Microcin-mediated Interactions Between *Klebsiella pneumoniae* and *Escherichia coli* Strains

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(Received 5 July 1983; revised 29 September 1983)

Amensal indirect interactions between a *Klebsiella pneumoniae* microcin-producing strain and several *Escherichia coli* strains, all of intestinal origin, were studied. Mixed batch cultures of both microcin-producing and microcin-sensitive strains showed that microcin production and excretion into the medium allowed the producer strain to prevail over sensitive strains, even when initial competition conditions were highly unfavourable for the producer. Mixed cultures also showed the production of a microcin-antagonist by the same microcin-producing strain when the nutrients in the medium had been depleted. The antagonist apparently promoted the viability of sensitive cells already damaged by microcin. These results have likely ecological implications.

INTRODUCTION

Quantitative features of microbial indirect relations mediated by inhibitory agents have been widely analysed (de Freitas & Fredrickson, 1978), but few experimental studies are available concerning antagonism between antibiotic-producing strains and other micro-organisms which share common habitats (Helling et al., 1981; Adams et al., 1979).

The human intestinal tract lodges a rather well-defined community of micro-organisms that show dynamic successions both in the species composition and in the relative abundance of each resident, under either normal or pathological conditions. These successions are frequently very fast and specific for some invaders, which are able in some way to displace other very closely related species by means of poorly understood mechanisms (Savage, 1977). Bacteriocin production has been suggested to be involved in amensal relations which would account for phenomena observed in this ecosystem such as implantation ability, strain displacements, colonization-resistance and many others. Nevertheless, no evidence supporting these hypotheses has so far been provided (Konisky, 1978).

The inhibitors called microcins are other candidates that may explain bacterial displacements in the intestinal microbial ecosystem. Microcins are low molecular weight antibiotics produced by *Enterobacteriaceae* isolated from human faeces (Asensio et al., 1976). Over 10% of the *Escherichia coli* and *Klebsiella* strains isolated from this source are microcinogenic. In accordance with their characteristics (low molecular weight compared with bacteriocins, production and activity in poor media, resistance to extremes of pH and certain proteases), it has been proposed that microcins are involved in the maintenance of homeostasis in the intestinal ecosystem (Baquero & Asensio, 1979; Aguilar et al., 1982a).

This paper introduces the study of indirect amensal interactions between a *Klebsiella pneumoniae* microcin-producing strain and microcin-sensitive *E. coli* strains, all of intestinal origin. Mixed batch cultures of producer and sensitive strains offer indications that interaction is mediated both by the inhibitory action of excreted microcin and by the release of a microcin-antagonist by the same microcin-producer strain when the nutrients in the culture are depleted.

† Deceased.
Table 1. Bacterial strains

<table>
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<th>Species</th>
<th>Strain</th>
<th>Relevant phenotype*</th>
<th>Origin</th>
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</thead>
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<td><em>Escherichia coli</em> K12</td>
<td>BM21</td>
<td>Prototrophic, NalR</td>
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<tr>
<td></td>
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<td>Thr, Leu, His, Pro, Arg, NalR</td>
<td>F. Moreno</td>
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<td>This work</td>
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<td>Prototrophic</td>
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<td>IER1</td>
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<td>This work</td>
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<td>KmR, Mcc+, Mccimm+</td>
<td>This work</td>
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<td>This work</td>
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</table>

* NaR, Resistance to nalidixic acid; RifR, resistance to rifamycin; KmR, resistance to kanamycin; MccR, resistance to microcin 492; Mcc+, microcin production; Mccimm+, immunity to microcin.
shaking at 37 °C until the mid-exponential phase (OD600 = 0.3) was reached. Then 8 ml of the culture was centrifuged at 2000g for 30 min, and the pellet was resuspended in 2 ml M9 medium without glucose. This standard suspension (approximately 10⁶ c.f.u. ml⁻¹) was stored at 5 °C until use. Such cells apparently maintained their metabolic situation at exponential conditions for at least 10 h.

Survival curves were constructed in the following way. Samples (4 ml) from a culture of microcin-producing strain in M9 medium were taken at suitable times during growth and mixed in 25 ml sterile Erlenmeyer flasks with 100 μl of the standard sensitive cell suspensions giving approximately 2.5 × 10⁷ c.f.u. ml⁻¹. Flasks were subsequently incubated at 37 °C with shaking and the changes in the viability of sensitive cells in the mixed culture were followed by plating samples at desired times on LB-nalidixic acid agar. When survival curves were made with supernatants, the culture of microcin-producing strain (taken at desired times), was centrifuged at 5 °C for 30 min at 2000g and the supernatant was either supplemented with nalidixic acid (40 μg ml⁻¹) to avoid growth of remaining K. pneumoniae cells, or filter-sterilized using polycarbonate membranes with 0.4 μm pore size (Bio Rad). A 4 ml portion of this supernatant was added to 100 μl standard sensitive cell suspension and the viability was followed as before.

Protease sensitivity was tested at a final protein concentration of 100 μg ml⁻¹. All proteases were purchased from Sigma. Trypsin was the type treated with diphenyl carbamyl chloride (DPCC) to avoid chymotrypsin contamination.

Microcin preparations. Crude cell-free preparations of microcin were obtained by the following procedure. M9 melted soft agar medium was inoculated at 42 °C with 4 × 10⁸ c.f.u. K. pneumoniae RYC492 ml⁻¹, and poured into sterile Petri dishes. The plates were incubated overnight at 37 °C and the agar was triturated and centrifuged at 20000g for 30 min. The supernatant was filter-sterilized as before; it showed an antibiotic activity of about 2000 arbitrary antibiotic units ml⁻¹ against E. coli BM21, as measured by the critical dilution method (Asensio et al., 1976). Further details concerning microcin purification and characterization will be published elsewhere (V. de Lorenzo, unpublished).

Microcin antagonist assay. A crude microcin preparation (0.25 ml) was spread on the surface of a Petri dish containing 25 ml M9 solid medium. The plate was then spray-seeded with a suspension of E. coli BM21 sensitive strain. Strains being tested for antagonist production were inoculated into the agar by means of toothpicks. Petri dishes were incubated at 37 °C for 40 h under aerobic or anaerobic conditions. Antagonist was considered present when the sensitive strain formed a circle of growth around the spot of the tested strain.

RESULTS

Mixed cultures E. coli/K. pneumoniae

Figure 1(a) shows the changes in the viability of the cells in a mixed culture of E. coli BM21 and K. pneumoniae RYC492 in minimal medium, with a starting ratio of microcin-producing to sensitive cells of 1:1. The microcinogenic strain titre rose regularly, whereas that of the sensitive strain fell in 3 h to reach its lowest level, three orders of magnitude below the initial conditions. From that point on, a rapid recovery of the viability of the sensitive cells was observed, which became stable in the following 3 h. When E. coli IER1 (RifR) was used as the sensitive strain, similar patterns were observed (data not shown). In all cases, no relevant changes of pH were detected so far in the culture medium during the development of mixed cultures (pH 6.9 to 6.6).

Figure 1(c) shows the same experiment performed in rich medium, BHI. Under these conditions, there was an inhibition in the growth of the sensitive strain without apparent killing of the cells. This could be due to a decreased sensitivity of the cells to microcin in rich media and/or to a lower concentration of the antibiotic. Examples of these two possibilities have been described for other microcins (Aguilar et al., 1982b).

Non-producing mutants, such as K. pneumoniae R302, were unable to produce any inhibitory effects on sensitive cells in either minimal or rich medium (Figs 1(b) and 1(d), respectively). The other hand, the patterns showed by the mixed cultures between microcin-producing K. pneumoniae RYC492 and microcin-resistant E. coli RK6 were fully similar to those described in Figs 1(b) and 1(d), in the sense that the growth of E. coli-resistant cells was not affected by the presence of microcin-producing strains. The same results were obtained when other Mcc⁻ or MccR mutants were used in mixed cultures (data not shown).

Mixed cultures were set up in minimal medium starting with the producing/sensitive cells ratio favourable to the latter (1:10 or 1:100). The plots in Fig. 2 show that the microcin-producing strain prevailed over the sensitive strain in the mixed culture even under such unfavourable initial conditions. Nevertheless, when the ratio producer/sensitive strain was yet
Fig. 1. Mixed culture of K. pneumoniae RYC492 and E. coli BM21 in different media. Strains were grown separately in either minimal or rich medium to reach an OD$_{660}$ of 0.1. Equal volumes of each strain were then mixed. Strain titres were obtained by plating samples of the mixed culture on LB-nalidixic acid (E. coli BM21 titre) and LB-kanamycin (K. pneumoniae RYC492 or R302 titre). Klebsiella pneumoniae R302 is a non-producing mutant (see Methods). (a) Mixed culture of K. pneumoniae RYC492 (■) and E. coli BM21 (○) in minimal medium. (b) Mixed culture of K. pneumoniae R302 (■) and E. coli BM21 (○) in minimal medium. (c) Mixed culture of K. pneumoniae RYC492 (■) and E. coli BM21 (○) in rich medium BHI. (d) Mixed culture of K. pneumoniae R302 (■) and E. coli BM21 (○) in rich medium BHI.

lower, i.e. 1:1000 (Fig. 2d), neither mortality of the sensitive strain nor prevalence of producer strain was detected, probably due to the low microcin concentration in the medium when the glucose had already been depleted by the growth of the sensitive strain.

The recovery of viability of the sensitive strain was observed in mixed cultures at times longer than 3 h (Fig. 1a). This recovery could not be attributed to the overgrowth of microcin-resistant cells, as indicated by analysis of the viable sensitive cells isolated from the mixed culture at late stages of the producer-strain growth. When the polyauxotrophic E. coli AB1157 strain was used as sensitive strain in conditions preventing its growth, viability recovery was still observed (Fig. 3). This indicated that recovery could not be attributed to cellular growth processes, but to some kind of rescue mechanism. This rescue seemed to be mediated by a microcin-antagonist which appeared in the medium when glucose was depleted. Experimental evidence supporting this hypothesis is shown below. This last experiment also showed that cell growth is not required for microcin action because killing took place whether or not the cells were growing.

**Survival curves**

Killing kinetics of sensitive cells were studied by constructing survival curves at shorter times. These curves were obtained by mixing subcultures of the microcin-producing strain, taken at different stages of growth, with a constant number of sensitive cells (Fig. 4). The highest
mortality was observed with subcultures of the microcin-producing strain obtained in its exponential phase of growth, whilst no mortality was observed with subcultures from the stationary phase. These results are consistent with those shown in Figs 1 and 2, in which it can be seen that the rescue began only when the mixed culture was about to reach the stationary phase. On the other hand (Fig. 4), a close parallelism can be observed in the mortality patterns of the microcin-sensitive strain in the presence or absence of producer cells. When the changes in the viability of the cells in this kind of mixed culture were followed for longer periods (Fig. 5), it was observed that recovery of viability was possible only when microcin-producing cells were present. When producer cells were removed from the culture by centrifugation, or their growth was inhibited at early stages of the exponential phase (when we presumed that there was no significant production of the microcin inhibitor), recovery was not detected or, in any case, was much slower than when microcin-producing cells were present (Fig. 5). This kind of experiment showed that the microcin-antagonist is a diffusible agent and that it is released into the medium by the same producer cells at late stages of growth.

Microcin activity was quickly inactivated by pronase and chymotrypsin (Fig. 6a), but not by trypsin (Fig. 6b). This last protease apparently exerted an activation effect, which is especially noteworthy in the situation plotted in Fig. 6(c). In this case, the subculture of the microcin-producing strain was obtained at the stationary phase of growth in which microcin activity can no longer be detected in the absence of the protease. This activation effect could be attributed to an inactivation of the microcin-antagonist by trypsin rather than to a direct action on the microcin itself, since purified microcin is insensitive to trypsin at the same concentration (V. de Lorenzo, unpublished). These experiments suggest that the antagonist could contain peptide linkages.
Fig. 3. Mixed cultures of *K. pneumoniae* RYC492 and polyauxotrophic *E. coli* AB1157. Mixed cultures were prepared as described in Methods. *Escherichia coli* AB1157 was grown in M9 medium supplemented with the amino acids required for growth up to an OD₆₆₀ of 0.1. Cells were then centrifuged, resuspended in the same volume of minimal medium without amino acids and mixed with the appropriate strain of *K. pneumoniae*. All the following mixed cultures were prepared in minimal medium. (a) Control mixed culture of *K. pneumoniae* RYC492 (●) and *E. coli* BM21 (○). (b) Mixed culture of *K. pneumoniae* RYC492 (●) and *E. coli* AB1157 (■). As control, the viability of *E. coli* AB1157 (△) was followed in minimal medium without amino acids. (c) Mixed culture of non-producing mutant *K. pneumoniae* R302 (■) and *E. coli* BM21 (○). (d) Mixed culture of *K. pneumoniae* R302 (■) and *E. coli* AB1157 (□).

Fig. 4. Survival curves of *E. coli* BM21 in cultures of *K. pneumoniae* RYC492 and their supernatants obtained at different stages of growth. Subcultures from a growing *K. pneumoniae* RYC492 culture in minimal medium were taken at different stages of growth. Standard suspensions of *E. coli* BM21 were mixed each time with the subculture and with the corresponding subculture supernatant. The viability of *E. coli* BM21 either in the subcultures with producer cells (●) or in the supernatants (○) was followed by plating samples in LB-nalidixic acid. Subcultures were taken at an OD₆₆₀ of: 0.3 (a), 0.8 (b) and 1.0 (c).
Fig. 5. Viability of *E. coli* BM21 in a culture of *K. pneumoniae* RYC492 and in its supernatant. *Klebsiella pneumoniae* RYC492 was grown to reach an OD<sub>660</sub> of 0.37. At that point, one half of the culture was centrifuged to remove producer cells. Standard suspensions of *E. coli* BM21 were added to the supernatant and to the culture with producer cells. Viability of *E. coli* BM21 was followed as described in Methods. ○, Titre of *E. coli* BM21 in the culture with producer cells; ●, the same, in the supernatant.

Fig. 6. Survival curves of *E. coli* BM21 in cultures of *K. pneumoniae* RYC492 treated with proteases. Standard suspensions of *E. coli* BM21 were mixed with subcultures of *K. pneumoniae* RYC492 in minimal medium in different conditions: (a) The subculture of *K. pneumoniae* RYC492 was taken at an OD<sub>660</sub> of 0.3. ○, Mortality of *E. coli* BM21 without proteases; ○, the same, plus 100 μg pronase ml<sup>-1</sup>; ■, the same, plus 100 μg chymotrypsin ml<sup>-1</sup>. (b) The subculture was taken as before at an OD<sub>660</sub> of 0.3. ○, Control, mortality of *E. coli* BM21 without enzymes; □, the same, plus 100 μg trypsin ml<sup>-1</sup>; Δ, viability of *E. coli* BM21 in minimal medium plus 100 μg trypsin ml<sup>-1</sup>, without producer cells. (c) The subculture was taken at the stationary phase at an OD<sub>660</sub> of 1.0. ○, Mortality of *E. coli* BM21 without protease; □, the same, plus 100 μg trypsin ml<sup>-1</sup>.

Plate assay of the microcin-antagonist

Further evidence for the production of a microcin-antagonist by the microcin producer strain was obtained as shown in Fig. 7. Mutants from *K. pneumoniae* RYC492 which failed to produce microcin were still able to release into the medium a diffusible agent which allowed the growth of sensitive cells around their spots on plates supplemented with microcin. Similar results were obtained when this assay was performed under anaerobic conditions.
Fig. 7. Production of microcin-antagonist by *K. pneumoniae* mutants which fail to produce M492. A Petri dish containing M9 minimal agar medium was supplemented with approximately 100 antibiotic units per ml of M492 obtained as described in Methods. Non-producing mutants of *K. pneumoniae* were then inoculated into the agar by means of a toothpick (anticlockwise from arrow: *K. pneumoniae* A301, A302, R301, R302, A303 and A305). The plate was spray-seeded with a suspension of the sensitive strain *E. coli* BM21 and incubated for 40 h. The central spot is a control of *K. pneumoniae* RYC492.

**Microcin activity in anaerobic conditions**

Plate assays showed that *K. pneumoniae* RYC492 produced microcin under anaerobic conditions. An inhibition zone was observed around the colony of the producer strain similar to that observed in the same experiment performed in aerobic conditions. The non-producing mutant *K. pneumoniae* R302 did not inhibit the sensitive strain *E. coli* BM21 under anaerobic conditions. Similarly, there was no inhibition zone when the resistant strain *E. coli* RK6 was tested for sensitivity to microcin. These controls ruled out the possibility that inhibition was due to causes other than microcin production and sensitivity to microcin in anaerobiosis.

**DISCUSSION**

The production of antibacterial substances by a large number of *Klebsiella* strains was occasionally described in the early studies on bacteriocins (Durlakowa et al., 1964). In most cases, these inhibitors have been indiscriminately named bacteriocins from *Klebsiella* or klebocins and have been classified (Maresz-Babczszyn et al., 1967) according to heterogeneous criteria such as resistance/sensitivity to high temperature, proteases or chloroform, and inducibility by UV light. Strain typing by means of these klebocins has been successfully used in clinical work for epidemiological studies (Buffenmyer et al., 1976). However, until now none of these *Klebsiella* bacteriocins has been purified or characterized and some of these substances formerly described as bacteriocins could actually be considered as microcins, since in current screening tests these two kinds of substances are virtually indistinguishable if differentiation assays (Asensio et al., 1976) are not performed. On the other hand, studies on indirect interactions between micro-organisms mediated by antibacterial substances have been rather limited (Adams et al., 1979) and no report is available in the literature concerning the interactions mediated by inhibitors produced by *Klebsiella*. The interest in this last point arises
mainly from the wide spread of *Klebsiella* nosocomial infections which might be attributable in some cases to imbalances in the bacterial ecosystems where this kind of micro-organism usually resides (Eickhoff, 1979).

When the microcin producer strain *K. pneumoniae* RYC492 was grown in liquid minimal medium mixed with an *E. coli* K12 strain, there was a net prevalence of the producer over the sensitive strain even when initial conditions were highly favourable to the latter. We ruled out the possibility that the prevalence of *K. pneumoniae* RYC492 was due to non-specific factors (pH changes, nutrient depletion, non-specific inhibitions) and consider the cause to be the release of a microcin into the medium, since non-producing mutants isolated from the RYC492 strain were unable to affect the growth of sensitive strains, and microcin-resistant cells (RK6 strain) grew unaffected by the presence of *K. pneumoniae* RYC492. When the producer strain reached the stationary phase, there was a recovery of viability in the sensitive strains. This was apparently due to microcin inhibition by a diffusible antagonist produced by the same *K. pneumoniae* RYC492 strain and not to the proliferation of resistance or any other kind of growth or cellular process in the sensitive cells.

Excretion into the medium by *Enterobacteriaceae* (and other micro-organisms) of biological inhibitors, together with antagonists of these inhibitors, has been described in some cases (Foulds & Shemin, 1969; Davie & Brock, 1966; Aguilar et al., 1982a) although no special significance has been ascribed to this fact. In our case, both antibacterial agent and antagonist are apparently synthesized and released into the medium by the same strain at different stages of its growth cycle. The microcin-antagonist exerts its action only when nutrients in the medium are depleted, but during the exponential growth of the producer strain the microcin activity appears to predominate. The antagonist apparently acts in cells already damaged by microcin, since this factor appears not to inactivate the microcin molecule itself.

A point which remains to be studied is the possible involvement of the microcin-antagonist in the immunity mechanisms of the microcin-producing strain against its own product. In this case, recovery of viability by the sensitive strain would be an effect derived from the self-protecting mechanism of producer cells.

The results of the experiments described in our *in vitro* amensalism/competition system suggest a mechanism of interaction between antibiotic-producer and sensitive strains which may have some significance in natural bacterial ecosystems. In this case, interaction would be mediated by the balance between the microcin and the microcin-antagonist, both released to the medium by the same producer strain in a ratio determined by the availability of nutrients in the system.

Both microcin production and sensitivity to the antibiotic were also observed under anaerobic conditions, as well as excretion of a microcin-antagonist by the same producer strain. This activity of the microcin in anaerobiosis is consistent with its proposed role in natural microbial interactions, although factors other than microcin production (namely generation times, activity of proteases and fermentation products) should be taken into account to relate our results to the phenomena observed in the intestinal ecosystem.

Further evidence to support the hypotheses above mentioned would require purification and characterization of the microcin-antagonist molecule as well as ecological work in more complex systems. These two topics are currently under study in this laboratory.

The authors are indebted to Drs F. Baquero, A. Aguilar and C. F. Heredia for critical reading of the manuscript; to Drs F. Moreno and J. L. San Millan for essential methodological suggestions and to N. Hopwood for correcting the manuscript. Technical assistance of Amalia Montes and Manuel Molina is also acknowledged. This work was supported by a grant from Fondo de Investigaciones Sanitarias de la Seguridad Social (1981).

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