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Conditions for the production of Agrocin 84 by *Agrobacterium radiobacter* K84

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SUMMARY

A chemically defined medium was developed that supported the growth of *Agrobacterium radiobacter* K84 and the production of agrocin 84. Various supplements were investigated for their effect on growth rate and production of agrocin 84 using a well-diffusion assay method. Mannitol was found to be a better substrate for growth of *A. radiobacter* K84 compared to the other sugar alcohols and sugars tested. By contrast, D-fructose was the better substrate for the production of agrocin 84. Biotin supplementation stimulated production of agrocin 84 but did not eliminate the diauxic lag seen with the basal medium. The opine, octopine, inhibited growth of *A. radiobacter* K84 and production of agrocin 84 as did coenzyme B_{12}. By contrast, the cytokinin, isopentenyl adenosine, was marginally stimulatory to production, as was vitamin B_{12}. Acetosyringone supplementation had a negligible effect on growth rate and production of agrocin 84.

INTRODUCTION

Crown gall, a neoplastic disease affecting many important fruit crops is caused by *Agrobacterium tumefaciens* [30]. Infection of orchards and vineyards with this microorganism has had significant worldwide economic impact on the agricultural industry [12]. Since the early 1970's, however, this disease has been amenable to biological control by exploiting unique properties of the non-pathogen *Agrobacterium radiobacter* strain K84 [13,14,23]. This bacterium can compete with the pathogen for binding sites on the plant wound [2,29] but more importantly can elaborate the bacteriocin, agrocin 84, which is inhibitory specifically to *A. tumefaciens* [14]. *A. radiobacter* K84 is potentially useful only in controlling the wide host range strains (biovars 1 and 2) of *A. tumefaciens*, but not the limited host range strain (biovar 3) which affects grapevines

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[10,24]. In addition, agrocin 84 appears to be effective only against the *A. tumefaciens* strains that carry a nopaline plasmid [5,16] and in certain strains capable of transporting and catabolizing agrocinopines [4,5].

The potential for biological control of this pathogen has resulted in an intense investigation, particularly at the genetic level, of both the pathogen and the biological control agent [6,8,26]. In spite of this concentrated activity little is known concerning factors controlling the biosynthesis of this valuable bacteriocin or its mechanism of action.

Agrocin 84, whose production is plasmid-encoded [28], has been described as a fraudulent adenine nucleotide [25]. It has been shown to inhibit growth [14], affect nucleic acid synthesis [3,22,33] and inhibit protein synthesis [22] probably as a terminator of DNA chain elongation [28] of *A. tumefaciens*.

In studying the physiology of *A. radiobacter* K84, Farrand's group reported that nopaline and agrocinopine A had no effect on the production of agrocin 84, nor did the *vir* gene inducer, acetosyringone, have an effect [26]. They also reported that ferric ion, glucose, arabinose, mannitol, adenosine or deoxyadenosine had no effect on the production of agrocin 84.

In earlier investigations, Kersters and DeLey [17] demonstrated the utilization of 2-keto-3-deoxy-6-phospho-D-gluconate (KDPG) by a number of *A. tumefaciens* and *A. radiobacter* strains, thus establishing the presence of the Entner-Doudoroff metabolic pathway in this genus. This observation was extended by Arthur et al. [1] who, in addition, demonstrated the presence of a pentose phosphate cycling system and the absence of the Embden-Meyerhof-Parnas pathway in *A. tumefaciens*. By implication, the Entner-Doudoroff pathway assumes importance as an anaplerotic system providing intermediates for the synthesis of normal nucleotides and unusual nucleotides such as agrocin 84.

In this study, a variety of parameters that affected growth of *A. radiobacter* K84 and production of agrocin 84 were investigated as a means of establishing a predictable, chemically-defined, environment in which to study further the physiology of *A. radiobacter* K84 and the biosynthesis of agrocin 84.

MATERIALS AND METHODS

Bacterials strains

Agrobacterial strains were kindly supplied by Dr. Mary-Dell Chilton of Ciba-Geigy Biotechnology Division, Research Triangle Park, North Carolina. The strains were maintained either on M-928 [20] slants and plates or in garden soil supplemented with calcium carbonate (1.0%) and dried blood (0.25%) [7].

Media

Medium M-928 [20] in liquid form was used to prepare inocula of these organisms. Growth and production of agrocin 84 was determined in a modified Stonier [32] medium. The modified Stonier's medium (FMS) contained in g/l: K_2HPO_4, 10.25 g; KH_2PO_4, 5.6 g; NH_4NO_3, 2.7 g; mannitol, 10 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.2 g; $CaSO_4$, 0.1 g; $Fe(NO_3)_3$, 5 mg; $MnCl_2$, 100 mg; $ZnCl_2$, 500 mg; and adjusted to pH 7.0. Other sugar alcohols and sugars were used in place of mannitol as indicated in the Results section. Supplements such as phenolics, cytokinins and vitamins were added to FMS media prepared with mannitol as the carbon source. The sugars and sugar alcohols were autoclaved separately and the supplements were sterilized by filtration through a nitrocellulose membrane (0.45 μm).

The procedure of Mayr-Harting et al. [21] was utilized to assay for agrocin 84 activity by using FMS medium solidified with Bacto-agar (0.8%). After autoclaving the medium it was allowed to cool in a 45°C water bath prior to the addition of mannitol (10 g/l), biotin (200 μg/l) and the indicator microorganism, *A. tumefaciens* C58.

Chemicals

D-(+)-octopine, nopaline, trans-zeatin, trans-zeatin riboside, isopentenyl adenosine, coenzyme B_{12}, vitamin B_{12}, *d*-biotin, *i*-erythritol, D-mannitol and potassium gluconate were purchased from Sigma Chemical Co., St. Louis, MO. Inositol, D-sorbitol, dulcitol, Bacto-agar and yeast extract were purchased from Difco, Detroit, MI. Glycerol, D-fructose and D-glucose were purchased from

Fisher Scientific. Acetosyringone was purchased from Aldrich Chemical Co., Milwaukee, WI. and sodium glutamate was purchased from Calbiochem, San Diego, CA.

Conditions of cultivation

Inocula for production media were prepared by inoculating 100 ml of liquid M-928 medium, in a 250-ml Erlenmeyer flask, with approximately 100 mg of soil culture of *A. radiobacter* K84 and incubated at 28°C in a gyratory incubator at 200 rpm.

After 48 h, cultures at a density of approximately 600 Klett units (540 nm filter), were harvested by centrifugation at 4500 × *g* for 30 min at 15°C. The pellet was washed twice in a similar manner with 100 ml of 0.85% NaCl solution. The washed cells were suspended in half the original volume with 0.85% NaCl solution.

Side arm nephlo flasks (300 ml, Bellco, Vineland, NJ.), containing 100 ml of FMS media (supplemented as indicated in the text), were inoculated with 5 ml of the washed cell suspension (1×10^6 viable cells/ml) and incubated as described above.

Assay procedures

Growth curves were developed from turbidimetric analyses using a Klett-Summerson colormeter with a 540 nm filter. Determinations were made every 6, 12 or 24 h as indicated in the text.

Production of agrocin 84 was determined by measuring inhibitory activity against *A. tumefaciens* C-58 in samples taken from production media every 6, 12 or 24 h. These samples were treated with half a volume of chloroform and the aqueous phase was used to load wells in bioassay plates.

Nuncatom bioassay plates (243 × 243 × 18 mm) were prepared with 150 ml FMS medium containing 0.8% agar, 300 mg of sodium glutamate and 30 μg of biotin. After the autoclaved medium was cooled in a 45°C waterbath and supplemented as described, 0.5 ml of an *A. tumefaciens* C-58 culture, grown to a Klett turbity of 600 units in M-928 medium, was added, vigorously mixed and poured. The plates were refrigerated overnight and holes were punched in the agar with a number 3 cork borer and filled with 50 μl of samples. The plates were incubated for 2 days at 28°C. The diameter of the zone of inhibition minus the diameter of the well (6.3 mm) was used as a parameter of production.

Growth and production in FMS medium (index medium) was analyzed statistically. The standard error of the mean was derived from the standard deviation. This value, in conjunction with '*t*' test analysis, was used to determine the confidence limits of the system for each time period. The effect on growth and production of substitutions or supplementations to FMS medium was not considered significant if the experimental values fell within the confidence limits of the index system. The mean values were within 5 to 12% of the extreme values of the confidence limits.

RESULTS

Effect of phosphate buffer concentration on growth and production

FMS media were prepared at four concentration levels of potassium phosphate buffer (10, 50, 100 and 200 mM) at pH 7.0. The media were inoculated and assayed as described in Materials and Methods. Growth at all four levels of buffer displayed a diauxic lag during the first 12 to 36 h. Media containing 50 or 100 mM phosphate buffer showed the best growth response (Fig. 1A). The final pH was determined on day 3 (Fig. 1B). Media prepared with 100 or 200 mM phosphate buffer resisted changes in pH while media containing 10 mM phosphate buffer became acidic (pH <4). Similarly, agrocin 84 activity was greatest at the higher phosphate concentrations (Fig. 1C). Production of agrocin activity coincided with the beginning of the exponential phase of growth and peaked at the end of this phase. In subsequent studies, FMS media prepared in 100 mM potassium phosphate buffer at pH 7.0 was chosen as the index medium. This level of phosphate buffer displayed the better growth response, resisted change in pH, and achieved peak levels of growth and agrocin 84 production sooner than the other media. Growth and production were followed over a five day period in this medium and repeated eight times in duplicate. These data were

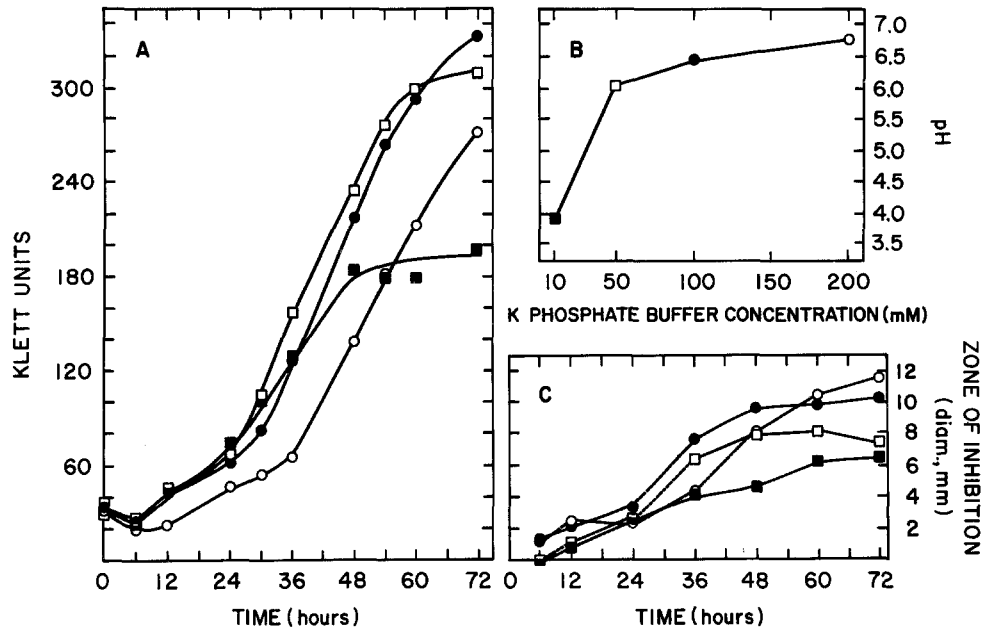

Fig. 1. The effect of phosphate buffer concentrations in FMS media on the (A) growth of *Agrobacterium radiobacter* K84, (B) final pH of the medium, and (C) production of agrocin 84. Potassium phosphate buffer concentrations at pH 7.0: 10 mM (■); 50 mM (□); 100 mM (●); and 200 mM (○).

analyzed statistically as described and used as a basis for comparison of the effects on growth and production of other modifications to the index medium.

As can be seen in Fig. 2, reproducibility of both growth and production during the earlier hours of incubation was quite variable; however, between the second and fourth day of incubation the variability was between 5 and 12% for each time period between 48 and 96 h.

Effect of carbon sources on growth and production

To determine the effect of carbon sources on growth and production, mannitol was replaced with an equivalent molar amount of other sugar alcohols (glycerol, inositol, sorbitol, dulcitol, erythritol), sugars (fructose, glucose), or potassium gluconate. Glycerol was added at twice the molar concentration (100 mM) to achieve an equivalent carbon load.

Fructose, glucose, glycerol and gluconate supported growth of *A. radiobacter* K84 and production of agrocin 84 (Fig. 3A,B,C,D). Like mannitol (Fig. 2), these carbon sources also showed a diauxic lag during the first 12 to 36 h of incubation, except gluconate, which displayed an extended lag phase of growth (Fig. 3D). The final pH fell between 6.3 and 6.5 with these carbon sources. Mannitol was superior to the other carbon sources, with peak growth values at 72 h of 320 ± 13.7 Klett units, followed by fructose, gluconate, glycerol and glucose. Inositol, sorbitol, dulcitol or erythritol did not support growth of *A. radiobacter* K84 or production of agrocin 84.

The carbon sources that supported growth also supported the production of agrocin 84 activity (Fig. 3A,B,C,D); however, fructose appeared to be the better substrate for agrocin production followed by glucose, glycerol, gluconate, and mannitol. The peak production levels achieved in the presence of fructose, glucose and glycerol may be statistically significant because they produced greater than 10.35 mm of inhibition, the upper value within the confidence limits of the index medium. Gluconate supplementation, on the other hand, produced about as much agrocin 84 activity as did mannitol.

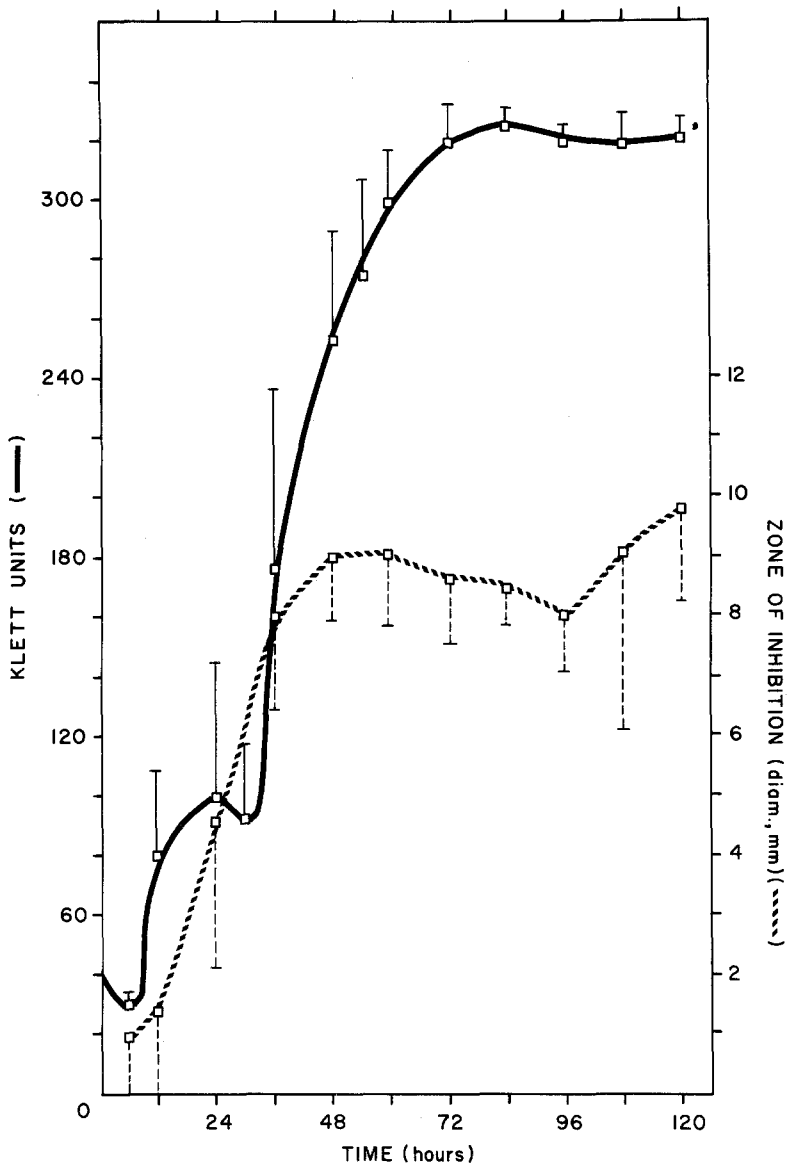

Fig. 2. Growth (——) of *Agrobacterium radiobacter* K84 and production (----) of agrocin 84 in FMS (index) medium. Klett readings of 50 and 100 correspond to optical densities of 0.410 and 0.840, respectively, at 540 nm measured in a Milton Roy Spectronic 601 spectrophotometer. At a Klett reading of 300 the viable cell count was 2.8×10^8/ml. The half-bars represent the upper or lower confidence limits for growth (——) and production (-------), respectively, of the mean values at at $p = 0.1$.

Both in the presence of mannitol or fructose, agrocin 84 activity was detected within the first 6 to 12 h of incubation, but with glucose and glycerol, activity was not detected until after 12 h of incubation, and after 24 h of incubation in the presence of gluconate.

In a comparative study, using AB media [22] which contains 25mM phosphate buffer, growth of *A. radiobacter* K84 was about 75% of that seen with FMS media with either glucose or mannitol as the carbon source. Production of agrocin 84 activity, however, was about half of that seen using FMS medium (Table 1). By contrast, glucose was similar to mannitol as a carbon source in AB media for

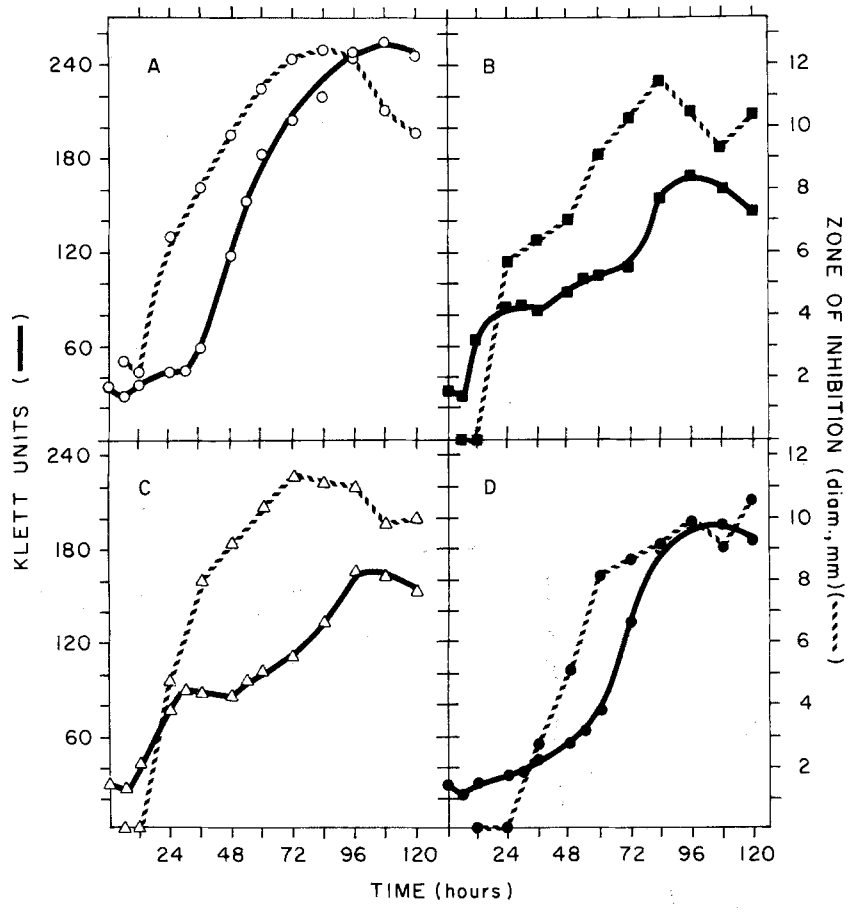

Fig. 3. The effect of (A) fructose (○), (B) glucose (■), (C) glycerol (△) and (D) gluconate (●) in FMS media on growth (——) of *Agrobacterium radiobacter* K84 and production (-----) of agrocin 84.

growth of the organism but glucose depressed the production of agrocin 84 activity. In addition, after 2 days incubation, AB media registered less than pH 5.0 from an initial value of 7.0, with either carbon source, while FMS media was at about pH 6.3 (Table 1). Since production occurs during active growth, acidification of the medium could account for diminished growth and hence diminished production of agrocin 84. Alternately, the sensitivity of the bacteriocin to acidic conditions could play a role in the apparent diminished yield.

The effect of opines on growth and production

The imino acid opines, nopaline and octopine produced by transformed plant cells, have been shown to serve as a nutrient source for the inciting *Agrobacterium* [16]. Since opines also affect the expression of other plasmid-encoded properties in *Agrobacterium* species [8], their effect on the plasmi-

Table 1

A comparison of AB and FMS media on the growth of *Agrobacterium radiobacter* K84 and production of agrocin 84 measured after 48 h incubation

Medium	Growth (Klett units)	Production (Zone of inhibition, diameter, mm)	Final pH
FMS with mannitol	240	9.6	6.3
FMS with glucose	115	5.0	6.3
AB with mannitol	186	4.4	4.4
AB with glucose	163	2.5	4.7

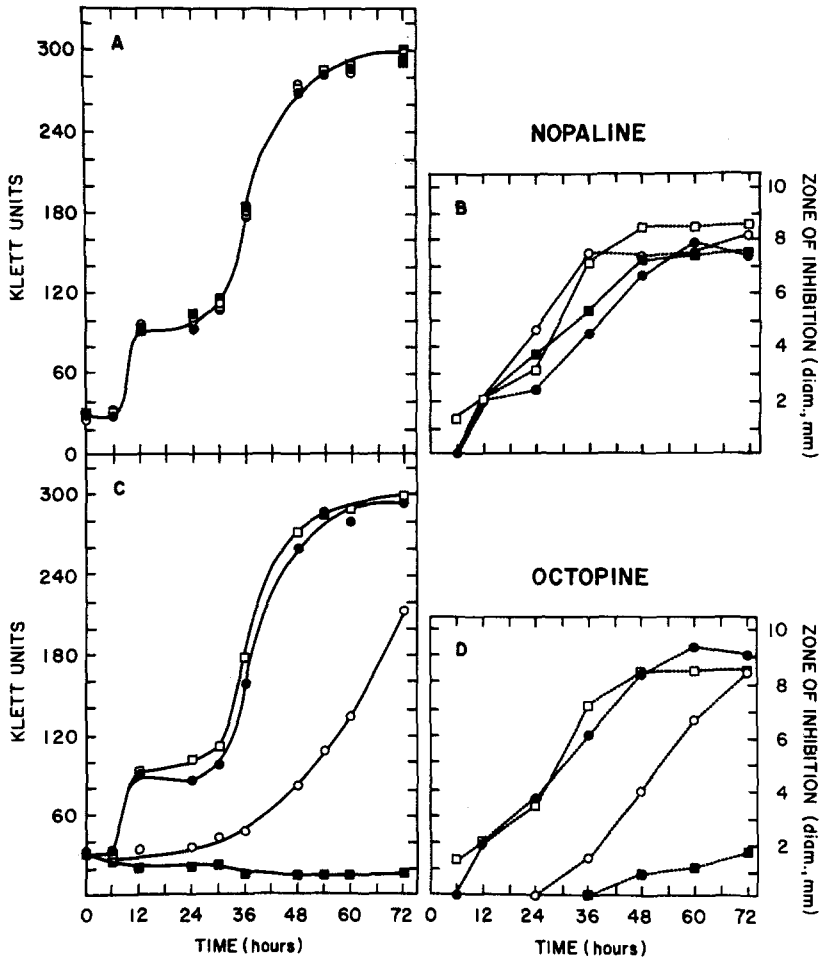

Fig. 4. The effect of nopaline (A, B) and octopine (C, D) at 1 μM (●), 10 μM (0) and 100 μM (■) on growth (——) of *Agrobacterium radiobacter* K84 and production (----) of agrocin 84 compared to control media (□).

dencoded property, agrocin 84 production [28], was also investigated.

It was found that supplementation of FMS medium with nopaline at three concentration levels (1, 10 and 100 μM) had no discernible effect on the growth of *A. radiobacter* K84, the diauxic lag or the production of agrocin activity (Fig. 4A and 4B). On the other hand, the addition of octopine at 100 μM inhibited growth completely and diminished agrocin production considerably (Fig. 4C and 4D). At a concentration of 10 μM, octopine inhibition was seen as a 36 h delay in the initiation of the exponential phase of growth and a similar delay in the production of agrocin. By 72 h, however, production levels were the same as control values. At 1 μM the effects on growth and production were negligible.

The effect of cytokinins on growth and production

The similarity in composition oof agrocin 84 [25] with the cytokinins prompted an investigation of their effects on production of this bacteriocin. One could speculate that the cytokinins could serve as substrate in the production of agrocin 84 or, because of similarity in structure, could inhibit the production of agrocin. The minor effects seen under these experimental conditions, using trans-zeatin, trans-zeatin riboside and isopentenyl adenosine at three concentrations, were inconclusive. The pro-

Table 2

The effect of various supplements to FMS media on growth of *Agrobacterium radiobacter* K84 and production of agrocin measured after 72 h incubation

	Growth, as % of control	Production, as % of control
A. Cytokinins		
trans-Zeatin		
1 μM	98.4	97.5
10 μM	88.9	100.0
100 μM	73.0	101.3
trans-Zeatin Riboside		
1 μM	96.8	90.0
10 μM	95.0	102.5
100 μM	95.0	105.0
Isopentenyl Adenosine		
1 μM	100.0	100.0
10 μM	109.5	108.8
100 μM	96.8	115.0
B. Vitamins		
Biotin		
0.4 μM	79.4	132.6
0.8 μM	87.4	127.9
2.0 μM	79.0	121.5
4.0 μM	79.0	123.3
Vitamin B-12		
1 μM	86.0	108.8
10 μM	101.6	111.3
100 μM	88.9	112.5
Coenzyme B-12		
1 μM	88.9	112.5
10 μM	22.9	65.0
100 μM	0.0	0.0
C. Phenolics		
Acetosyringone		
1 μM	109.0	103.6
10 μM	101.0	93.5
100 μM	98.8	92.6

duction values generated fell within the margin of error of the system, even though there was a progressive increase in these values with concentration, particularly with isopentenyl adenosine (Table 2).

The effect of vitamins on growth and production

Biotin has been shown to be required by some strains of *Agrobacterium* [19] while the B_{12}'s were investigated for their putative effect on nucleotide metabolism. The supplementation of FMS medium with biotin, vitamin B_{12} or coenzyme B_{12} revealed that coenzyme B_{12} was inhibitory to growth of the microorganism and production of agrocin 84 with increasing concentrations of the coenzyme (Table 2). By contrast, vitamin B_{12} supplementation resulted in consistently elevated production values; however, these values fell within the margin of error of the index system therefore cannot be considered significant. The effect with biotin was more dramatic. The overall effect of biotin supplementation at four concentrations was a minor inhibition of growth and a 20 to 30% stimulation of agrocin ac-

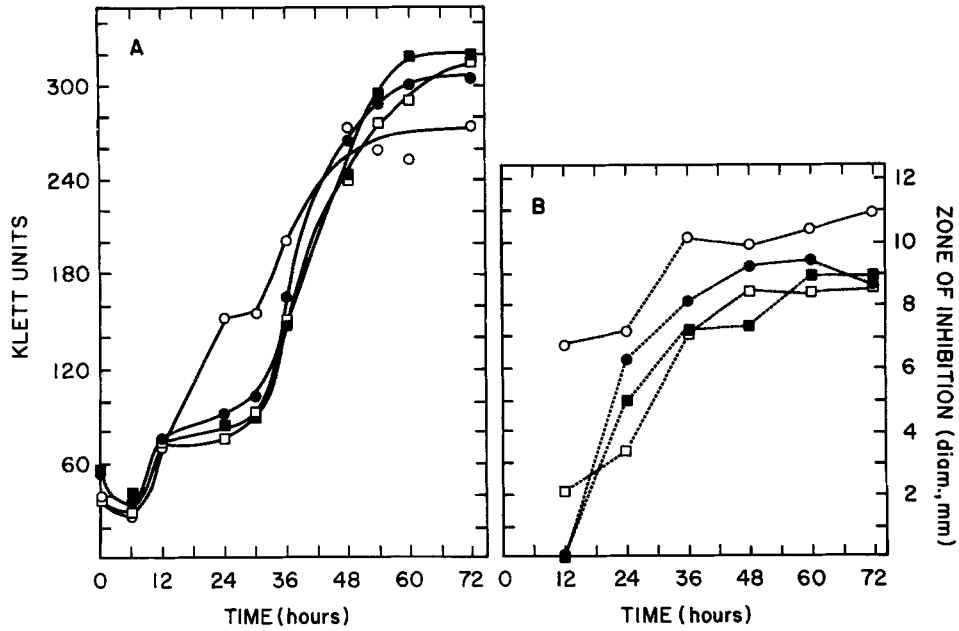

Fig. 5. The effect of 1 μM biotin (0), vitamin B_{12} (●) and coenzyme B_{12} (■) supplementation on growth (A) of *Agrobacterium radiobacter* K84 and production (B) of agrocin 84 compared to control media (□).

tivity (Table 2). At 1 μM, biotin supplementation delayed the diauxic lag seen with unsupplemented media or media supplemented with B_{12}'s at an equivalent concentration (Fig. 5A). Higher levels of biotin, however, did not eliminate the diauxic lag (data not shown). Biotin supplementation resulted in an initial burst of production and an overall 30% stimulation of production after 72 h (Fig. 5B).

The effect of acetosyringone

Acetosyringone has been shown to act as an inducer of virulence (*vir*) genes in *A. tumefaciens* resulting in, among other things, the synthesis of cytokinin [11]. Since agrocin 84 appears to be a structural analog of cytokinin, conceivably, acetosyringone could also act to induce the DNA sequences responsible for agrocin 84 synthesis. To test this hypothesis, FMS medium was supplemented with acetosyringone at three concentrations. At a concentration of 1 μM, the effect on growth and production was negligible. At the higher concentrations (10 and 100 μM) a slight depression occurred suggesting acetosryringone had little effect on growth of *A. radiobacter* K84 or production of agrocin 84 (Table 2).

The effect of glutamate on growth and production

Adding an additional nitrogen source to FMS medium in the form of sodium glutamate at 1,2,5 and 10 mg/ml of medium did not eliminate the diauxic lag; however, at 10 mg/ml sodium glutamate was slightly inhibitory to growth. The effect on production of agrocin activity was negligible at all concentrations of glutamate. By omitting mannitol but not NH_4NO_3 from the medium, sodium glutamate as both a carbon and nitrogen source did not support growth or production (data not shown).

DISCUSSION

Under all experimental conditions described in this study, production of agrocin activity was always growth related in that production coincided

with the exponential phase of growth. The growth and production kinetics seen mitigate against agrocin as a secondary metabolite (non-growth associated production), even though agrocin displays antibiotic activity [34]. Its antibiotic activity, however, is host-specific [2] suggesting a bacteriocin, although its chemical nature suggests otherwise [25].

Agrocin 84 is sensitive to inactivation above pH 8.0 and below pH 4.0 [9,33]. The apparent stimulation of production observed at the higher concentrations of phosphate buffer could be explained as protection from inactivation during extended periods of incubation (Fig. 1). Similarly, the poorer response seen in AB medium, or in FMS media prepared with lower buffer concentrations, could be due to the acidification of the medium which could result in diminished growth, hence diminished production. (Table 1).

The effect of other modifications of the basal medium provided some insight into avenues to be explored in discerning the mechanism of biosynthesis of agrocin 84. For instance, the growth response seen with mannitol, in comparison with the effects of glucose, fructose, gluconate and glycerol, was suggestive of the utilization of the Entner-Doudoroff pathway (Figs. 2 and 3) [18]. Enzymes associated with the Entner-Doudoroff pathway have been demonstrated in a number of agrobacterial strains [17]. The non-responsiveness to sorbitol could indicate a transport problem with this sugar alcohol since both sugar alcohols were processed similarly by other microorganisms utilizing the Entner-Doudoroff pathway [18]. Similarly, the diauxic lag seen could reflect the time required to induce the appropriate transport systems.

The effect with erythritol appeared anomalous, since *A. radiobacter* K84 is a biovar 2 strain; however, for purposes of physiological classification a more complex medium is generally used [15]. When the Kerr and Panagopoulos medium was used (data not shown), growth could been seen after a week of incubation at 28°C. In the chemically defined medium used in this study erythritol could not serve as a carbon source. The factors implicating the Entner-Doudoroff pathway are currently under investigation.

A. radiobacter K84 can utilize nopaline as a carbon and nitrogen source [26], but under the experimental conditions employed, the small amounts of nopaline added did not affect either growth or production (Fig. 4A and 4B). Octopine additions, on the other hand, had a more dramatic effect (Fig. 4C and 4D). Not only was octopine not utilized, but the inhibition seen for both growth and production was proportional to the concentrations of octopine used. Consequently, the suppression of growth in the presence of octopine is better explained as inhibition of metabolism rather than as an inability of the nopaline-agrobacteria to utilize octopine as a carbon and nitrogen source.

Agrocin 84 and the cytokinins share similarities in composition and structure suggesting a similarity in biosynthetic pathways and genetic loci. As such, the cytokinins could serve either as substrate for agrocin 84 biosynthesis and thus stimulate production, or alternately, because of shared properties could inhibit enzymes responsible for the biosynthesis of agrocin 84. Similarly, acetosyringone, which not only has been implicated in triggering the passage of bacterial DNA into the plant cell [31], has also been shown to induce *vir* genes with subsequent expression of the *ipt* cytokinin gene in T-DNA [11]. The results reported indicated that acetosyringone did not stimulate a related gene (Table 2). Acetosyringone did not stimulate production, verifying a previous report [26]; this observation suggests the constitutive nature of the agrocin locus or the genetic unrelatedness of cytokinin and agrocin biosynthesis. DNA homology studies between these loci could help to clarify this question.

The agrocin 84 molecule contains deoxyarabinose [25] which may require a B_{12} derivative to effect the rearrangement of a precursor to the deoxy sugar. The coenzyme form of the vitamin was profoundly inhibitory to growth and production while vitamin B_{12} itself was only suggestive of a positive effect. Perhaps these cofactors imposed an allosteric or feed-back restriction on growth and production. Biotin supplementation, on the other hand, although it did not eliminate the diauxic lag seen under basal conditions, did increase the ultimate yield of agrocin activity by approximately 30%. The ex-

act role of these vitamins in the biosynthesis of agrocin 84 requires further explorations.

We have developed a completely synthetic medium that can be used to explore further the biosynthesis of this important bacteriocin. The effect of biotin implicated the carboxylations required in this biosynthesis. Moreover, the growth and production responses seen in the presence of certain sugars and sugar alcohols warrants further excursions into the Entner-Doudoroff pathway. The inhibitory effects seen with octopine may, under certain circumstances, compromise the use of *A. radiobacter* K84 as a biological control agent.

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REFERENCES

1 Arthur, L.O., L.A. Bulla, Jr., G. St. Julian and L.K. Nakamura. 1973. Carbohydrate metabolism in *Agrobacterium tumefaciens*. J. Bacteriol. 116: 304–313.

2 Cooksey, D.A. and L.W. Moore. 1982. Biological Control of Crown Gall with an Agrocin Mutant of *Agrobacterium radiobacter*. Phytopathology 72: 919–921.

3 Das, A., S. Stachel, P. Ebert, P. Allenza, A. Montoya and E. Nester. 1986. Promoters of *Agrobacterium tumefaciens* Tiplasmid Virulence Genes. Nucleic Acid Res. 14: 1355–1364.

4 Ellis, J.G. and P.J. Murphy. 1981. Four New Opines from Crown Gall Tumors: Their Detection and Properties. Mol. Gen. Genet. 181: 36–43.

5 Engler, G., M. Holsters, M. Van Montague, J. Schell, J.P. Hernalsteens, and R. Schilperoort. 1975. Agrocin 84 sensitivity: A plasmid Determined Property in *Agrobacterium tumefaciens*. Mol. Gen. Genet. 138: 345–349.

6 Formica, J.V. 1988. Crown Gall Neoplasms. In: The Pathobiology of Neoplasia. (A.E. Sirica. ed.), pp. 497–512, Plenum, NY.

7 Formica, J.V. and M.J. Waring. 1983. Effect of Phosphate and Amino Acids on Echinomycin Biosynthesis by *Streptomyces echinatus*. Antimicrob. Ag. Chemothr. 24: 735–741.

8 Gheysin, G.P. Dhaese, M. Van Montague and J. Schell. 1985. In: Advances in Plant Gene Research (B. Hohn and E.S. Dennis, eds.), vol. 2, pp. 11–47, Springer Verlag, Wien.

9 Heip, J., G.C. Chatterjee, J. Vanderkerckhove, M. Van Montague and J. Schell. 1975. Purification of the *Agrobacterium radiobacter* 84 Agrocin. Arch. Int. Physiol. Biochem. 83: 974–976.

10 Hendson, M., L. Askjaer, J.A. Thomson and M. Van Montague. 1983. Broad host range agrocin of *Agrobacterium tumefaciens*. Appl. Environ. Microbiol. 45: 1526–1532.

11 John, M.C. and R.M. Amasino. 1988. Expression of an Agrobacterium Ti Plasmid Gene Involved in Cytokinin Biosynthesis is Regulated by Virulence Loci and Induced by Plant Phenolic Compounds. J. Bacteriol. 170: 790–795.

12 Kennedy, B.W. and S.M. Alcorn. 1980. Estimates of U.S. Crop Losses to Procaryotic Plant Pathogens. Plant Dis. 64: 674–676.

13 Kerr, A. 1972. Biological Control of Crown Gall. Seed Inoculation. J. Appl. Bacteriol. 35: 493–497.

14 Kerr, A. and K. Htay. 1974. Biological Control of Crown Gall Through Bacteriocin Production. Physiol. Pl. Path. 4: 37–44.

15 Kerr, A. and C.G. Panagopoulos. 1977. Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. Phytopath. Z. 90: 172–179.

16 Kerr, A. and W.P. Roberts. 1976. Agrobacterium: Correlations Between and Transfer of Pathogenicity, Octopine and Nopaline Metabolism and Bacteriocin 84 Sensitivity. Physiol. Pl. Path. 9: 205–211.

17 Kersters, K. and J. Delay. 1968. The occurrence of the Entner-Doudoroff pathway in bacteria. Antonie van Leeuwenhoek. 34: 393–408.

18 Lessie, T.G. and P.V. Phibbs, Jr. 1984. Alternate Pathways of Carbohydrate Utilization in Pseudomonads. Ann. Rev. Microbiol. 38: 359–387.

19 Lippencott, J.A. and B.B. Lippencott. 1975. The Genus *Agrobacterium* and plant tumorigenesis. Ann. Rev. Microbiol. 29: 377–407.

20 Loper, J.E. and C.I. Kado. 1979. Host Range Conferred by the Virulence-Specifying Plasmid of *Agrobacterium tumefaciens*. J. Bacteriol. 139: 591–596.

21 Mayr-Hartung, A., A.J. Hedges, and R.C.W. Berkely. 1969. Methods for Studying Bacteriocins. In: Methods of Microbiology. Vol. 7A. pp. 315–422, (J.R. Norris and D.W. Ribbons, eds.), Academic Press, London.

22 McCardell, B.A., and C.F. Pootjes. 1976. Chemical Nature of Agrocin 84 and its Effect on a Virulent Strain of *Agrobacterium tumefaciens*. Anitmicrob. Ag. Chemothr. 10: 498–502.

23 New, P.B. and A. Kerr. 1972. Biological Control of Crown Gall: Field Measurements and Glasshouse Experiments. J. Appl. Bact. 35: 279–287.

24 Ondrey, M. and J. Vlasak, 1987. Sensitivity of Different *Agrobacterium* strains to Agrocin 84. Folia Microbiol. 32: 239–243.

25 Roberts, W.P. and M.E. Tate, and A. Kerr. 1977. Agrocin 84 is a 6-*N*-Phosphoramidate of an Adenine Nucleotide Analogue. Nature 265: 379–381.

26 Ryder, M.H., J.E. Slota, A. Scarim, and S.K. Farrand. 1987.

Genetic Analysis of Agrocin 84 Production and Immunity in *Agrobacterium* spp. J. Bacteriol. 169: 4184–4189.

27 Sciaky, D., A.L. Montoya and M-D. Chilton. 1978. Fingerprints of *Agrobacterium* Ti Plasmids. Plasmid 1: 238–253.

28 Slota, J.E., and S.K. Farrand. 1982. Genetic Isolation and Physical Characterization of pAgK84, the Plasmid Responsible for Agrocin 84 Production. Plasmid 8: 175–186.

29 Smith, V.A. and J. Hindley. 1978. Effect of Agrocin 84 on Attachment of *Agrobacterium tumefaciens* to Cultured Tobacco Cells. Nature 276: 498–500.

30 Smith, E.F. and C.O. Townsend. 1907. A plant-tumor of Bacterial Origin. Science. 25: 671–673.

31 Stachel, S.E., B. Timmerman and P. Zambryski. 1986. Generation of Single-Stranded T-DNA Molecules During the Initial Stages of T-DNA transfer from *Agrobacterium tumefaciens* to Plant Cells. Nature 322: 706–712.

32 Stonier, T. 1960. *Agrobacterium tumefaciens*. II. Production of Antibiotic Substance. J. Bacteriol. 79: 889–898.

33 Thompson, R.J., R.H. Hamilton and C.F. Pootjes. 1979. Purification and Characterization of Agrocin 84. Antimicrob. Ag. Chemother. 16: 293–296.

34 Wang, D.I.C., C.L. Cooney, A.L. Demain, P. Dunnell, A.E. Humprey and M.D. Lilly. 1979. Fermentation kinetics. In: Fermentation and Enzyme Technology. pp. 57–97, John Wiley & Sons, NY.