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Lactococcus lactis-expressing listeriolysin O (LLO) provides protection and specific CD8$^+$ T cells against *Listeria monocytogenes* in the murine infection model

Mohammed Bahey-El-Din[a,b], Pat G. Casey[a,c], Brendan T. Griffin[b], Cormac G.M. Gahan[a,b,c,*]

[a] *Department of Microbiology, University College Cork, Cork, Ireland*
[b] *School of Pharmacy, University College Cork, Cork, Ireland*
[c] *Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland*

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ABSTRACT

Lactococcus lactis has previously been proposed as a vaccine platform for the safe delivery of heterologous antigens. Here we utilized *L. lactis* as a live vector for expression of listeriolysin O (LLO), a major *Listeria monocytogenes* antigen and virulence factor. A variety of plasmid constructs were designed to permit either constitutive or nisin-inducible expression of secreted or non-secreted LLO in *L. lactis*. Recombinant strains were subsequently tested in a murine model for vaccination efficacy against *L. monocytogenes* infection. CD8$^+$ T lymphocytes specific for the LLO_{91-99} epitope were detected when strains were administered via the intraperitoneal (IP) but not the oral route. Challenge with live *L. monocytogenes* revealed different levels of protection among the three vaccine strains tested with the nisin-inducible LLO-secreting *L. lactis* strain providing the greatest protection against secondary infection. This work highlights the usefulness of the GRAS (*G*enerally *R*egarded *A*s *S*afe) organism *L. lactis* as the basis of a live vaccine vector against *L. monocytogenes*. The work suggests that LLO-expressing *L. lactis* strains may also have the potential to act as a platform for directing other co-expressed antigens towards the cytosolic MHC class I pathway for enhanced stimulation of the CD8$^+$ T-cell response.

1. Introduction

Listeria monocytogenes, the causative agent of listeriosis, is a food-borne pathogen which can infect immunocompromised individuals causing meningitis and septicemia [1]. Pregnant women are particularly susceptible, developing flu-like symptoms followed by chorioamnionitis and miscarriage [1]. Although *L. monocytogenes* infections are generally sporadic in the community, several large-scale common-source outbreaks have occurred worldwide [2]. Once listeriosis is established in an infected host the mortality rate is high (20–30%), and in terms of human mortality *L. monocytogenes* is considered to be among the most significant bacterial causes of food-borne diseases [3]. Moreover, *L. monocytogenes* also causes infections of cattle and sheep and can result in appreciable losses through foetal infection and spontaneous abortions in these animals. Infection of farm animals is also considered to pose a zoonotic threat to humans who may be exposed to *L. monocytogenes* through contaminated milk or meat products [4].

L. monocytogenes is an intracellular pathogen which has a unique mechanism of cellular infection. Upon phagocytosis, the pathogen secretes a hemolysin, listeriolysin O (LLO), that forms pores in the phagosomal membrane enabling the bacterium to access the host cell cytoplasm [3]. Through the expression of other specific virulence factors (including ActA, Hpt, and PlcB), *L. monocytogenes* replicates and moves within the cytosol and can spread from cell to cell without exposure to the extracellular milieu [5]. Investigation of the immune response against *L. monocytogenes* showed that in addition to its role in intracellular pathogenesis, LLO is a major immunodominant listerial antigen. Indeed protective immunity against *L. monocytogenes* is dependent on cytotoxic CD8$^+$ cell-mediated immunity against epitopes of the two major virulence factors LLO and P60 [6]. The central role of LLO in the listerial infection cycle, coupled to the significant antigenicity of this protein indicates that LLO may have potential as the basis of a vaccine against *L. monocytogenes*. Live bacterial vectors such as attenuated *Bacillus anthracis* and *aroA*$^-$ *Salmonella typhimurium* strains have previously been engineered to express LLO for vaccination against *L. monocytogenes* [7–9]. In addition, several attenuated *L. monocytogenes* mutants have been investigated as possible vaccines against listeriosis or as heterologous antigen delivery systems for vaccination against cancer and

* Corresponding author at: Department of Microbiology, University College Cork, Western Road, Cork, Ireland. Tel.: +353 21 4901363; fax: +353 21 4903101.
E-mail address: c.gahan@ucc.ie (C.G.M. Gahan).

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Table 1
Bacterial strains and plasmid vectors

Strain or plasmid name	Description	Reference or source
Escherichia coli Top10	Chemically competent intermediate host, plasmid free	Invitrogen
E. coli M15 (pREP4)	Expression host for pQE30-cloned genes, containing the repressor plasmid pREP4 (Kanamycin resistant; Km^R) for suppression of basal protein expression under uninduced conditions	Qiagen
E. coli BL21	Chemically competent *E. coli*, used in this study as an intermediate host for pQE30, plasmid free	Novagen
Listeria monocytogenes EGDe serovar 1/2a	Wild type *L. monocytogenes*	[65]
Luminescent *L. monocytogenes* EGDe	Constitutively luciferase-expressing *L. monocytogenes* EGDe (carrying pPL2luxPhelp on its chromosome through single cross-over integration), Cm^R	[42]
Lactococcus lactis subspecies *cremoris* MG1363	Plasmid free *Lactococcus* strain	[66]
L. lactis NZ9700	Nisin producer strain	[29]
L. lactis NZ9000	*L. lactis* subsp. *cremoris* MG1363 carrying nisRK on the chromosome	[29]
L. lactis NZ9000 Δ*htrA*	*L. lactis* NZ9000 with a chromosomal deletion in the *htrA* promoter and the 5′-end region of *htrA*	[32]
pQE30	Expression vector using phage T5 promoter and adding an N-terminal six-His tag to the expressed protein, Amp^R	Qiagen
pNZ8048	*E. coli–L. lactis* shuttle vector containing PnisA promoter and start codon in NcoI site, Cm^R	[29]
pNZ44	pNZ8048 derivative containing P44 promoter instead of PnisA promoter, Cm^R	[31]
pNZP44:CYTO-LLO	Modified pNZ8048 containing P44 promoter with downstream His-tagged *hly*, Cm^R	This study
pNZP44:SEC-LLO	Modified pNZ8048 containing P44 promoter with downstream secretion signal of Usp45 protein and His-tagged *hly*, Cm^R	This study
pNZPnisA:CYTO-LLO	Modified pNZ8048 containing PnisA promoter (NcoI site eliminated) with downstream His-tagged *hly*, Cm^R	This study
pNZPnisA:SEC-LLO	Modified pNZ8048 containing PnisA promoter (NcoI site eliminated) with downstream secretion signal of Usp45 protein and His-tagged *hly*, Cm^R	This study

other pathogens [10–13]. However, although the efficacy of these approaches was clearly demonstrated, all the abovementioned vectors are based upon live attenuated pathogens which may have the potential for reversion to a virulent state.

Lactococcus lactis is a GRAS (*G*enerally *R*egarded *A*s *S*afe) microorganism that is widely used in the food industry. The development of numerous inducible and constitutive expression systems for *L. lactis* has enhanced the use of this organism as a cellular factory for expression of various heterologous proteins of biotechnological interest [14]. This work has extended into the use of *L. lactis* as a live vaccine vector for delivery of heterologous antigens. Several antigens such as tetanus toxin fragment C (TTFC) [15], *Helicobacter pylori* Cag12 antigen [16], *Giardia lamblia* cyst wall protein 2 [17], and SARS-coronavirus nucleocapsid protein [18] have been successfully expressed in *L. lactis* with promising immunological outcomes following administration in mice. Moreover, *L. lactis* has also been used as a vehicle for delivery of therapeutic bioactive substances (including interleukin-10 (IL-10) and trefoil factors) to reduce inflammation and enhance recovery in murine models of colitis [19,20]. Indeed, an *L. lactis* strain expressing human IL-10 and utilizing a novel biological containment strategy has recently been the subject of clinical trials in patients with Crohn's disease [19]. Since *L. lactis* is a non-pathogenic, non-invasive and non-commensal food-grade bacterium, it is particularly attractive as a basis for safe delivery of antigens or bioactive molecules. Furthermore, the Gram-positive bacterium *L. lactis* has a relatively small genome size with few exoproteins and unlike proposed Gram-negative hosts, does not produce endotoxin [21–23].

In the present work, we successfully expressed *L. monocytogenes* LLO in *L. lactis* to act as a potential live vaccine vector. The P44 constitutive promoter and the PnisA nisin-inducible promoter were employed to express LLO in different compartments (intracellular and secreted). LLO was expressed in an active form in all cases except the constitutive intracellular form. Investigation of the immune response upon vaccination of BALB/c mice via the intraperitoneal (IP) and oral routes revealed different levels of LLO-specific $CD8^+$ T cells, IgG antibodies and protection against challenge with *L. monocytogenes*. The current work demonstrates the application of *L. lactis* in the development of potential vaccine platforms against *L. monocytogenes*.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

A summary of bacterial strains and plasmids used in this study is shown in Table 1. Luria–Bertani (LB) broth was used for *Escherichia coli* cultures while M17 broth (Oxoid) supplemented with 0.5% glucose (i.e. GM17) was used for *Lactococcus*. For *L. monocytogenes*, brain heart infusion (BHI) broth (Oxoid) was used. Technical agar (Merck) was added (1.5%, w/v) when solid media were required. Incubation temperatures were 30 °C for *L. lactis* and 37 °C for *L. monocytogenes* and *E. coli*. When required, ampicillin (Amp) was used at a concentration of 100 μg/ml for *E. coli* while chloramphenicol (Cm) was used at 10 μg/ml for *E. coli*, and *L. lactis* and at 7.5 μg/ml for luminescent *L. monocytogenes*. All cell culture media and reagents were obtained from Gibco unless otherwise stated.

2.2. Cloning of LLO in modified pNZ8048 plasmid vectors

Four plasmids were constructed for the expression of LLO in *L. lactis*: the constitutive pNZP44:SEC-LLO and pNZP44:CYTO-LLO plasmids, and the nisin-inducible pNZPnisA:SEC-LLO and pNZPnisA:CYTO-LLO plasmids (Fig. 1). Plasmids pNZP44:SEC-LLO and pNZPnisA:SEC-LLO were designed to secrete LLO using the N-terminal secretion signal of the Usp45 protein which is a secreted lactococcal protein [24]. The use of the Usp45 secretion signal has previously been described to direct the secretion of heterologous proteins in *L. lactis* [25,26]. Plasmids pNZP44:CYTO-LLO and pNZPnisA:CYTO-LLO lack a secretion signal and were thus designed