Potential biotechnological applications of a *Lactococcus lactis* strain lacking cell wall hydrolase that persists in the gastrointestinal tract

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Abstract

Lactococcus lactis has a long use in traditional biotechnology for making fermented dairy and other food products. A *L. lactis* mutant lacking the cell wall hydrolase acmA, and growing in long chains, is shown to survive in the normal mice for several weeks, unlike wild type *L. lactis*. Persistent *L. lactis* has greater potential for safe, long term delivery of vaccines and therapeutics.

Introduction

Lactococcus lactis is a Gram positive coccus that has been used for thousands of years in traditional biotechnology for making cheese, buttermilk and other fermented food products. It is typically ovoid with a diameter of 0.5-1µm, grows best at 30°C in pairs or short chains, is not invasive and does not colonise the gastrointestinal tract (GIT) except in germ-free mice [1-3]. Because *L. lactis* is generally regarded as safe for ingestion, it is being developed for use in modern biotechnology as a carrier or vector for eliciting systemic and mucosal immunity against pathogens through oral immunisation [3, 4] and for delivering therapeutics to the GIT, e.g. IL-10 in colitis [5]. However the transitory nature of the organism in the GIT limits exposure to antigen or therapeutic, and often requires that the foreign protein expression is induced *in vitro* before feeding.

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A mutant form of *L. lactis* strain NZ9000 that lacks the cell wall hydrolase AcmA (*L. lactis*-Δ*acmA*) grows as very long chains in culture because the cells fail to separate properly after division [6]. We examined the survival of *L. lactis*-Δ*acmA* in mice in the context of the use of *L. lactis* expressing heterologous proteins for mucosal immunisation.

Materials and Methods

Wild type *L. lactis*-NZ9000 expressing the malaria protein MSA2 [4] and mutant *L. lactis*-Δ*acmA* were from the Department of Genetics, University of Groningen, Groningen, The Netherlands. Balb/c mice were from the Medical Research Institute, Colombo, Sri Lanka and were age-matched for each experiment. The bacteria were grown in M17 medium (Difco, MD, USA) containing 1% glucose at 30°C as standing cultures and stocks were stored frozen in a viable state at -80°C in glycerol as described [4].

Mice were fed orally with 4.5 x 10^9 bacteria in 20% sucrose through a plastic micropipette, while 0.5 x 10^9 bacteria were introduced into the nasal cavities with a plastic pipette at the same time. This procedure was adopted because of a concomitant investigation of the immune response to MSA2. The procedure was repeated for two

consecutive days (delivery on days 0-2) and then again after 3 weeks (days 20-22) and finally after a further 4 weeks (days 49-51). Faecal pellets were freshly collected, beginning with the day after the first feeding, from two groups of two marked mice fed the mutant and wild type *L. lactis* respectively. The pellets were weighed, homogenised in sterile PBS (1 ml per 100mg wet weight of faeces) and 50µl aliquots of serial ten-fold dilutions in PBS plated on M17 agar. Chloramphenicol (5 $\Delta g.ml^{-1}$) was added to the plates for selecting wild type *L. lactis*, since this strain carried a resistance gene (5). The extracts from mice fed *L. lactis*-Δ*acmA* were plated without the antibiotic as the mutant lacked the resistance gene. The plates were incubated for 16h at 30°C and the colonies counted. The colonies were examined under the microscope to determine the morphology of the cells. The cells were also Gram-stained to confirm identity.

Results

The plates from faeces of wild type *L. lactis* contained a small proportion of unrelated colonies of a single, clearly distinguishable morphotype, probably a yeast. However such contaminants were rare in plates of faecal extracts from mice fed of *L. lactis*-Δ*acmA*, probably because of the higher numbers of *L. lactis*-Δ*acmA* excreted. No lactococcal colonies were obtained from faeces of control mice not fed *L. lactis*. The *L. lactis* colony forming units (cfu) in the faecal pellets of one representative mouse from each group are shown in Fig 1. Similar results were obtained from the other mice fed the mutant and wild type *L. lactis*. The mouse fed wild type *L. lactis* excreted viable bacteria for 2-5 days after commencement of feeding with peaks reaching approximately 10^4-10^5 cfu per 100mg faeces on the second and third days of feeding. The mouse fed *L. lactis*-Δ*acmA* showed continuous excretion of bacteria at approximately 10^5 cfu/100mg faeces over the 62 days of observation, with peaks reaching approximately 10^9 cfu/100mg faeces on the second and third days of feeding. It was estimated that the average wet weight of faeces produced by this cohort of Balb/c was 100mg per day.

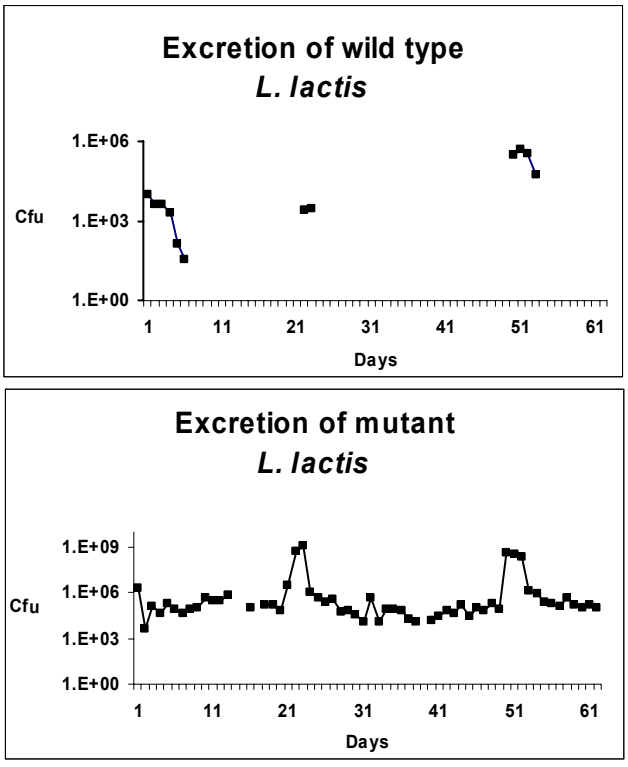
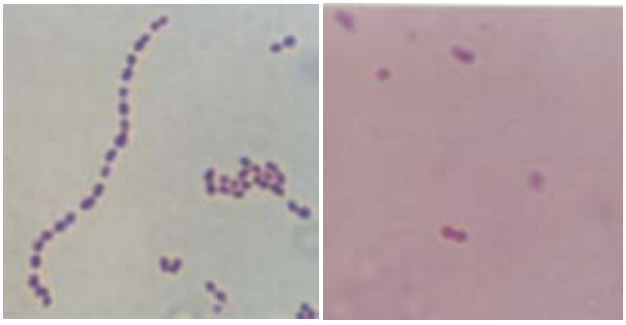


Figure 1. Graphs showing the excretion of wild type and Δ*acmA*-*L. lactis* from one mouse of each group. The results are expressed as colony forming units (cfu) of *L. lactis* per 100 mg of faecal pellet which were collected and analyzed every day for 62 days. When no *L. lactis* was detected this is shown without a data point in the graph except for days 14,15 & 17 for the mouse fed Δ*acmA*-*L. lactis* when the faecal pellets were not collected for analysis.

Gram staining confirmed that the colonies enumerated were typical of wild type *L. lactis* and *L. lactis*-Δ*acmA* (Fig 2). The mice were sacrificed on day 63 and their spleens, mesenteric lymph nodes and GIT examined. These were normal morphologically and histologically, except for a tendency for larger mesenteric lymph nodes and more prominent Peyer's patches in mice fed either strain of *L. lactis*, compared to unfed controls. We also examined serum antibody production to lactococcal antigens in mice similarly fed wild type and mutant *L. lactis*. Antibody levels increased with each set of feedings, but were not significantly different between the two *L. lactis* strains (data not shown).



ΔacmA *L. lactis* wild type *L. lactis*

Figure 2. Photomicrographs of Gram-stained samples of Δ*acmA*-*L.* lactis and wild type *L. lactis* colonies from plated faecal extracts, viewed at x 1000 magnification. The morphology is characteristic of the mutant and wild type strains described previously [6].

Discussion

It has been reported that *L. lactis* is quite resistant to gastric acidity but susceptible to trypsin and other factors in the duodenum, and relatively less affected in the lower GIT of rats [7]. *L. lactis* colonisation in germ-free mice predominantly occurs in the caecum [9]. *L. lactis* does not colonise normal mouse GIT [8] and does not translocate to the mesenteric lymph nodes and spleen, unlike endogenous gut bacteria [9]. Experimental findings on feeding wild type *L. lactis* to humans are compatible with the rodent findings, and show that the bacterium is killed and lysed in the GIT with <2% of the ingested bacteria being excreted live in faeces [2]. Our findings confirm that wild type *L lactis* is eliminated from normal mice within a few days and only a very small proportion, estimated in the order of 0.002%, is detectable as live bacteria in faeces. In contrast, *L. lactis*-Δ*acmA* persists and is shed from the GIT continuously for at least 3 weeks after introduction, with approximately 10% of ingested bacteria detected in faeces as live bacteria. The actual numbers of live bacteria in the faeces may be greater, and dependent on the sensitivity of the extraction and detection procedure.

The constant rate of excretion of live *L. lactis*-Δ*acmA* is consistent with the hypothesis that mutant persists in the lower GIT and is able to multiply there. The long chains of *L. lactis*-Δ*acmA* may facilitate trapping in the GIT. However the possibility that the bacterium survives in the nasal cavity and is then shed into the GIT, although unlikely, cannot be entirely excluded on the present data. Our results also suggest that *L. lactis*-Δ*acmA*, despite its enhanced persistence, does not produce more changes in host animals than wild type *L. lactis*.

We anticipate that *L. lactis*-Δ*acmA*, and similar mutants of other food grade bacteria, will be useful for safely improving the delivery of probiotics, therapeutics and vaccines to man and animals.

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