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Biological control of *Agrobacterium* species *in vitro*

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Isolates of *Agrobacterium radiobacter* var. *radiobacter* and *A. radiobacter* var. *tumefaciens* biotype 1 and 2 were screened on agar for sensitivity to bacteriocin 84 produced by *A. radiobacter* var. *radiobacter* strain 84 (Keane *et al.* 1970: *Australian Journal of Biological Science 23:* 585–95) using a modification of Stonier's (1960: *Journal of Bacteriology 79:* 889–98) bacteriocin screening technique. In addition, isolates of *A. radiobacter* var. *radiobacter* were screened for production of broad spectrum bacteriocins similar to bacteriocin 84. The influence of medium, incubation temperature, and incubation period on bacteriocin 84 production and expression were also studied.

Keywords *Agrobacterium radiobacter* var. *tumefasciens; Agrobacterium radiobacter* var. *radiobacter* strain 84; bacteriocins; biological control

INTRODUCTION

Biological control of crown gall disease caused by *Agrobacterium tumefaciens* (Smith & Town.) Conn. in field trials has been reported by Kerr (1972), Kerr & Htay (1974), New & Kerr (1972), Dhanvantari (1976), Schroth & Moller (1976), Moore (1977), Kerr & Panagopoulos (1977), and Spiers (1980). Kerr & Htay (1974) concluded that the biological control mechanism operated through the production of a bacteriocin and described simple laboratory tests based on the methods of Stonier (1960) for determining if pathogenic strains were subject to biological control. Kerr & Htay (1974) noted the presence of pathogens insensitive to bacteriocin 84 and speculated that it should be possible to isolate further bacteriocin producing strains capable of inhibiting such pathogens.

The aims of the present study were:

1. To determine the susceptibility of local isolates of *Agrobacterium* species to bacteriocin 84 produced by *A. radiobacter* var. *radiobacter* strain 84 (Keane *et al.* 1970) on agar.
2. To investigate factors influencing production and expression of bacteriocin 84 *in vitro*.
3. To identify local bacteriocin-producing strains.

MATERIALS AND METHODS

Effects of medium on bacteriocin 84 production

Stonier's (1960) method was used with modification. Plates of the medium under evaluation were swabbed with the producer strain (*A. radiobacter* var. *radiobacter* strain 84) and incubated at 28°c for 48 h. The producer strain was then wiped from the agar surface using a flamed slide and 1.5 cm diameter plugs were punched from the agar with a cork-borer. On removal the plugs were inverted and inserted singly into corresponding holes punched in the centre of basal medium (BM) plates (Stonier 1956). The plates of BM were then overlaid with a continuous lawn of phosphate buffer agar (Stonier 1960) containing the indicator strain (GC4), sensitive to bacteriocin 84 prepared by adding one 3 mm loopful of inoculum obtained directly from Tech agar (BBL) to 5 ml of buffer agar. The plates were then incubated at 28°c for 48 h. A clear inhibition zone surrounding the central plug was considered to be an indication of bacteriocin production.

Bacteriocin production was evaluated on the following media: BBL-MacConkey agar (MA); King's B (KB); Tech agar; tryptone glucose yeast extract agar (TGYA); eosin methylene blue agar (EMB); Czapek Dox agar (CDA); antibiotic assay agar (AA); base agar (BA); Difco Bacto-1% water agar (WA); 1% water agar + 1% yeast extract (WAY); nutrient agar (NA); citrate agar; malonate agar (MLA); Todd Hewitt agar (THA); tryptic soy agar (TSA); oxoid yeast extract agar (YEA); yeast mannitol agar (YMA); yeast extract salts agar (YS) (Dye 1968); basal medium (BM) (Stonier 1956); Patel's agar (Patel 1926); ketolactose agar (Bernaerts & De Ley 1963); and New & Kerr's agar (1970).

Effects of base medium, inoculation technique, and post-inoculation temperature on expression of bacteriocin 84 activity

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TABLE 2 — Effects of media, basal medium, inoculation technique, and post-inoculation temperature on expression of bacteriocin 84 activity (diam. inhibition zone in cm)

Medium	Tight whorl	Spot inocul. 0.05 ml	Agar plug in self 28°c	Agar plug in Tech 28°c	Agar plug in BM 28°c	Agar plug in BM 20°c	Total inhibition zone over the 6 tests
YMA	4.5	4.0	4.5	4.5	6.5	7.5	31.5
CDA	6.0	6.0	5.0	4.0	4.5	5.0	30.5
BM	5.0	5.0	4.5	5.0	4.5	5.0	29.0
Tech	4.7	5.2	4.0	4.0	5.0	6.0	29.0
YESA	6.0	4.5	4.0	3.5	5.0	5.5	28.5
YEA	4.5	5.0	3.5	4.0	5.2	5.6	27.8
Ketolactose	4.5	5.2	3.5	3.0	5.0	6.0	27.2
AA	3.5	4.0	4.0	4.0	5.5	6.0	27.0
MA	4.0	4.0	3.0	4.0	5.0	6.0	26.0
TGYA	4.0	4.0	4.0	3.0	5.0	6.0	26.0
NA	4.5	5.0	3.0	3.0	5.0	5.5	26.0
EMB	3.0	4.0	4.0	3.5	4.5	5.0	24.0
BA	4.0	4.0	3.5	3.0	3.5	4.0	23.0
Patels	0.0	0.0	0.0	5.0	6.0	7.0	18.0
TSA	0.0	0.0	0.0	4.0	6.0	7.0	17.0
Citrate	0.0	0.0	0.0	4.0	5.0	6.0	15.0
KB	0.0	0.0	0.0	0.0	7.0	7.5	14.5
THA	0.0	0.0	0.0	0.0	6.5	7.0	13.5
New & Kerr	—	—	—	4.0	4.5	4.5	13.0
MLA	0.0	0.0	0.0	3.0	4.0	4.5	11.5
WAY	—	—	—	2.0	2.5	3.0	7.5
WA	0.0	0.0	0.0	2.0	2.5	3.0	7.5

TABLE 1 — Nomenclature and origin of isolates tested for bacteriocin 84 sensitivity

Strains	Number	Biotype
1. Ag 1–32, 34–62, 64–72 75–78, 80–100	95	1
Ag 33, 63, 73, 74, 79	5	1–2
2. GD 1–45	45	1
3. GC 1–26	26	1
4. NAG 2–11, 16, 18, 20–22	15	1
NAG 17	1	2
NAG 1, 12–15, 19	6	1–2
5. NK 6	1	1
NK 9, 13, 15–17, 21, 31–34, 36–37, 39, 41, 43–48	19	2
NK 1–5, 7–8, 10–12, 14, 18–20 22–30, 35, 38, 40, 42, 49–50	30	1–2
6. NKRS 1–3, 5–20	19	2
NKRS 4	1	1–2

1. AG — isolates from nursery soil obtained on dilution plates of Kado & Heskett (1970) and Clark (1969).
2. GD — isolates obtained from galls on *Salix matsudana* × *S. alba* seedlings on Kado & Heskett's (1970) selective medium.
3. GC — isolates obtained from galls on *Salix matusdana* × *S. alba* seedlings on Clark's (1969) selective medium.
4. NAG — isolates obtained from nursery soil on dilution plates of New & Kerr's (1970) selective medium.
5. NK — isolates obtained from nursery soil on dilution plates of New & Kerr's (1970) selective medium.
6. NKRS — isolates obtained from soil under galled rose bushes from the Esplanade, Palmerston North on New & Kerr's (1970) selective medium.

medium on bacteriocin 84 production and expression were compared by inserting agar plugs into the parent medium, BM, and Tech. The agar plug inoculation technique was compared with Stonier's (1960) spot inoculation method and in addition plates were inoculated centrally with a loop in a tight whorl. The effects of post-inoculation temperatures of 20° and 28°C were also evaluated using plugs from the different media inserted into BM.

Effects on medium, incubation time, and incubation temperature on bacteriocin 84 activity

The effects of incubation temperature and period were assessed on Tech, BM, and YEA at 20°, 28°, 32°, and 34°C. After 1, 2, 3, 5, and 7 days agar plugs were removed and inserted into BM overlaid with isolate GC4 and incubated at 28°C. Treatments were replicated 4 times.

Screening isolates for bacteriocin 84 sensitivity

Two hundred and sixty-two isolates were screened by growing strain 84 on Tech agar at 28°C for 48 h. A total of 182 biotype 1, 39 biotype 2, and 41 biotype 1–2 isolates (Spiers 1979) (Table 1) were screened. The plates were then scraped and several plugs per plate were removed, inverted, and inserted centrally into BM plates and overlaid with the strain being tested and incubated at 28°C for 48 h.

Non-pathogenic isolates not sensitive to bacteriocin 84 were tested similarly for production of bacteriocins that inhibited the following five strains:

1. *A. radiobacter* var. *radiobacter* biotype 2 strain 84 (Keane *et al.* 1970)
2. *A. radiobacter* var. *tumefaciens* biotype 2 strains NK6
3. *A. radiobacter* var. *tumefaciens* biotype 1 strains GC4, GC12, GC16.

Isolates found to inhibit these strains were screened for inhibition of an additional 30 pathogenic biotype 1 isolates, viz, Ag 1–10, GC 1–10, and G–C 1–10.

RESULTS

Effects of medium on bacteriocin 84 production

The influence of medium on bacteriocin 84 production is shown in Table 2. All media tested supported bacteriocin 84 production. On some media bacteriocin 84 activity was expressed only when agar plugs were inserted into another medium, e.g., citrate MLA, KB, THA, TSA, WA, and Patel's agar. The best media for bacteriocin 84 production were, YMA, ketolactose, YEA, MA, TGYA, NA, YESA, Tech, CDA, EMB, AA, BA, and BM. Of these media Tech was chosen for screening isolates for bacteriocin 84 sensitivity.

Effects of basal medium, inoculation technique and post-inoculation temperature on expression of bacteriocin 84 activity

For most media, bacteriocin activity was expressed better when plugs were inserted into Stonier's (1956) BM rather than into the parent medium or Tech. Inhibition zones were larger when the plates of BM were incubated at 20°C instead of 28°C after inoculation with the indicator strain (Table 2). Stonier's (1960) spot inoculation technique gave comparable results to the tight whorl and the agar plug technique except on citrate, malonate, YEA, King's B, THA, TSA, WA, and Patel's agar. These media gave no expression using Stonier's (1960) spot inoculation and the tight whorl inoculation methods (Table 2). The agar plug technique was easier to use than Stonier's (1960) spot technique for routine bacteriocin screening tests. With the technique it was possible to obtain up to 10 bacteriocin 84 containing plugs which in turn could be used to screen 10 different isolates. By reducing the plug size more isolates could have been screened.

Effects of medium, incubation time, and incubation temperature on bacteriocin activity

The effects of medium, incubation time, and temperature on bacteriocin 84 production are given in Table 3. The effects of temperature and incubation period on bacteriocin 84 production were influenced by media. For example, zero bacteriocin 84 production was recorded on BM and YEA after incubation

TABLE 3 — Effects of medium, incubation temperature, and incubation period on expression of bacteriocin 84 activity (diam. inhibition zone in cm)

	BM				TECH				YEA		
Incubation Period (days)	20°c	28°c	32°c	34°c	20°c	28°c	32°c	34°c	20°c	28°c	34°c
1	0.0	3.0	3.0	3.0	5.4	4.5	4.0	4.0	0.0	4.5	3.0
2	4.0	5.0	4.6	4.2	5.0	5.0	4.5	4.5	3.0	6.0	6.0
3	5.0	4.5	4.5	4.5	3.0	3.0	3.0	3.0	5.0	5.5	5.5
4	4.5	4.0	4.0	4.0	3.0	3.0	3.0	3.0	5.0	5.3	5.0
5	4.2	4.0	4.0	4.0	3.0	3.0	3.0	3.0	5.3	5.3	5.3
6	4.0	4.0	4.0	4.0	3.0	3.0	3.0	3.0	5.3	5.5	5.5

at 20°c for one day and high production was recorded on Tech. On all media regardless of temperature bacteriocin 84 production declined after reaching a peak with increasing incubation time viz, BM 4 days, Tech and YEA 2 days. In general, the effects of temperature and incubation period on bacteriocin 84 production were related directly to the amount of growth of strain 84.

Screening of isolates for bacteriocin 84 sensitivity

The sensitivity of *Agrobacterium* isolates to bacteriocin 84 is listed in Table 4. Twenty-four percent of the biotype 1 isolates were insensitive to bacteriocin 84; this include 2% of the pathogenic and 63% of the non-pathogenic isolates. Five percent of the non-pathogenic biotype 2 isolates tested were inhibited by bacteriocin 84 and the one pathogenic isolate was not inhibited. Twenty five percent of the biotype 1–2 isolates were sensitive to bacteriocin 84. Inhibition zones formed by isolates sensitive to bacteriocin 84 were uniformly 4.0 cm in diameter.

Screening of isolates for bacteriocin production

None of the 64 non-pathogenic biotype 1 isolates tested produced bacteriocins, and 2 of the 38 biotype 2 isolates produced bacteriocins which inhibited all 33 pathogenic biotype one and the 1 pathogenic biotype two isolate tested. Twelve of the biotype 1–2 isolates tested inhibited some of the pathogenic isolates; however, the inhibition zones formed were transient and were overgrown within 5 days. The reasons for this are obscure, since transient inhibition zones were also formed when the plugs were chloroformed before insertion, thus eliminating competition from any remaining producer bacteria as a possible explanation.

TABLE 4 — Sensitivity of isolates to bacteriocin 84

	Biotype 1	Biotype 1–2	Biotype 2
Number of isolates examined	187	36	39
Pathogenic (%)	66	0	2.5
Inhibition by Bacteriocin 84 (%)	76	26	5
Inhibition of pathogenic isolates (%)	98	—	0
Inhibition of non-pathogenic isolates (%)	37	26	5

DISCUSSION

The relationship between biological control and bacteriocins was first established by Kerr & Htay (1974) who showed that there was a correlation beteen pathogenicity and bacteriocin sensitivity. Roberts & Kerr (1974) later confirmed this relationship and, since all bacteriocins described to date were proteins and functioned through attachment to receptor sites, assumed that the same mechanism applied to bacteriocin 84. They concluded that tumor-inducing bacteria must therefore have a specific molecular configuration on the cell surface acting as the receptor site for bacteriocin 84. When this configuration was altered, pathogenicity was lost. Moore (1977) supported Kerr & Htay (1974) and similarly concluded that strain 84 prevented infection through a bacteriocin rather than by exclusion of the pathogen from the cell infection site as proposed by Lippincott & Lippincott (1969). During field trials infection was controlled completely only when pathogens sensitive to bacteriocin 84 were present (Spiers 1980). On agar plates 98% of pathogenic biotype 1 *Agrobacterium* were inhibited by bacteriocin 84. The correlation between non-pathogenic isolates and bacteriocin resistance was not complete since 38% of these biotype 1 isolates were sensitive to bacteriocin 84. In comparison only 5% of the non-pathogenic biotype 2 isolates were sensitive. Roberts & Kerr (1974) noted the rare occurrence of both non-pathogenic strains sensitive to bacteriocin 84 and pathogenic strains resistant to bacteriocin 84, and speculated that these two groups must have either a different cell surface molecular configuration or alternatively have the same molecular configuration but are immune to bacteriocin 84.

The agar plug modification of Stonier's (1960) bacteriocin screening technique enabled isolates to be screened against uniform levels of bacteriocin 84 using minimal amounts of media. The technique permitted optimum expression of bacteriocin 84 activity in that it utilised different media for bacteriocin production and expression. It also eliminated

the need to chloroform plates to kill remaining producer bacteria. Little is published on factors influencing production of bacteriocin 84, which is surprising because this compound is important in biological control. *In vitro* tests showed that although bacteriocin 84 was produced on all media, for certain media bacteriocin activity was expressed only when agar plugs were inserted into another medium. The reasons for this are unknown, since if none was produced on the first medium, or if bacteriocins were inactivated after formation, no subsequent expression would have occurred. Bacteriocin 84 formation was influenced by incubation medium and incubation temperature and period. Bacteriocin production was directly related to growth of the producer bacterium, and factors reducing growth, viz, unfavourable media or incubation temperatures or insufficient incubation period, also adversely affected bacteriocin production. Bacteriocin production peaked coincident with peak bacterium growth, declined as growth entered the lag phase, and then remained static. Within a particular incubation regime the inhibition zones formed were of uniform diameter. In addition, the diffusion rate of bacteriocin 84 was influenced by the basal medium and post inoculation temperature.

During this study only 2 of the 38 biotype 2 isolates produced effective bacteriocins. These isolates now require field evaluation. There appear to be few isolates that can produce effective broad spectrum bacteriocins similar to bacteriocin 84. None of the 64 biotype 1 isolates produced bacteriocins. Moore (1977) reported that 3 strains out of 32 isolates of *A. radiobacter* biotype 2 examined effectively prevented infection of mazzard cherry seedlings. Kerr & Panagopoulos (1977) reported that 17 out of 221 strains of *A. radiobacter* biotype 1 showed only limited bacteriocinic activity, whereas none of the 399 strains of *A. radiobacter* biotype 2 were bacteriocinic. These reports indicate that an international collection would be assembled of non-interacting bacteriocinic strains of *A. radiobacter* var. *radiobacter* biotype I and II for controlling crown gall disease. If this were done it may reduce the likelihood of infection by pathogens resistant to bacteriocins.

Further work is necessary to investigate factors influencing bacteriocin production *in vivo* and to clarify the relationship between pathogenicity and bacteriocin resistance and non-pathogenicity and bacteriocin sensitivity.

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REFERENCES

Bernaerts, M. J.; De Ley, J. 1963: A biochemical test for crown gall bacteria. *Nature 197:* 406–7.

Clark, A. G. 1969: A selective medium for the isolation of *Agrobacterium* species. *Journal of Applied Bacteriology 32:* 348–51.

Dhanvantari, B. N. 1976: Biological Control of crown gall of peach in southwestern Ontario. *Plant Disease Reporter 60:* 549–51.

Dye, D. W. 1968: A taxonomic study of the genus *Erwinia*. I. The "amylovora" group. *N.Z. Journal of Science 11:* 590–607.

Kado, C. I.; Heskett, M. G. 1970: Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas,* var. *Xanthomonas*. *Phytopathology 60:* 969–76.

Keane, P. J.; Kerr, A.; New, P. B. 1970: Crown gall of stone fruit II. Identification and nomenclature of *Agrobacterium* isolates. *Australian Journal of Biological Science 23:* 585–95.

Kerr, A. 1972: Biological control of crown gall: seed inoculation. *Journal of Applied Bacteriology 35:* 493–7.

Kerr, A.; Khin Htay, 1974: Biological control of crown gall through bacteriocin production. *Physiological Plant Pathology 4:* 37–44.

Kerr, A.; Panagopoulos, C. G. 1977: Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. *Phytopathologische Zeitschrift 90:* 172–9.

Lippincott, B. B.; Lippincott, J. A. 1969: Bacteria attached to a specific wound site as an essential stage in tumor initiation by *Agrobacterium*. *Journal of Bacteriology 97:* 620–8.

Moore, L. W. 1977: Prevention of crown gall on *Prunus* roots by bacterial antagonists. *Phytopathology 67:* 139–44.

New, P. B.; Kerr, A. 1970: A selective medium for *Agrobacterium radiobacter* Biotype 2. *Journal Applied Bacteriology 34:* 233–6.

————1972: Biological control of crown gall: Field measurements and glasshouse experiments. *Ibid. 35:* 279–87.

Patel, M. K. 1926: An improved method of isolating *Pseudomonas tumefaciens* Sm. and Town. *Phytopathology 16:* 577.

Roberts, W. P.; Kerr, A. 1974: Crown gall induction: Serological reactions, isozyme patterns and sensitivity to mitomycin C and to bacteriocins, of pathogenic and non-pathogenic strains of *Agrobacterium radiobacter*. *Physiological Plant Pathology 4:* 81–92.

Schroth, M. N.; Moller, W. J. 1976: Crown gall controlled in the field with a non-pathogenic bacterium. *Plant Disease Reporter 60:* 275–8.

Spiers, A. G. 1979: Isolation and characterisation of *Agrobacterium* species. *N.Z. Journal of Agricultural Research 22:* 631–00.

————1980: Biological control of *Agrobacterium* species on *Salix*. *Ibid. 23:* 139–42.

Stonier, T. 1956: Labelling crown gall bacteria with P^{32} for radioautography. *Journal of Bacteriology 72:* 259–68.

————1960: *Agrobacterium tumefaciens* Conn. II. Production of an antibiotic substance. *Ibid. 79:* 889–98.