Survival of a Recombinant Rumen Bacterium in the Rumen of Sheep

Yasuo KOBAYASHI^a, Masaki YAMADA and Mayu YAMAMOTO^b

Faculty of Bioresources, Mie University, Tsu-shi 514-8507, Japan

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Abstract Survival of the recombinant rumen bacterium *Butyrivibrio fibrisolvens* NO4, expressing an exogenous xylanase and erythromycin resistance, was assessed *in vitro* and *in vivo*. Selective enumeration of the recombinant strain from indigenous rumen bacteria was performed using an erythromycin-supplemented anaerobic medium. When incubated *in vitro* with mixed rumen bacteria and protozoa obtained from sheep, the numbers of the recombinant strain stabilised at 10^3 /ml (48 h). When incubated with mixed microbes from the goat rumen, strain NO4 disappeared 48 h after inoculation. The presence of protozoa in the culture tended to decrease the numbers of strain NO4 ten-fold compared to bacteria alone. *In vivo*, numbers declined rapidly after inoculation, and the recombinant strain was no longer detectable in the sheep rumen after 144 h.

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Recent developments in genetic engineering are applicable to rumen microbes, allowing improvements to their metabolic capabilities, *e.g.* fibrolysis, protein synthesis and detoxification⁶⁾. One such application is close to be at the animal production stage³⁾. Studies tracking *Prevotella ruminicola*²⁾ and a *Ruminococcus albus* transconjugant⁸⁾, inoculated into the rumen, suggested that rapid elimination or poor establishment occurs with bacteria that have been maintained in culture for a considerable time. However, Gregg *et al.*³⁾ observed that inoculated wild and recombinant *Butyrivibrio fibrisolvens* in the rumen of sheep were detectable by PCR even at 10^{5-7} dilutions. Identification of factors important for survival and establishment of specific bacterial strains (including recombinants) in the rumen is required. The present paper describes the survival of the recombinant rumen anaerobic bacterium *Butyrivibrio fibrisolvens* NO4 in simple batch culture and the sheep rumen.

Materials and Methods

Butyrivibrio fibrisolvens NO4⁵⁾ expressing a *Eubacterium ruminantium* xylanase gene (*xynA*) and a pAM β 1-originated erythromycin resistance gene (*erm*) was used. NO4 was anaerobically grown until mid logarithmic phase (approximately, OD₆₀₀=0.5) in a

reduced CCA medium¹⁾, and used directly for *in vitro* inoculation. A rumen cannulated sheep and goat (weighing 45 and 21 kg) were used as rumen fluid donors for *in vitro* experiments. The animals were fed a cubed alfalfa hay and concentrate diet (400 g:100 g (sheep) and 200 g:50 g (goat) respectively) twice daily.

Rumen fluid was collected prior to morning feeding, strained through 2 layers of surgical gauze and centrifuged (500 × g, 2 min). The supernatant was centrifuged (20,000 × g, 15 min) to collect bacteria and another aliquot of the rumen fluid was incubated with an acetate buffer at 37 °C to separate protozoa. Bacteria and protozoa fractions were washed twice with an anaerobic dilution solution⁹⁾, resuspended in McDougal's artificial saliva and combined if necessary (bacteria (B) and bacteria plus protozoa (BP)). Both B and BP were mixed with 2 g ground alfalfa hay, for the simple batch culture (100ml in volume) experiments. Samples were taken at 0, 2, 4, 6, 8, 12, 24 and 48 h after inoculation of strain NO4 to enumerate total bacteria, strain NO4 and protozoa.

The donor sheep was used as the recipient for strain NO4 inoculation *in vivo* (managed according to Mie University safeguard for recombinant DNA experiments). NO4 cells harvested from a 2 litre culture by centrifugation and washed twice with anaerobic dilution solution were

Present address: ^aGraduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo-shi 060-8589, Japan

^bGraduate School of Medicine, Osaka University, Suita-shi 565-0871, Japan

Corresponding: Yasuo KOBAYASHI (fax: +81(0)11-706-2550, e-mail: kyas@anim.agr.hokudai.ac.jp)

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resuspended in 200 ml dilution solution for inoculation. The inoculum was added to the rumen by a tube and peristaltic pump (5 min duration). Rumen fluid was collected at 0, 2, 4, 6, 12, 24, 48, 72, 144, 240 and 336 h after inoculation, strained with 2 layer of gauze and used for microbial counting.

Total bacteria and protozoa were counted by culturing with CCA medium and light microscopy, respectively. Enumeration of strain NO4 was carried out using roll tube culturing with CCA medium containing 400 mg/ml erythromycin (*see* Results and Discussion). Colonies from erythromycin-supplemented roll tubes were confirmed to be NO4 by slot blot hybridization¹⁰ using a *xynA* fragment as a digoxigenin-labelled DNA probe⁵.

Fig.1. Fate of the recombinant rumen bacterium incubated with mixed rumen microbes with [A] or without [B] protozoa from sheep.

Results and Discussion

Growth of indigenous rumen bacteria was gradually inhibited as erythromycin concentration increased in the culture medium (data not shown). As no growth was

observed with 400 mg/ml of erythromycin, this concentration was used to selectively enumerate strain NO4. Colonies on erythromycin-supplemented roll tubes were confirmed to carry the chimeric plasmid coding for *xynA* by slot blot hybridization (data not shown). The recombinant strain maintained resistance to erythromycin even after 10 day culture passages (approximately 50 generations) without erythromycin selection pressure. This indicates that the current protocol is suitable for enumerating NO4 during relatively long-term experiments.

Numbers of strain NO4 in batch culture experiments with rumen microbes of sheep origin are shown in Fig. 1. NO4 was stably maintained at 10^3 /ml throughout 48 h in the mixed culture containing protozoa. Although numbers of NO4 maintained similarly in batch culture lacking protozoa, the value was approximately 10 fold higher (10^4 /ml). Protozoa appear to have a negative effect on the maintenance of the recombinant strain. Predatory action of the protozoa against NO4 may be a possible explanation. Changes in indigenous bacteria by protozoa addition are negligible: perhaps due to the bacteria originating from the same source as the protozoa.

Results from batch cultures using microbes of goat rumen origin are shown in Fig. 2. Strain NO4, initially present at 10^4 /ml, declined in number rapidly and disappeared within 48 h. The results suggest certain ruminal characteristics unfavorable for NO4 exist, that are specific to the goat rumen. Differences in protozoal and bacterial diversity between the goat and sheep may be key factors. In fact, additional diplomonads and entodina were observed in the goat rumen (data not shown).

Fig.2. Fate of the recombinant rumen bacterium incubated with mixed rumen microbes from goat.

The fate of the NO4 *in vivo* is shown in Fig.3. When initially introduced into the sheep rumen, NO4 rapidly reduced in number from 10^6 /ml (0 h) to 10^2 /ml (24 h). Although this number (10^2 /ml) was maintained until 72 h, no more culturable recombinant existed by 144 h. As the NO4 persisted for 48 h *in vitro* (Fig.1), the cause of the disappearance within 144 h may be due to washout of the strain NO4 from the rumen. The ability of the NO4 to attach to feed particles has not been assessed, but this characteristic may allow decreased washout of the recombinant strain. Other speculations have been made in relation to the poor survival of newly introduced bacteria in the rumen^{2, 4, 7, 8, 11}.

Results indicate that the sheep rumen is more favorable for the survival of NO4 compared to goat. The unidentified limiting factors may relate to the protozoa population, bacteria diversity, phages, trace nutrients and antibacterial compounds such as bacteriocins. Exploring these factors will allow the conditions required for successful establishment of strain NO4 in both the rumen of sheep and goats to be clarified further.

Fig.3. Fate of the recombinant rumen bacterium inoculated into the rumen of sheep (*in vivo*).

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