P]agrocine 84 at 25 °C by *A. radiobacter* strains K57A (▲) and K57 (●). (Agrocine 84: 5×10^{-7} M, 15000 c.p.m. ³²P)

Fig. 3. Effect of temperature on the uptake of [³²P]agrocine 84 by *A. radiobacter* strain K57A: ●, control, incubated in Stonier's medium at 25 °C; ▲, incubated at 0 °C for 1 h and then transferred to 25 °C (arrow). (Agrocine 84: 5×10^{-7} M, 40000 c.p.m. ³²P)

Fig. 4. Inhibition of [³²P]agrocine 84 uptake by *A. radiobacter* strain K57A at 25 °C: ●, control (no inhibitor); △, 100 μM-dicyclohexylcarbodiimide; ■, 10 mM-dinitrophenol; ▲, 10 mM-sodium azide. (Agrocine 84: 5×10^{-7} M, 40000 c.p.m. ³²P)

Fig. 5. Agrocin 84 and its degradation products extracted from *A. radiobacter* strain K57A. Bacteria were incubated with [^{32}P]agrocin 84 for 2 h, washed four times with Stonier's medium and then sonicated. The extract was centrifuged to remove broken cell components and the supernatant was subjected to electrophoresis in $\text{Na}_2\text{B}_4\text{O}_7$; mobilities were determined relative to the markers 2-deoxyadenosine (0.0) and orange G (1.0). The electrophoretogram was scanned for ^{32}P and bioassayed for agrocin 84 activity.

Table 1. *Equilibrium dialysis binding of agrocin 84 to cytoplasmic and periplasmic fractions of Agrobacterium radiobacter*

Cytoplasmic and periplasmic fractions of *A. radiobacter* were prepared as described in Methods (using 500 μg carbenicillin ml^{-1} and 100 μg lysozyme ml^{-1} in experiment 1, and 500 μg carbenicillin ml^{-1} only in experiment 2). Binding of agrocin 84 was determined by equilibrium dialysis.

Strain	Fraction	^{32}P bound [c.p.m. (mg protein) $^{-1}$]	
		Expt 1	Expt 2
K57A	Cytoplasmic	1990	2336
K57A	Periplasmic	7885	5000
K57	Cytoplasmic	1845	2328
K57	Periplasmic	709	0

uptake of agrocin 84. Dicyclohexylcarbodiimide at 100 μM also reduced the uptake of agrocin 84, but not to the same extent as the other inhibitors which were used at higher concentrations.

Transport of agrocin 84 into sensitive bacteria

Sensitive bacteria which had previously been incubated in the presence of [^{32}P]agrocin 84 for 2 h at 25 $^{\circ}\text{C}$ were washed and then sonicated. Nearly 90% of the radioactivity remained in the supernatant fraction after centrifugation at 37000 g for 30 min to remove walls and membrane components (Sykes, 1971). Centrifugation at 160000 g for 4 h did not alter this distribution. Electrophoresis of the supernatant fraction yielded a major peak corresponding to free unbound agrocin 84 (Fig. 5). This peak also retained biological activity. Other minor peaks which have not yet been characterized were also found, in particular a non-mobile peak.

An agrocin 84 binding protein

As measured by equilibrium dialysis, agrocin 84 was bound to whole cell extracts of both sensitive and insensitive bacteria, but the specific activity of binding was about twofold higher for the sensitive strain. Removal of membrane particles and organelles by centrifugation at 200000 g for 2 h did not alter the specific activity of binding. When the cell extracts of both sensitive and insensitive strains were incubated with 1 mg protease ml^{-1} for 3 h at 37 $^{\circ}\text{C}$, which resulted in an 80% reduction in protein level, binding was eliminated indicating that agrocin 84 was binding to a protein component.

Localization of the agrocin 84 binding protein

To determine the location of the agrocin 84 binding protein, protein extracts from the periplasmic space and cytoplasm of *A. radiobacter* were made and tested by equilibrium dialysis. Table 1 shows the specific activity of binding of agrocin 84 to periplasmic and cytoplasmic fractions of both sensitive and insensitive strains.

DISCUSSION

Selective inhibition (Fig. 1) of the converted pathogenic *A. radiobacter* strain K57A by agrocin 84, while growth of its unconverted non-pathogenic precursor strain K57 is unaffected, confirms the earlier work on selectivity of agrocin 84 using plate bioassays (Roberts & Kerr, 1974). In addition, Fig. 2 shows that this extraordinarily selective growth inhibition, which is characteristic of bacteriocins (Reeves, 1972), is a consequence of a selective uptake of agrocin 84 by the sensitive pathogenic strain K57A.

At 0 °C (Fig. 3) no uptake of agrocin 84 was observed, demonstrating that an actively metabolizing cell is an essential pre-requisite for uptake. This observation was further supported by studies (Fig. 4) with the respiratory inhibitors sodium azide and dinitrophenol which are known to interfere with oxidative phosphorylation and which also markedly inhibit the uptake of agrocin 84. Likewise dicyclohexylcarbodiimide also inhibits the uptake of agrocin 84 and is known to interfere with ATPase function (Altendorf & Zitzmann, 1975).

Equilibrium dialysis studies of the binding of [³²P]agrocin 84 showed that periplasmic protein extracts from the sensitive strain K57A bound agrocin 84, whereas there was little or no binding (depending on the extraction method used) to periplasmic protein extracts of the insensitive strain. Agrocin 84 was bound equally well to cytoplasmic extracts from both sensitive and insensitive strains; this probably represents non-specific binding.

Our results provide no evidence for irreversible binding of agrocin 84 to wall components or any other organelle; this is in marked contrast to most other known bacteriocins such as the colicins (Reeves, 1972). Since the bulk of the intracellular bacteriocin is unbound (Fig. 5), it is likely that the periplasmic binding protein(s) (Table 1) are concerned with transport into the bacterium, and as such the observed binding would be a reversible phenomenon; unpublished results supports this suggestion.

The unique selectivity of agrocin 84 possibly resides in the presence of specific binding protein(s) in the sensitive strain K57A which is released into the surrounding medium in the presence of carbenicillin and is presumably periplasmic in origin. It is generally accepted that periplasmic proteins are involved in the uptake of a variety of low molecular weight substances (Boos, 1974). We suggest that agrocin 84 is the first recorded member of a new class of highly selective low molecular weight nucleotide bacteriocins. These nucleotide bacteriocins may be characterized by uptake of the bacteriocin rather than adsorption to the cell surface and by the presence in sensitive strains of specific receptor protein(s). Subsequent to uptake the intact bacteriocin may be metabolized intracellularly.

As the difference between non-pathogenic strains and converted pathogenic strains is known to be due to the presence in the latter of a large plasmid, it seems likely that the specific receptor protein(s) in the pathogenic strain K57A is plasmid-coded. During the preparation of this manuscript, the isolation of two plasmid-coded periplasmic proteins from *A. tumefaciens* C-58, a virulent strain sensitive to agrocin 84, was reported. The avirulent plasmid-free derivatives of this strain, which is not sensitive to agrocin 84, did not possess these proteins (Sonoki & Kado, 1978).

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