

# Efficacy of a *Lactococcus lactis* $\Delta$ *pyrG* vaccine delivery platform expressing chromosomally integrated *hly* from *Listeria monocytogenes*

Mohammed Bahey-El-Din,<sup>1,2,4</sup> Pat G. Casey,<sup>2,3</sup> Brendan T. Griffin<sup>1</sup> and Cormac G.M. Gahan<sup>1-3,\*</sup>

<sup>1</sup>School of Pharmacy; <sup>2</sup>Department of Microbiology; <sup>3</sup>Alimentary Pharmabiotic Centre; University College Cork; Cork, Ireland; <sup>4</sup>Department of Pharmaceutical Microbiology; Faculty of Pharmacy; Alexandria University; Alexandria, Egypt

**Key words:** *Lactococcus lactis*, biological containment, listeriolysin O, *pyrG*, *hly*, vaccine

**Abbreviations:** LLO, listeriolysin O; CTP, cytidine triphosphate; UTP, uridine triphosphate; IP, intraperitoneal; Em, erythromycin; Cm, chloramphenicol; SOE, splicing by overlap extension; SEC, Usp45 secretion signal; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; ELISPOT, enzyme-linked immunospot test; APCs, antigen presenting cells; CDM, chemically defined medium

*Listeria monocytogenes* is a significant food-borne pathogen and the causative agent of listeriosis, a disease which manifests as meningitis in immunocompromised adults or infection of the fetus and miscarriage in pregnant women. We have previously used *Lactococcus lactis*, a GRAS (Generally Regarded As Safe) organism, as a vaccine vector against listeriosis by engineering plasmid-mediated expression of the immunodominant antigen from *L. monocytogenes*, listeriolysin O (LLO). However, the environmental release of an engineered vaccine vector carrying a replicating plasmid during clinical usage may raise safety concerns. Here we describe the integration of the LLO gene (*hly*) into the *L. lactis* chromosome through homologous double crossover to allow stable expression, in order to avoid the use of antibiotic selection markers and to eliminate the requirement for a plasmid-based system. The approach was designed to simultaneously eliminate the *pyrG* gene encoding the CTP synthase which is responsible for converting UTP to CTP in a unique step in the de novo pyrimidine synthesis in *L. lactis*. This gene was targeted in order to restrict bacterial replication outside of the host (biological containment). The resulting cytidine auxotroph was able to secrete LLO constitutively and could elicit LLO<sub>91-99</sub>-specific CD8<sup>+</sup> T lymphocytes in the murine infection model. Moreover, protection against lethal challenge with *L. monocytogenes* was accomplished after intraperitoneal (IP) vaccination with the constructed strain. The implications for the use of cytidine auxotrophy in biological containment are discussed.

## Introduction

*Listeria monocytogenes* is a food-borne pathogen that can lead to meningitis in immunocompromised adults or fetal infection and potentially miscarriage in pregnant women.<sup>1</sup> Listeriolysin O (LLO) is the major virulence factor of *L. monocytogenes* and enables the escape of the pathogen from the phagosome to the cytoplasm of infected host cells. This unique mechanism depends on the ability of LLO to interact with cholesterol in the phagosomal membrane where it oligomerizes and creates pores through which bacteria can escape to the cytosol.<sup>2</sup> Being an intracellular microorganism, *L. monocytogenes* evades host antibody-mediated immunity and protection against infection is mainly accomplished through cytotoxic cell-mediated immunity.<sup>3,4</sup> The development of cytotoxic CD8<sup>+</sup> immunity against major listerial antigens (including LLO) is particularly important for protection against listeriosis.<sup>4</sup>

*Lactococcus lactis* is a Gram-positive bacterium that is widely used in the food industry. Due to its GRAS (Generally Regarded

As Safe) status, *L. lactis* has been extensively investigated as a vaccine vector by expressing heterologous antigens of various pathogens.<sup>5</sup> Recently we investigated *L. lactis* as a potential vaccine vector against listeriosis by expressing LLO constitutively or inducibly in various cellular compartments.<sup>6,7</sup> In the latter study the LLO gene (*hly*) was cloned in plasmid vectors and transformed into *L. lactis*. However, ultimately the clinical use of a plasmid-borne system will raise safety concerns about the release of vectors expressing antibiotic resistance markers which have a risk of transfer to pathogens in the environment. Since promising immunological outcomes were obtained using plasmid-mediated expression of LLO in *L. lactis*,<sup>6</sup> we endeavoured in the present study to stably integrate *hly* into the chromosome of *L. lactis* for constitutive expression of LLO. The strong constitutive lactococcal P23 promoter<sup>8</sup> was chosen to drive LLO expression and a construct was designed to replace the lactococcal *pyrG* gene using the pORI280/pVE6007 integration system.<sup>9,10</sup> The *pyrG* gene was targeted because it encodes the CTP synthase responsible

\*Correspondence to: Cormac G.M. Gahan; Email: c.gahan@ucc.ie

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for the unique de novo pathway that converts UTP to CTP in *L. lactis*.<sup>11</sup> Consequently, when *pyrG* is knocked out, a requirement for cytidine is established and cytidine has to be added in culture media for bacterial survival.<sup>11</sup> It was previously reported that mutations in the thymidylate synthase (*thyA*) gene of bacteria led to strict thymidine or thymine auxotrophy and rapid cell death in thymidine-free media (thymine-less death).<sup>12</sup> The previous concept was utilized by Steidler et al.<sup>13</sup> to create a biologically contained *L. lactis* that secretes hIL-10 (human interleukin 10) to treat inflammatory bowel disease (IBD) by the oral route. When the hIL-10 expression cassette replaced *thyA* in *L. lactis*, a strict thymidine auxotroph was created which rapidly died upon thymidine starvation and is therefore 'biologically contained'.<sup>13</sup> This biological containment property is proposed to prevent the dissemination of genetically modified bacteria in the environment where pyrimidine is limiting.

In brief, in the present work we created a cytidine auxotroph of *L. lactis* that constitutively secretes LLO from an integrated construct. In vivo vaccination using the created strain was investigated in mice where it resulted in an LLO-specific CD8<sup>+</sup> response and protection upon challenge with wild type *L. monocytogenes*. This construct will provide a platform for the development of future vaccines and for delivery of further heterologous antigens.

## Results

**Success of replacement recombination in *L. lactis* MG1363 chromosome and production of LLO.** We constructed a pORI280 plasmid vector carrying the *hly* gene from *L. monocytogenes* under the influence of the P23 promoter and flanked by appropriate sites for integration in place of the *pyrG* gene of *L. lactis* (Figs. 1 and 2). Integration of pORIP23:SEC-LLO (single crossover) in the *L. lactis* MG1363 chromosome followed by excision of pORI280 along with *pyrG* (including its native promoter), (i.e., double crossover), was confirmed by a number of PCR reactions (Fig. 3A) using primers outlined in Figure 2. The resulting strain, *L. lactis* MG1363  $\Delta$ *pyrG* (P23:SEC-LLO), was examined for LLO secretion by TCA-precipitation of the supernatant followed by western blot using primary rabbit anti-LLO antibodies (Diatheva, Italy). A specific band of LLO was obtained at the expected protein size (about 57 KDa) (Fig. 3B). Culture supernatant of *L. lactis* MG1363  $\Delta$ *pyrG* (P23:SEC-LLO) had complete hemolytic units (CHU) of 8 confirming the secretion of biologically active LLO.

**Deletion of *pyrG* gene has a bacteriostatic rather than bactericidal effect on the cytidine auxotroph *L. lactis* MG1363  $\Delta$ *pyrG* (P23:SEC-LLO).** The *pyrG* gene is reported to encode the enzyme CTP synthase.<sup>11</sup> This enzyme is responsible for the conversion of UTP to CTP and it is the only pathway for the de novo synthesis of CTP. We examined if *pyrG* deletion would result in a strictly auxotrophic cytidine mutant and if this deletion would have a bacteriostatic or bactericidal effect on the resulting mutant in cytidine deprived culture medium. Upon growing *L. lactis* MG1363  $\Delta$ *pyrG* (P23:SEC-LLO) in CDM lacking cytidine, there was a slow decline of the bacteria from 6.5 log CFU/ml to about 5 log CFU/ml over a 15 day period (Fig. 4A).

This finding indicates that the absence of cytidine from the culture medium is generally bacteriostatic rather than bactericidal. When the same mutant was grown in CDM containing 20  $\mu$ g/ml cytidine, a phase of initial growth occurred starting from 6.5 log CFU/ml up to 8 log CFU/ml in the first 24 h, followed by a slow decline until it reached about 6.5 log CFU/ml again on day 15. This suggests that upon consumption of the provided cytidine from the CDM culture medium, the resulting cytidine starvation condition is bacteriostatic (Fig. 4A).

Survival of the cytidine auxotroph *L. lactis* MG1363  $\Delta$ *pyrG* (P23:SEC-LLO) was also examined in autoclaved soil as an attempted simulation of a natural environmental situation. No significant difference in survival was observed between the cytidine auxotroph and the wild type MG1363 strain (Fig. 4B). This indicates that cytidine auxotrophy does not lead to a dramatic cell death in conditions of cytidine starvation and suggests that other approaches (such as deletion of the *thyA* locus) are necessary for full biological containment.<sup>13</sup>

**CD8<sup>+</sup> T lymphocytes specific for the H2-K<sup>d</sup>-restricted LLO<sub>91-99</sub> epitope are elicited by *L. lactis* MG1363  $\Delta$ *pyrG* (P23:SEC-LLO) following IP immunization.** The ELISPOT assay was used to analyze the development of LLO<sub>91-99</sub>-specific CD8<sup>+</sup> cells. The mouse mastocytoma cells P815-1-1 were used as antigen presenting cells (APC) as they express restricted H2-K<sup>d</sup> MHC class I molecules, so any resulting spots are due to LLO-specific CD8<sup>+</sup> cells.<sup>22</sup> The cellular immune response was examined 4 weeks after the last IP booster. Groups injected with *L. lactis* MG1363  $\Delta$ *pyrG* (P23:SEC-LLO) or the positive control *L. monocytogenes* EGDe showed significant LLO<sub>91-99</sub>-specific spots ( $p < 0.05$ ) (Fig. 5A). On the contrary, no specific spots were observed with groups treated with the wild type *L. lactis* MG1363 or the PBS-treated group (Fig. 5A).

**Protection against *L. monocytogenes* challenge following IP vaccination.** Mice were challenged intraperitoneally with *L. monocytogenes* EGDe 4 weeks after the final vaccine booster. Three days following challenge, mice were euthanized and the listerial count was determined in the spleens. Bacterial count results following the IP vaccination revealed significant protection ( $p < 0.05$ ) as evidenced by the low listerial count in spleens of mice vaccinated with *L. lactis* MG1363  $\Delta$ *pyrG* (P23:SEC-LLO) (Fig. 5B). In contrast, high listerial counts were observed in the organs of the PBS-treated groups or groups treated with the control strain *L. lactis* MG1363 (Fig. 5B). These results reflect the previous ELISPOT results (Fig. 5A) where *L. lactis* MG1363  $\Delta$ *pyrG* (P23:SEC-LLO) could elicit LLO<sub>91-99</sub>-specific spots.

## Discussion

*L. lactis* has been repeatedly examined as a safe and effective vaccine delivery platform for the delivery of various heterologous antigens to the immune system.<sup>5</sup> In our previous work, we successfully expressed listeriolysin O (LLO) of *L. monocytogenes* in *L. lactis* under inducible and constitutive conditions.<sup>6</sup> The *hly* gene of LLO was expressed on plasmid vectors and immunizations with the constructed strains elicited specific protective immune responses against listeriosis in the murine infection













