FACTORS AFFECTING MICROCIN E492 PRODUCTION

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The production of the antibiotic polypeptide microcin E492 by *Klebsiella pneumoniae* RYC492 has been studied in respect to the composition of the culture medium. The nature of both the carbon and nitrogen sources had a dramatic effect on the levels of the microcin detected in the supernatants of the liquid cultures. Gluconeogenic precursors such as lactate or citrate led to microcin titers 10 to 100 fold higher than those obtained with glucose. However, neither glycerol nor cAMP stimulated the production, ruling out a typical type of catabolic repression by glucose. Limitation of growth by phosphate or nitrogen led to a marked decrease in production. Treatment with mitomycin C did not result in an enhancement of microcin levels.

It is well known that the production of most antibiotics is dependent on the composition of the culture medium in which the producer microorganism is grown. In many cases, choice of the culture conditions for optimizing antibiotic production has been largely empirical. However, research efforts have been devoted in the last few years to elucidate the regulatory mechanisms involved at the molecular level in the control of the biosynthesis of antibiotics[1]. Most of the work performed in this sense has been centered on antibiotics produced by fungi and actinomycetes. For these, a number of biochemical and genetic controls have been described to explain (and eventually, to increase) the levels at which the antibiotics are produced in defined media. Carbon and nitrogen catabolite regulation, phosphate regulation and induction appear to be general regulatory mechanisms that control the onset of antibiotic biosynthesis (see ref 1 for review).

A number of antibiotics produced by Gram-negative bacteria have been described in the last years, namely, several new β-lactams[2] and the antibacterials termed microcins[3]. The microcins are antimetabolites and medium size antibiotic peptides (MW 500~5,000) produced by enterobacteria, mainly *Escherichia coli*, but also *Klebsiella* and other bacterial genera[3,4]. Microcins share some common features with colicins, which are antibiotic proteins produced by *E. coli* strains carrying colicinogenic plasmids[5]. However, colicins have a much higher molecular size (MW 27,000~80,000) and unlike microcins, their production is normally dormant except under conditions which activate the SOS repair mechanisms[5]. The production of some colicins is also dependent on the composition of the medium[6] and in several cases, mechanisms of catabolic repression of the colicin production have been suggested[7].

In order to compare the factors which affect the production of microcins with those of other antibiotics and of colicins, we have screened a number of carbon and nitrogen sources to optimize the production of the *ca.* 5,000 MW microcin produced by *K. pneumoniae* RYC492, termed microcin E492[8,9]. We have also tried to induce it with mitomycin C and cAMP and studied the influence of phosphate. The results suggest that microcin production by *K. pneumoniae* RYC492 is regulated by some type of carbon and nitrogen catabolite control. Its characteristics are different with those of both the classical antibiotics and the colicins.

Methods

Bacterial Strains and Media

The microcin E492 producer strain *K. pneumoniae* RYC492 was previously described[8,9]. *E. coli* K12 (BM21 strain, nalidixic acid resistant[8]) and *E. coli* B (NCIB9484 strain) were used as sensitive strains. *E. coli* RK6 is an *E. coli* BM21 derivative mutant resistant to microcin E492[8] which was used as a control of specificity for the production of microcin. All the media used have been previously described: minimal M9 medium[8], Simmons citrate[9], MOPS[10] and 121-salts[11]. 0.2% (w/v) of the appropriate carbon source was added to M9 medium in the corresponding experiment. Ammonium sulfate in M9-glucose medium was replaced where indicated by 400 μg/ml of equivalent nitrogen as either proline or Casamino Acids.

Microcin Extraction and Titration of Antibiotic Activity

Unless indicated, the producer strain was incubated overnight at 37°C with vigorous shaking in the liquid medium as specified in each of the experiments. A hundred ml of each of the supernatants of the cultures were then passed through an octadecylsilica Bondapak C18 microcolumn (Waters) on which the antibacterial was quantitatively retained[9]. The microcin was eluted from the microcolumn with a stepwise gradient (the antibiotic is found in the fractions between 60 and 90% methanol). The activity was measured as antibiotic units per ml (AU/ml) by the critical dilution method[6] on *E. coli* BM21 and *E. coli* B in standard conditions[8]. In all cases, the activity of the microcin was tested against the microcin-resistant mutant *E. coli* RK6 in order to rule out unspecific antibiotic activity due to inhibitory substances other than the microcin which could be eventually present in the active preparations.

Kinetics of Microcin Action in Glucose Medium

The changes in the *in vivo* activity of the microcin present in a minimal-glucose culture were followed by taking 4 ml-aliquots at 30 minutes intervals from the growing culture of the producer strain and mixing them with 2.5×10^7 cells/ml of an standard inoculum of the sensitive *E. coli* BM21 strain. The mixtures were subcultured under the same conditions and the mortality of the sensitive strain was monitored by plating aliquots at 0, 15 and 30 minutes after mixing, on Petri dishes containing rich agar medium and 40 μg/ml of nalidixic acid[8]. As microcin E492 shows single-hit kinetics[8,9], the survival of the sensitive strain at 30 minutes ($\log N_0/N_{30}$) was considered proportional to the activity of the microcin present in the medium[12].

Induction Assays

Either 1 μg/ml of mitomycin C or 1 mM cAMP were added in the mid-exponential phase ($OD_{660} = 0.3 \sim 0.5$) of growth to a growing culture of the producer strain in minimal M9 medium. Samples (50 ml) were taken from the culture at 30, 60 and 120 minutes after the addition and were processed as described above to determine the levels of microcin present in the cultures.

Results and Discussion

Microcin E492 is excreted into the medium together with a reversible microcin inhibitor; the onset of inhibitor formation requires the depletion of the carbon source in the culture[8]. Fig. 1 (A) shows that the accumulation of microcin in the supernatants of the cultures in glucose medium was approximately parallel with the growth of the producer strain, to reach its highest value in the stationary phase. No significant increases in the accumulated microcin were detected during this stage. Fig. 1 (B) shows, however, that the maximum *in vivo* activity was detected in the middle of the exponential stage and a dramatic decrease followed this maximum with activity virtually disappearing by the stationary phase due to the onset of production of microcin inhibitor. A comparison between Figs. 1 (A) and 1 (B) shows that the procedure used to extract the microcin virtually separates the antibiotic from its inhibitor. In fact, using that method, we were able to recover 100% active microcin at late stages of growth of the

Fig. 1.

(A) Production kinetics of microcin E492 in glucose minimal medium.

The microcin present in the supernatants of a growing culture of the strain *K. pneumoniae* RYC492 was extracted at the different times and titrated as described in the text on *E. coli* BM21.

(B) Kinetics of action *in vivo* of microcin E492.

The activity of the antibacterial is expressed as survival of *E. coli* BM21 after 30 minutes of mixed culture with the microcin-producing strain ($\log_{10} N_0/N_{30}$). See text for details.

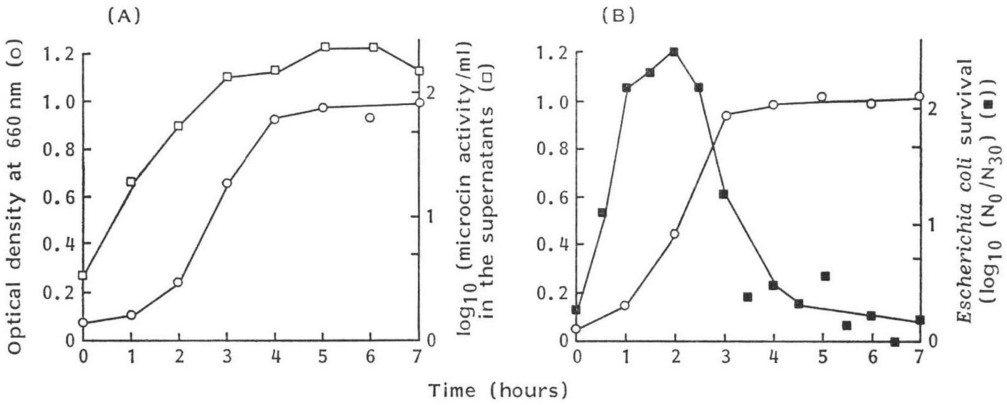


Fig. 2. Microcin production with different sources of carbon and nitrogen.

(A) The producer strain was grown in minimal medium with 0.2% of the carbon source indicated until reaching the stationary phase. The microcin present in the supernatants was extracted as described in the text and its antibiotic activity was measured over *E. coli* BM21. *E. coli* B was in all cases 10 fold more sensitive to microcin E492 than *E. coli* BM21 strain. The antibiotic yields are represented assuming the value of 0.38 mg/ml of cell dry weight per unit of optical density at 660 nm.

(B) Same, using M9-glucose as basal medium with the indicated nitrogen sources. The values of pH were approximately constant throughout the incubations.

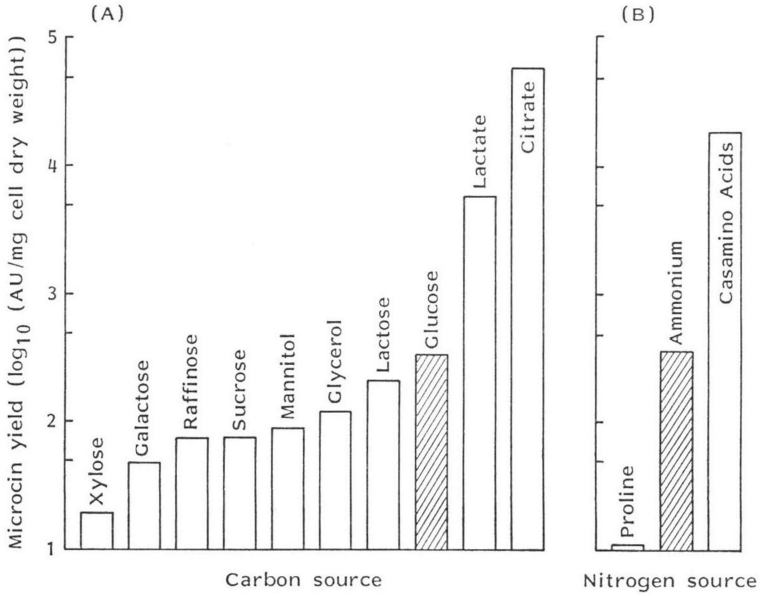
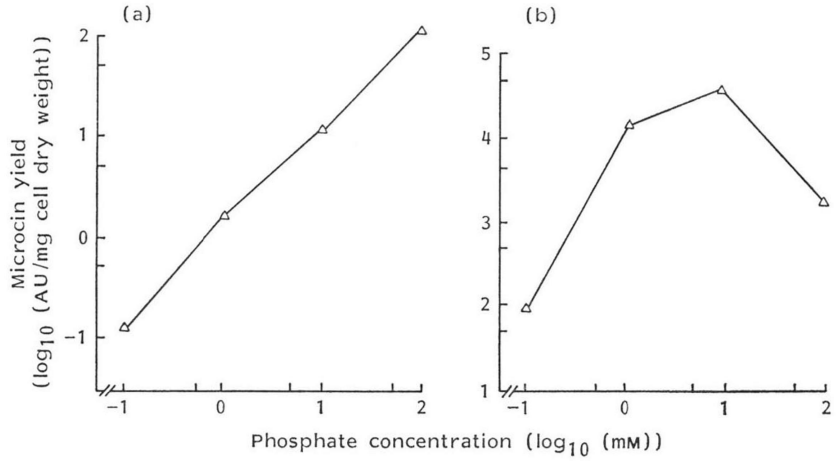


Fig. 3. Effect of phosphate concentration on microcin production.

The producer strain was grown in either of the defined media MOPS (a) or 121-salts (b) with the phosphate concentrations indicated and 0.2% glucose as the sole carbon source. The microcin was extracted from the supernatants as indicated in the text and the antibiotic activity was measured with *E. coli* BM21.



producer cells, where no activity was any longer detected with the *in vivo* assay of Fig. 1 (B), due to the presence of the inhibitor[8]. This allows to study the parameters which affect the production regardless of this inhibitor. The data of Fig. 1 (A) can be used to show that the specific titer of microcin expressed as AU/mg of cell dry weight is approximately constant throughout growth (*ca.* 1,000 AU/mg). This suggests that the production of the antibiotic takes place mostly during the growth of the cells. This has permitted us to compare directly the levels of the microcin present in the supernatants of the cultures of the producing strain grown under different conditions. In all cases, minimal media of defined chemical composition have been used since the production of this microcin is lower when rich media are employed[8].

Fig. 2 (A) shows the different levels of microcin obtained using a variety of substrates as the sole carbon source. There was a striking increase in production in media with lactate or citrate. This promotion of microcin production may not be correlated only with carbon regulation but since these substrates are good gluconeogenic precursors, a mechanism of catabolic repression by glucose could be proposed to explain this behavior. However, in the same Fig. 2 (A) it can be seen that other gluconeogenic substrates like glycerol or some carbohydrates did not lead to similar increases but, on the contrary, restrained the production of microcin. Moving back to Fig. 1 (A), it is noticeable that depletion of glucose in the culture did not significantly enhance the titers of the antibiotic in the medium. Furthermore, the addition of 1 mM cAMP to the culture with either glucose or glycerol did not result in an increase of the microcin yield (not shown). These data seems to exclude a typical glucose effect although they certainly suggest some kind of carbon catabolite control. Whether the regulation is due to carbon catabolite repression, inhibition or inactivation is still undetermined. However, since the culture conditions were comparable in all cases, we think that these changes in the production of microcin deal with a likely regulatory effect.

Another point of interest was to determine the effect of the partial starvation of nitrogen on the microcin production. For this purpose, we changed the ammonium sulfate of the M9-glucose medium

by an equivalent amount of nitrogen from proline. This amino acid is slowly metabolized by the producer strain and its use as the sole nitrogen source in the medium results in a virtual nitrogen limitation for growth. Thus, we observed a much lower growth rate of the producer strain in the medium with proline (not shown). Fig. 2 (B) shows that the growth of *K. pneumoniae* RYC492 in this medium resulted in a complete inhibition of microcin production. On the contrary, Casamino Acids increased the production. Although Casamino Acids are also a mixture of carbon sources, these results suggest that the easier the utilization of the nitrogen source, the higher are the levels of microcin produced. This contrasts with the production of many antibiotics whose synthesis increases with the exhaustion of the nitrogen source[13]. In the same way, phosphate appears to increase the production of microcin (Fig. 3). This also contrasts with the production of antibiotics like candicidin or tetracyclin, whose syntheses are initiated by phosphate depletion[1].

Production of microcin E492 was not induced neither by 1 μg/ml of the DNA-damaging antibiotic mitomycin C or by UV radiation, cold shock of other treatments which induce the production of colicins (not shown). These results are in agreement with the criterion that the main difference between microcins and colicins (apart from MW) is that the former are not induced by agents which activate the SOS response[14].

The study of the nutritional conditions which affect the production of antibiotics and colicins do not always throw light on the intimate mechanisms involved in regulation of their synthesis. The term 'production' includes in the case of colicins (and eventually in microcins) at least three different steps with possible independent regulatory mechanisms: (a) biosynthesis, (b) excretion across the membranes into the medium and (c) readsorption by the outer-membrane receptors of the producer cells. The level of the antibacterial found in the cultures is always the result of these simultaneous parameters, although in some cases, one of them can virtually determine the general process of production. Therefore, the likely regulatory effect produced by carbon and nitrogen sources and by phosphate on microcin production cannot be attributed solely to an effect on the biosynthesis.

Recent studies carried out with colicin E2[15] have shown that the levels of colicin detected in the medium are dramatically affected by changes in colicin E2 receptor activity on the cell surface. It remains to be determined whether or not the nutritional status of the producer cells could determine the number of receptors whose activity would remove the antibacterial from the culture medium. In this sense, the relationship between the nutritional conditions of the microcin-producing cells and the activity of the microcin attachment sites is presently under study.

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