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Milbemycin production by *Streptomyces* sp.: the effect of carbohydrates

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SUMMARY

Milbemycin production by *Streptomyces hygroscopicus* RB4569D was examined in media containing different carbohydrates. Total milbemycin titer could be increased by substitution of fructose for glucose and by selection of the appropriate starch type. Total titer could be further enhanced by increasing the concentration of fructose and/or starch in the medium. Rates of carbohydrate utilization were shown to be independent of their initial concentration and increased titers in high carbohydrate media were shown to be due to a prolonged production phase rather than an increased accretion rate. The pattern of individual milbemycin components was governed by the carbon:nitrogen ratio of the medium rather than carbohydrate concentration and there was a critical C:N ratio below which no milbemycin was produced.

INTRODUCTION

Milbemycins are a large family of related 10-membered macrolide antibiotics wtih anthelmintic activity. First reported to have been isolated from *Streptomyces hygroscopicus* subsp. *aureolacrimosus* as part of an insecticidal screening program [16], further milbemycins have since been isolated from the original strain [13], its mutants [12,17] and a number of other Streptomycetes. These include *S. cyanogriseus* subsp. *noncyanogenus* [2], *S. thermoarchaensis* [14,18] and *S. hygroscopicus* E225 [7]. Milbemycins have also been isolated from a hybrid organism obtained by protoplast fusion of *S. avermitilis* and *S. hygroscopicus* [11]. A closely related group of compounds, the avermectins, was discovered as a result of screening for compounds with anthelmintic activity [1] and the structure of a number of different avermectins has now been elucidated [5].

While an empirical view of secondary metabolism suggests that nutrient limitation is the primary control factor in antibiotic production, this can be further differentiated into control by carbon, nitrogen or phosphate limitation [4]. Carbon regulation of antibiotic biosynthesis by catabolite repression has been the subject of several reviews [3,9]. Glucose is the most commonly cited regulatory carbon source but others including glycerol, citrate and glutamate have been shown to exert control over the biosynthesis of some antibiotics [4].

This paper shows that milbemycin accretion by *S. hygroscopicus* RB4569D is influenced both by the type and amount of carbon present in the medium and that the profile of milbemycin produced is affected by the carbon:nitrogen ratio of that medium.

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MATERIALS AND METHODS

A seed medium containing glucose (20 g L^{-1}), Collofilm dextrin (20 g L^{-1}) (Amylum N.V., Burchtstraat 10, B9300, Aalst, Belgium), Arkasoy 50 (10 g L^{-1}) (British Arkady Co, Old Trafford, Manchester, M16 ONJ, UK), $CaCO_3$ (5 g L^{-1}), casein (BDH Light white soluble) (2 g L^{-1}) and $MgSO_4 \cdot 7H_2O$ (1 g L^{-1}) (pH adjusted to 6.3 with 50% HCl) was sterilized in 50-ml aliquots in 250-ml conical flasks.

A primary seed flask was inoculated with 1 ml of liquid nitrogen preserved vegetative culture and incubated at 28 °C, 240 rpm (5 cm throw). After 48 h secondary seed flasks were each inoculated with primary seed (2 ml) and incubated as above. After 48 h, 250-ml shaken flasks each containing 50 ml of final stage medium were inoculated with secondary seed (2 ml) and incubated as above. For studies on the effects of carbohydrates a basal medium (F1) was used containing Arkasoy 50 (10 g L^{-1}), $CaCO_3$ (5 g L^{-1}), casein (2 g L^{-1}) and $MgSO_4 \cdot 7H_2O$ (1 g L^{-1}) in addition to different concentrations of a range of carbohydrates. With the exception of soluble starch (BDH, Broom Road, Poole, Dorset, BH12 4NN, UK), all other starches used (see Table 1) were obtained from Tunnel Avebe Ltd, Otterham Quay, Rainham, Gillingham, Kent, ME8 7UU, UK.

The carbon:nitrogen ratio of the different media was calculated assuming that carbohydrates contain 40% carbon

TABLE 1

Effect of starch type on milbemycin production by *S. hygroscopicus* RB4569D after 14 days incubation in F1 media containing 20 g L^{-1} glucose and 20 g L^{-1} starch

Starch type[a]	Milbemycin titer (mg L^{-1}) after 14 days			
	VM44866	MX	VM44857	Total
Glucose media				
Collofilm dextrin	9.9	28.8	35.6	74.3
Perfectamyl B1102	22.6	117.1	57.6	197.3
Perfectamyl D6	16.8	81.5	75.4	173.7
Prejel PA5	11.0	65.2	59.9	136.1
Amylogum CL5	6.8	35.9	31.8	74.6
Paselli D40	3.2	26.8	47.2	77.2
BDH soluble	15.0	95.5	97.6	208.1

[a]Collofilm dextrin obtained from potatoes. Pefectamyl B1102 is a potato starch dextrin. Perfectamyl D6 is a predried natural potato starch. Prejel PA5 is a pregelatinized, oxidized potato starch. Amylogum CL5 is an esterified potato starch. Paselli D40 is a product of dextrose manufacture. BDH soluble starch. Cat. no. 10271.

and that protein contains 16% nitrogen and 40% carbon; Arkasoy 50 contains 50% protein and 32% carbohydrate.

Growth was assessed by measuring the packed cell volume (PCV) in 10 ml whole broth centrifuged at 1200 × *g* for 15 min at 5 °C.

Carbohydrate assays were performed on a Cobas Bio Autoanalyser. Fructose in whole broth was assayed using a Boehringer Glucose/Fructose Test Kit No. 139106 adapted for use in the Autoanalyser. Free glucose was measured using a Roche Diagnostica Glucose Test Kit No. 10970. Starch was measured as glucose released after acid hydrolysis of whole broth at 121 °C. A 0.5-ml aliquot of whole broth and 1.5 ml 1 N HCl were mixed in a glass universal bottle and autoclaved at 121 °C for 15 min. Samples were neutralized with 1.5 ml 1 N NaOH and diluted with deionized water to a 1 in 50 dilution of whole broth. Glucose was measured as above and the starch concentration was expressed as glucose concentration after hydrolysis minus the initial free glucose concentration.

Milbemycins were extracted by adding 4 ml acetone to 2 ml whole broth and mixing intermittently for >1 h followed by filtration through Whatman GF/A and GF/F glass fiber filter papers. Milbemycins were quantified by HPLC using a Waters Wisp 710B autosampler fitted with a C_{18} μbondapak 5-μm column using a methanol/water (84/16) solvent system at a flow rate of 1.5 ml min^{-1}. Column eluates were monitored at 244 nm.

Triplicate shake flasks were sacrificed at each time point, samples were taken from each flask and assayed separately. All data shown are the means of triplicate samples at each time point.

RESULTS

S. hygroscopicus RB4569D is known to grow on a variety of carbon sources and preliminary experiments showed that total milbemycin titers of between 50 and 70 mg L^{-1} were produced on F1 medium containing 20 g L^{-1} glucose and 20 g L^{-1} Collofilm dextrin. The major milbemycins produced by this organism are VM44866, VM48130, VM48633 and VM44857. The HPLC system used for this work does not adequately resolve VM48130 and VM48633 and throughout this work the major milbemycins produced by this organism will be referred to as VM44866, MX (VM48130 and VM48633) and VM44857.

Milbemycin production medium contains two main sources of carbon, a monomeric sugar (glucose or fructose) and starch. Results in Table 1 show that, in glucose-containing medium, the type of starch affects the production of all three major milbemycins. Higher titers were produced after growth on natural starches while any chemical modification of the starch during manufacture reduced subsequent production of milbemycins. Replacement of glucose by fructose in the three highest producing media shown in Table 1 increased the production of VM44866, VM44857 and MX (Table 2); total milbemycin production in fructose-containing media was approximately double that in glucose-containing media.

The time-course of milbemycin accretion in fructose/B1102 (20/20) medium is shown in Fig. 1. Accretion begins at 60 h after biomass (measured as PCV) has stopped increasing, starch utilization has begun and the culture has pigmented to a dark olive green color. Total milbemycin titer increases linearly until 210 h when the fructose and starch are depleted and carbon limitation causes a rise in pH and a tailing off of milbemycin accretion. Fermentation profiles in other fructose and starch-containing media (Table 2) are similar to those shown in Fig. 1 (data not shown).

Table 3 shows that total milbemycin titer can be enhanced by increasing the initial concentrations of either fructose or starch. In the media tested here, carbohydrate utilization rates remain unaffected by their initial concentration (apart from medium containing 50 g L^{-1} fructose) and so carbon limitation is delayed in high carbohydrate media. Milbemycin accretion rates during the linear (i.e. non-carbon limited) production phase are also independent of initial carbohydrate

TABLE 2

Effect of starch type on milbemycin production by *S. hygroscopicus* RB4569D after 14 days incubation in F1 media containing 20 g L^{-1} fructose and 20 g L^{-1} starch

Starch type[b]	Milbemycin titer (g L^{-1}) after 14 days			
	VM44866	MX	VM44857	Total
Perfectamyl B1102	77.1	202.5	81.5	361.2
Perfectamyl D6	60.6	211.7	78.3	350.6
BDH soluble	77.3	270.8	70.1	418.3

[b] See Table 1 for details of starch type.

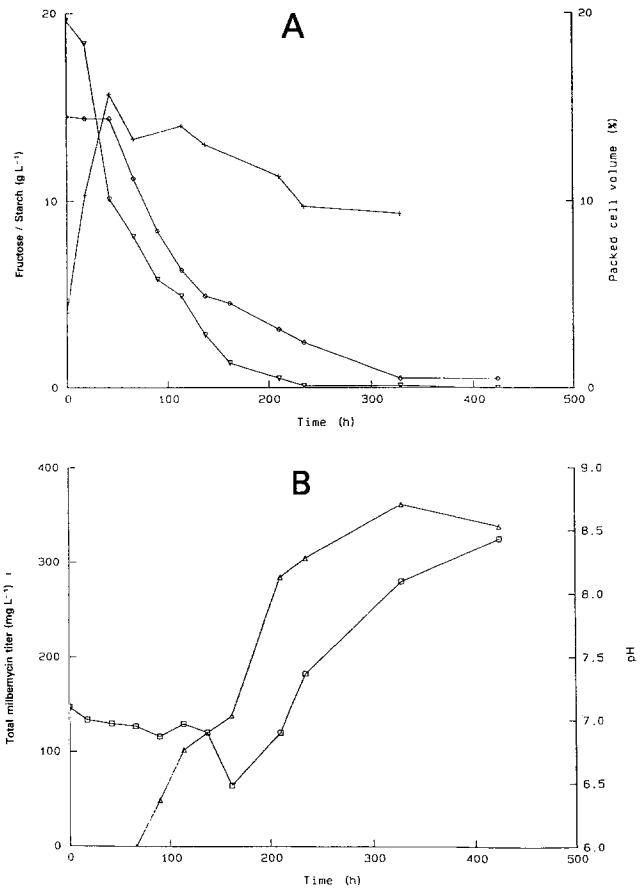

Fig. 1. Fermentation profile of *S. hygroscopicus* RB4569B in production medium containing (A) 20 g L^{-1} fructose and (B) 20 g L^{-1} Perfectamyl B1102 as carbon sources. (△) milbemycin titer mg L^{-1}, (□) pH, (▽) fructose g L^{-1}, (◇) starch g L^{-1}, (+) packed cell volume %.

concentration (see Table 3). Thus, in high carbohydrate media increased milbemycin titers result from a prolonged production phase rather than an increased accretion rate.

This is illustrated in Fig. 2 which shows time-courses of total milbemycin accretion and the corresponding pH profiles in different media. Thus, in fructose/B1102 (20/20) medium, milbemycin accretion stops after 250 h when the pH rises. In higher carbohydrate media accretion continues until at least 400 h as the pH remains approximately 7.0 prior to carbon limitation (data not shown).

Table 4 shows the total milbemycin titer produced in different media and the proportion of individual components within that total. These results show that while the total milbemycin titer is affected primarily by the carbohydrate content of the media the pattern of the individual components is dependent on the carbon:nitrogen (C:N) ratio of the medium. At high C:N ratios there is a trend towards an increasing proportion of VM44857 and a decreasing proportion of MX. This applies whether the C:N ratio is modified by altering the initial level of fructose, starch or Arkasoy. These results also indicate that, irrespective of total carbohydrate content, there is a critical C:N ratio of approximately 14 below which milbemycins are not produced.

At C:N ratios close to this critical value the total milbemycin titer is very much reduced and the concentrations of the individual components do not comply with the trend observed at higher C:N ratios. This is likely to be due to the higher pH in these media causing abnormal accretion profiles.

DISCUSSION

The major milbemycins produced by this isolate of *S. hygroscopicus* RB4569D are similar to those produced by an earlier isolate of this organism, E225 [7]. In common

TABLE 3

Effect of carbohydrate concentration on milbemycin production and substrate utilization rates by *S. hygroscopicus* RB4569D in F1 media containing different carbon sources

Initial carbohydrate concentration in medium		Fructose utilization rate	Starch utilization rate	Total milbemycin titer (424 h)	Milbemycin accretion rate
Fructose (g L^{-1})	BDH starch (g L^{-1})	(g L^{-1} h^{-1})	(g L^{-1} h^{-1})	(mg L^{-1})	(mg L^{-1} h^{-1})
10	20	0.093	0.082	234	1.86
20	20	0.090	0.108	595	2.00
30	20	0.093	0.087	605	2.21
40	20	0.090	0.090	757	2.15
50	20	0.060	0.065	851	2.43
Fructose (g L^{-1})	*B1102* (g L^{-1})				
20	20	0.096	0.087	442	2.03
20	30	0.098	0.108	589	2.13
20	40	0.094	0.092	651	2.24
20	50	0.097	0.111	834	2.64

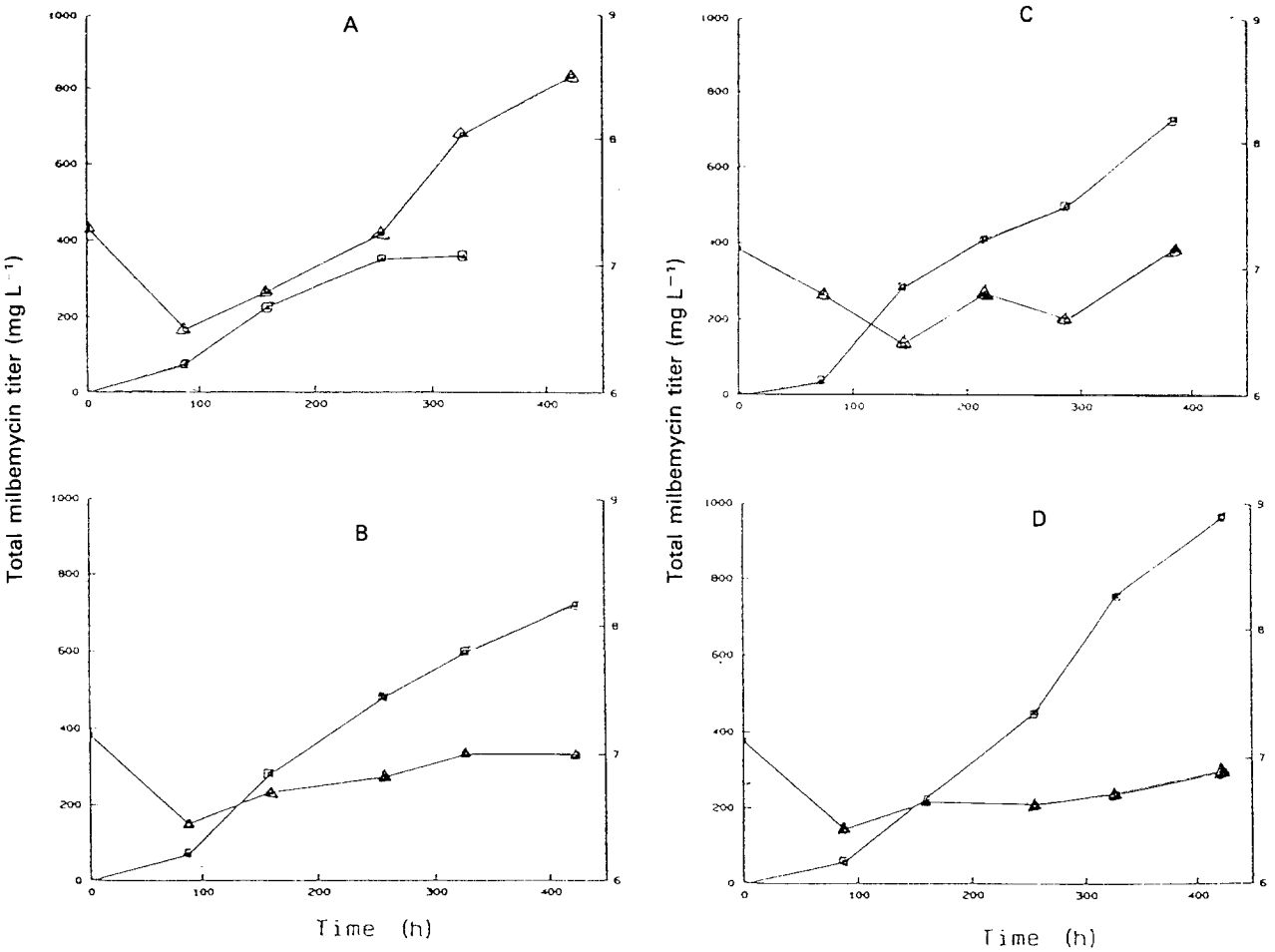

Fig. 2. Time-courses of total milbemycin accretion (□) by *S. hygroscopicus* RB4569D in production media containing different concentrations of fructose and Perfectamyl B1102. The pH profiles (△) are also shown. (A) 20 g L^{-1} fructose, 20 g L^{-1} B1102; (B) 40 g L^{-1} fructose, 20 g L^{-1} B1102; (C) 20 g L^{-1} fructose, 50 g L^{-1} B1102; (D) 40 g L^{-1} fructose, 40 g L^{-1} B1102.

with other producing strains this isolate grows well on a number of different carbon sources which have also been shown to affect milbemycin production (see Tables 1 and 2); titers of the major milbemycins are considerably enhanced by growth on fructose compared to growth on glucose. This may be due to the removal of catabolite repression caused by glucose which is known to repress antibiotic production, transport systems and sugar catabolism in a number of *Streptomyces* [3,4]. Glucose is also known to repress α-amylase, the first enzyme of starch catabolism and initial levels of α-amylase in production medium containing 20 g L^{-1} fructose are greater than those in medium containing 20 g L^{-1} glucose (data not shown). High levels of milbemycin production seem to be closely linked to starch catabolism; accretion does not begin until after pigmentation of the culture which, in this medium, appears to be correlated to starch catabolism and is delayed in glucose-containing (i.e. catabolite-repressed) medium.

The type of starch and method of preparation also affects total milbemycin titer (Table 1) and final titers may be related to the breakdown of starch to residual 'limit dextrins'. The α-amylase from *S. hygroscopicus* SF1084 has been described as 'unusual' in converting starch to maltose at 75% yield [6] but there is no information available on the α-amylase, or on maltose uptake systems in this isolate.

Carbohydrate metabolism is known to be involved in the production of avermectin by *S. avermitilis*. The addition of glucose at an early stage in the fermentation suppressed avermectin production by reducing the activity of 6-phosphogluconate dehydrogenase which restricted the supply of precursors of macrolide biosynthesis. However, glucose feeding at a late stage in the fermentation enhanced avermectin production 2-fold [8]. A pathway for avermectin biosynthesis has been proposed [15] in which the oleandrose units of the aglycone backbone are derived from glucose. Little is known of the pathway of milbemycin biosynthesis but it is possible that initial growth of *S. hygroscopicus* on glucose has a similar effect on enzyme activity and that growth on fructose removes any restriction on the supply of precursors for milbemycin biosynthesis.

While this could account for the earlier onset of accretion in fructose-containing media, the results in Fig. 2 show that increased titers in high carbohydrate media are the result of delayed carbon limitation allowing a prolonged production

TABLE 4

Effect of initial carbon:nitrogen ratio on the pattern of individual milbemycins produced by *S. hygroscopicus* RB4569D. C:N ratio was adjusted by altering the initial concentration of either fructose, starch or Arkasoy 50

Medium type[c] Fru (g L^{-1}), Starch (g L^{-1}), Ark (g L^{-1})	C:N ratio	Time (h)	Milbemycins			
			Total mg L^{-1}	% VM44866	% MX	% VM44857
Altered fructose						
Fru 20, BDH 20, Ark 10	17.9	424	595	18.9	64.7	16.3
Fru 30, BDH 20, Ark 10	21.4	424	605	23.5	58.4	18.1
Fru 40, BDH 20, Ark 10	25.0	424	757	24.9	36.3	38.8
Fru 50, BDH 20, Ark 10	28.6	424	851	19.6	30.6	49.8
Altered starch						
Fru 20, BDH 20, Ark 10	17.9	384	464	17.2	59.9	23.0
Fru 20, BDH 50, Ark 10	28.6	384	726	22.9	44.8	32.0
Fru 20, B1102 20, Ark 10	17.9	384	443	18.7	56.5	24.8
Fru 20, B1102 50, Ark 10	28.6	384	835	23.6	40.0	36.5
Fru 20, D6 20, Ark 10	17.9	384	335	18.1	57.9	23.9
Fru 20, D6 50, Ark 10	28.6	384	680	24.4	41.6	33.9
Altered Arkasoy						
Fru 20, W80 20, Ark 20	11.9	424	T	–	–	–
Fru 20, W80 20, Ark 15	14.2	424	50	12.0	44.8	43.2
Fru 20, W80 20, Ark 10	17.9	424	222	23.3	59.1	17.5
Fru 20, W80 50, Ark 30	13.7	424	0	–	–	–
Fru 20, W80 50, Ark 25	15.5	424	91	16.5	51.3	27.0
Fru 20, W80 50, Ark 20	18.1	424	459	18.8	73.9	7.3
Fru 20, W80 50, Ark 15	22.1	424	577	29.4	58.4	12.2
Fru 20, W80 50, Ark 10	28.6	424	593	33.7	33.7	32.5
Fru 20, W80 50, Ark 5	42.2	424	390	24.6	24.3	51.1

[c] Fru = Fructose, BDH = BDH soluble starch, B1102 = Perfectamyl B1102, D6 = Perfectamyl D6, W80 = Avedex W80, Ark = Arkasoy 50.
In addition to the components shown the media contained $CaCO_3$ (5 g L^{-1}), Casein (2 g L^{-1}) and $MgSO_4 \cdot 7H_2O$ (1 g L^{-1}). T = trace.

phase. This applies whether the carbohydrate content is increased by additional fructose or by additional starch. In both cases the rates of carbohydrate utilization and the milbemycin accretion rate remain relatively constant (Table 3) and the pH remains below 7.5 until the carbon sources are depleted (Fig. 2). It is likely that even higher titers could be obtained by fructose feeding as has been demonstrated with glucose for increased avermectin production [8]. Some form of pH control may also allow a more efficient conversion of carbon to milbemycin.

Although the total milbemycin titer can be increased by increasing the total carbohydrate content of the medium, the results in Table 4 show that the pattern of individual milbemycins is dependent on the C:N ratio of the medium and that there is a critical C:N ratio below which milbemycin production is reduced. However, the results in Table 1 suggest that the low molecular weight carbon source can also affect the proportions of the different milbemycins. A similar effect has been observed in *S. avermitilis* in which the C:N ratio of the medium was found to affect both the specific productivity of avermectin and the composition profile of the different components [10].

Overall these results show that total milbemycin production by *S. hygroscopicus* RB4569D can be increased by both the type and amount of carbohydrate present in the medium. The profile of individual milbemycins within that total is affected by the C:N ratio of the medium. In these respects milbemycin production by the organism is similar to the production of avermectin by *S. avermitilis*. However, increased production in fructose-containing media has not been reported previously for either milbemycin or avermectin production.

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