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In vivo transfer of R factors between *Escherichia coli* strains inoculated into the rumen of sheep

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SUMMARY

Substantial transfer of R factors occurred *in vivo*, under certain conditions, in the rumen of adult sheep in the absence of any antibiotic treatment. A starvation period of 24–48 hr. was required to produce the conditions necessary, when even quite low inocula (*ca.* 10^3 cells) of donor and recipient *E. coli* could grow within the rumen and reach a population density sufficient for transfer to take place. The results indicate that under the same conditions R factors may be transferred between organisms in the lower intestinal tract also. Without the starvation period, the inoculation of even massive numbers (10^{10} cells) of the same organisms resulted in almost no detectable transfer.

Some of the experimental animals on which a starvation period was imposed became carriers of either the inoculated recipient *E. coli*, or of R factor bearing coliforms, and these formed 1–10% of the total coliform population of the faeces for at least 6 weeks.

INTRODUCTION

The transfer of antibiotic resistance by R factors from donor to recipient bacteria can be shown readily *in vitro*, but it has proved much more difficult to demonstrate such transfer *in vivo*, at least in normal, adult animals without some form of antibiotic treatment (Smith, 1971; Jarolmen, 1971). Nevertheless, unless large-scale transfer of R factors occurs in animals, it is difficult to account for the widespread presence of antibiotic-resistant bacteria in farm livestock (Smith, 1971). All experimental demonstrations of *in vivo* transfer to date have been achieved using monogastric animals and chickens, but ruminants also harbour resistant organisms and may prove to be an important reservoir from which bacteria containing R factors are carried to the human population. Certainly, beef for human consumption has been shown to be contaminated with antibiotic-resistant *E. coli* in the U.S.A. (Babcock, Berryhill & Marsh, 1973) and in England (Walton, 1970). Therefore it seems mechanisms must exist whereby R factors, and plasmids generally, can be easily transferred between bacterial cells *in vivo* in many animals, including ruminants. Without such mechanisms it is difficult to understand how plasmids, which are postulated to carry 'mobile' genetic material in a few cells of a bacterial population (Clowes, 1973), could survive in nature or why such entities even exist. As the gut is a normal habitat of certain organisms belonging to the

Enterobacteriaceae it would be expected that, at least under some conditions, transfer could occur readily within the gastro-intestinal tract of animals.

Anderson (1968) described the conditions necessary for the transfer of R factors *in vitro*. Briefly, these include the compatibility of the donor and recipient organisms, the mobility of the R factor in the donor strain, and the population density of the donor and recipient bacterial cells (which determines the frequency of chance contact). In addition, the donor, and preferably both donor and recipient, must be in an active metabolic state, i.e. capable of protein synthesis. If similar conditions ever occurred within an animal host, then transfer of R factors *in vivo* should also take place readily.

Experiments have shown (Brownlie & Grau, 1967; Grau, Brownlie & Smith, 1969) that the dietary regimen is most important in determining the fate of salmonella cells inoculated into the rumen of cattle and sheep. If a short starvation period was imposed on the animals, the salmonellas (and any adventitious coliform organisms already present) could grow in the rumen and attain fairly large numbers. This paper describes the *in vivo* transfer of an R factor, in the absence of antibiotic treatment, between suitable strains of *E. coli* inoculated into the rumen of sheep subjected to a short period of starvation.

MATERIALS AND METHODS

Bacterial cultures

Two strains of *E. coli* were isolated from the faeces of different sheep. An R factor, conferring resistance to streptomycin (25 μg./ml.) and sulphadimidine (500 μg./ml.), was transferred to one from a laboratory stock culture, and this was used as the donor organism. A chromosomal mutant, resistant to nalidixic acid (50 μg./ml.), was obtained from the other strain. *In vitro* tests showed the R factor to be transferred at a high frequency from this donor to the recipient. When 10^6 cells of each were inoculated into 10 ml. nutrient broth (Oxoid) and incubated at 37 °C. for 24 hr., exconjugant organisms, i.e. recipient cells into which an R factor had been transferred and resistant to the three antibiotics, accounted for 2–4 % of the final number of either donor or recipient organisms present (*ca.* $2{\cdot}0 \times 10^8$ cells each).

Plating media

Total coliform counts were made using MacConkey agar plates (Oxoid). The donor, recipient and exconjugant organisms were also counted on this medium with the required antibiotics added. When low counts were expected, each of five plates was surface inoculated with 0·2 ml. of the rumen fluid or faecal suspension (10 g. faeces blended in 90 ml. of 0·1 % peptone water for 30 sec. on the high-speed setting of a Sunbeam blender). Thus, one organism/ml. rumen fluid, or 10/g. faeces, could be detected. Otherwise, 0·1 ml. of the rumen fluid or faecal suspension, or of a series of 10-fold dilutions of them, was used. The plates were incubated at 37° C. for 18–24 hr.

Animals

Merino wethers (3–4 years old) were used. Each animal was used in only one experiment and was always pre-tested to ensure that no antibiotic-resistant bacteria were present in either the rumen or faeces. As far as could be ascertained none of the animals had ever been treated with antibiotics, and certainly not within the previous 6 months. The animals were confined in separate metabolism cages in such a way that they could not ingest any of their faecal material and were fed lucerne chaff (1 kg./day), free of antibiotics. Each experiment was performed on at least two animals.

Animal inoculation

Cells from cultures grown in nutrient broth for 24 hr. at 37° C. were inoculated directly into the rumen using a sterile sampling device passed down the oesophagus, and washed through with 100 ml. of 0·1 % peptone water. The donor and recipient organisms were inoculated at least 15 min. apart with separate sampling devices to ensure no transfer could occur during this time.

Samples

Approximately 30 ml. of liquor was withdrawn from the rumen at the required intervals by means of a sterile sampling device and transferred to a sterile, capped jar. Within 10 min. all agar plates for the determination of donor, recipient and exconjugant organisms were inoculated to minimize any significant amount of transfer occurring after the sample was taken.

Freshly voided faecal samples were collected each morning in a sterile plastic bag (30 × 46 cm.) clipped to the wool of the animals hind quarters. Plates were always inoculated from the faecal suspension within 5 min. of blending so that negligible transfer could occur during this time.

RESULTS

The results of each of the duplicated experiments were similar, and the figures shown represent one of the animals used each time.

When large numbers of donor and recipient organisms (*ca.* $2{\cdot}0 \times 10^8$ cells) were inoculated into animals on a full feed diet, no exconjugants were detected in either the rumen or faeces (Fig. 1*a*). Even when massive doses (cultures in 100 ml. of nutrient broth incubated at 37° C. for 24 hr.) of donor and recipient organisms (*ca.* $2{\cdot}0 \times 10^{10}$ cells) were inoculated into animals on a full feed ration, only low numbers of exconjugant cells (less than 10/ml.) were transiently detected in the ruminal fluid and none were found in the faeces (Fig. 1*b*). In both cases, the inoculated organisms decreased rapidly and could not be detected in the rumen after 3 days or in the faeces after 5 days.

However, if a 3-day starvation period was imposed on the animals from the time they were inoculated with the donor and recipient organisms (*ca.* $2{\cdot}0 \times 10^8$ cells), quite different results were obtained. Initially, both organisms decreased in the rumen as before, but, after the animals had been without food for about 24 hr.,

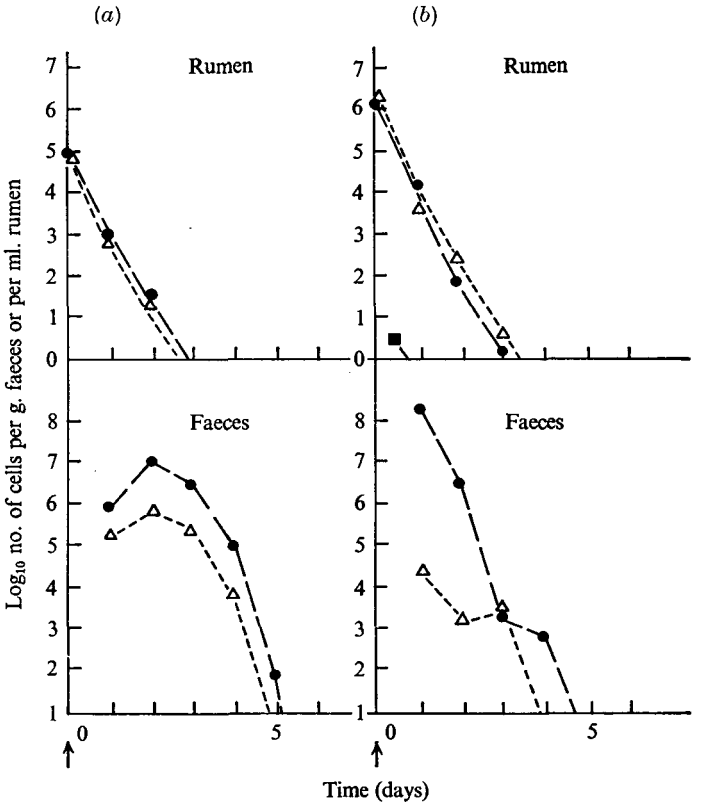

Fig. 1. Number of exconjugant cells (■) detected in rumen and faeces of sheep maintained on a full-feed diet after inoculation (↑) with *ca.* $2{\cdot}0 \times 10^8$ cells (*a*) or *ca.* $2{\cdot}0 \times 10^{10}$ cells (*b*), of donor (●) and recipient (△) organisms.

the tendency for coliform bacteria to die out rapidly in the rumen ended and they began to multiply instead. Some 4 hr. later, exconjugant organisms were detected indicating that the transfer of R factors from donor to recipient bacteria had occurred within the rumen. After about another 10 hr. this growth phase ceased and the numbers of donor, recipient and exconjugant cells in the rumen began to fall again. When the animals were fed again after the 3-day starvation period, however, another period of rapid growth began and lasted about 10 hr. With continued feeding, the coliform bacteria in the rumen again decreased and were no longer detectable after a further 11 days.

Exconjugant organisms were isolated from the faeces of both animals at 24 hr., approximately 4 hr. before such cells were detected in the rumen. About 3 days after the exconjugant cells disappeared from the rumen, they also disappeared from the faeces. However, it appeared that both the donor and recipient organisms could sometimes become lodged as part of the microflora of the lower intestinal tract of the experimental animals. In one animal, large numbers of coliforms containing the R factor (10^4–10^6 cells/g. faeces) were continually excreted for at least 6 weeks. In the second animal high numbers of recipients (10^6–10^8/g.) resistant to nalidixic acid were excreted for at least the same length of time (Fig. 2). Obviously,

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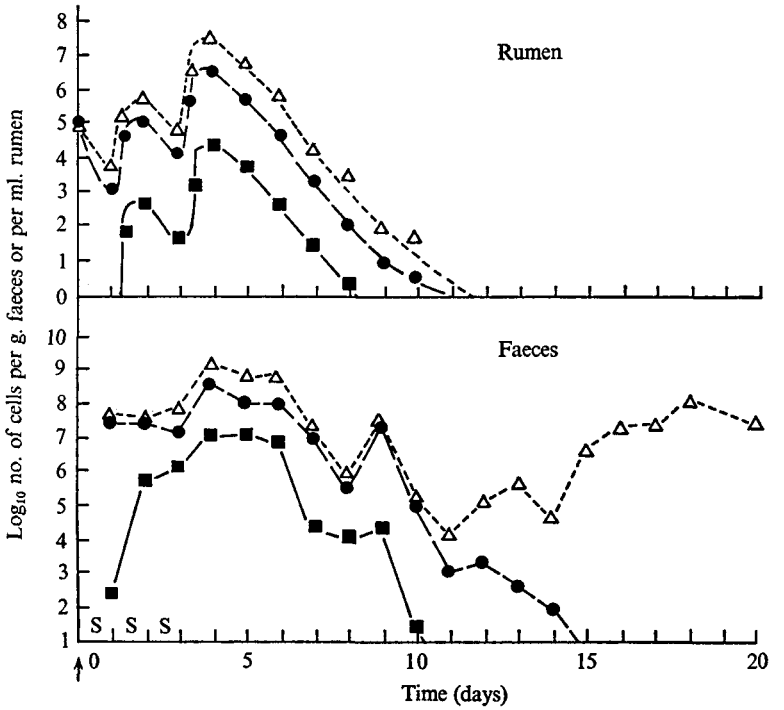

Fig. 2. Number of exconjugant cells (■) detected in rumen and faeces of a sheep starved for 3 days (S) and re-fed after inoculation (↑) with *ca.* $2{\cdot}0 \times 10^8$ donor (●) and recipient (△) organisms.

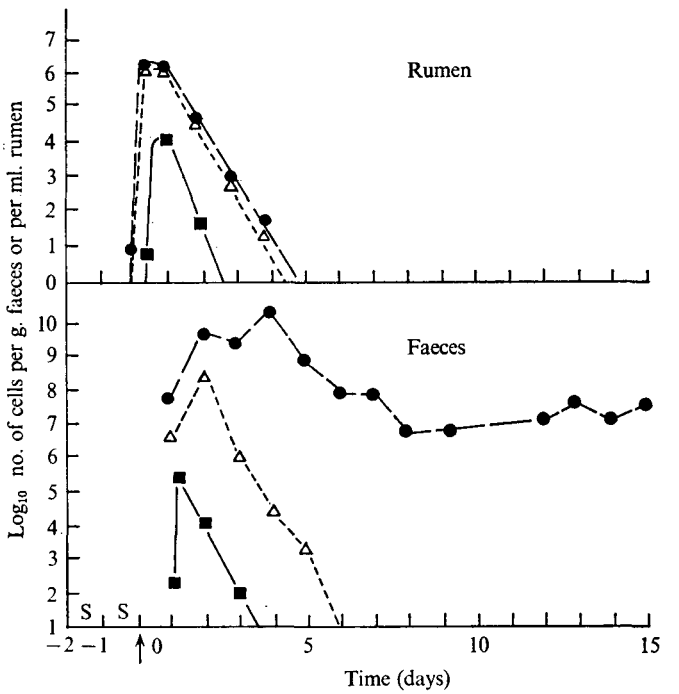

Fig. 3. Number of exconjugant cells (■) detected in rumen and faeces of a sheep inoculated (↑) with *ca.* 10^4 donor (●) and recipient (△) organisms and immediately re-fed after a 2-day starvation period.

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both animals had become permanent, or at least prolonged, excretors of one or other of these organisms. After 6 weeks the animals were released and not tested again.

Experiments were also carried out with four sheep which were starved for 2 days inoculated with small numbers of donor and recipient bacteria (in two cases with *ca.* 10^4 organisms and in the other two cases with *ca.* 10^3 organisms giving less than one viable cell/ml. rumen fluid) and immediately re-fed.

With each animal, growth of the inoculated coliforms began immediately and quite large numbers of exconjugant cells were present in the rumen within 10 hr. They were also detected in the faeces after 24 hr. On continued feeding of the experimental animals, the exconjugant organisms disappeared from the rumen in 4 days and from the faeces in 6 days. The numbers of donor and recipient bacteria also decreased and could no longer be detected in the rumen after 9 days. In three of the four sheep, both organisms also disappeared from the faeces by 15 days. However, in the remaining animal (Fig. 3), high numbers of coliform organisms containing the R factor (10^6–10^8/g. faeces) were excreted continuously for at least a further 6 weeks at which time the experiment was terminated.

In another three similar experiments, sheep were inoculated after a 2-day starvation period with *ca.* 10^4 of both donor and recipient cells and re-fed, but no exconjugant organisms were detected. In each case, large numbers of natural coliforms (*ca.* 10^3 cells/ml.) were already present in the rumen. These also multiplied rapidly after the animal was re-fed, outgrowing the inoculated organisms which consequently only reached populations of *ca.* 10^2–10^4 cells/ml., probably too few for cell-to-cell contact to occur.

DISCUSSION

Anderson (1968) described the conditions necessary for the transfer of R factors to take place between bacterial cells *in vitro*. The results reported here show that, provided similar conditions can be reproduced within the gastro-intestinal tract of an animal, transfer also takes place quite readily *in vivo*. The method used to achieve this with adult sheep was by withholding food for 24–48 hr. On a full-feed diet, no transfer occurred unless extremely large numbers of both donor and recipient bacteria were inoculated. It is not known how often, under natural conditions, both donor and recipient cells would be present in the rumen of one animal. However, if this should occur, and they are able to grow to large numbers, there appears to be no reason why transfer should not readily take place. Some natural conditions under which *E. coli* or other Enterobacteriaceae would be able to multiply within the gastro-intestinal tract of an animal can be readily envisaged. A short period of starvation, as was imposed in the present studies, is probably suffered frequently by both wild and domestic animals such as in times of food shortage, during recovery from fright or wounds, or due to periods of illness. Also, it is characteristic of some pathogenic organisms, e.g. salmonellas, to proliferate rapidly after invasion of the gastro-intestinal tract of a host animal.

None of the animals used during these experiments was exposed at any stage to antibiotics, and as far as could be ascertained, never had been. Therefore, the R

factor transfer took place *in vivo* in the absence of any special selective pressure, and possession of the R factor conferred no extra survival value on the host bacterial cells. This indicates that probably any plasmid, not only R factors, could be transferred under similar conditions. If a selective pressure was applied as well, undoubtedly far more exconjugant organisms containing the plasmid would be formed.

The theory has been advanced (Clowes, 1973) that plasmids contain a pool of '*mobile*' *genetic material within a bacterial population. In the absence of a* selective pressure this genetic information confers no special survival value on the host organism but is actually a slight metabolic burden, and the host cell is at a growth disadvantage compared with cells not containing the plasmid (Anderson, 1973). Nevertheless, because the genetic information may sometimes be required, it is carried constantly by a few cells and sometimes transferred to other cells to ensure it is maintained within the bacterial population. The results of the experiments reported here support this concept and show how, under certain conditions which may not be uncommon in nature, this genetic information can be exchanged between cells of the Enterobacteriaceae in their natural habitat within the gastro-intestinal tract of an animal.

It was noticed that when low numbers of donor and recipient organisms (*ca.* 10^4 cells giving less than 10 cells per ml. rumen fluid) were inoculated into animals after a 48 hr. starvation period, no exconjugants were obtained if large numbers of adventitious coliforms ($> 10^3$/ml.) were already present in the rumen. Under these conditions, the inoculated organisms were probably unable to grow to a sufficient population density to come into contact with each other. The R factor could have been transferred from the inoculated donor cells to some of these adventitious coliforms, but as neither contained a specific chromosome marker they could not be distinguished apart.

Sometimes, the experimental animals became permanent, or at least prolonged, excretors of either the inoculated recipient organism or of coliforms containing the R factor. In the three cases where this happened, these organisms formed a significant part of the total coliform population of these faeces (1–10 %). The recipient contained a non-transferable chromosomal marker and could be easily identified, but it was impossible to determine whether the R factor-containing organisms were from the population introduced into the rumen or part of the natural flora already present into which the R factor had been transferred.

Exconjugants were sometimes detected in the faeces before they were detected in the rumen. This implies that R factors can also be transferred *in vivo* in the lower gastro-intestinal tract of animals, an aspect which is being further investigated.

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