Short communication: Labeling *Listeria* with anaerobic fluorescent protein for food safety studies

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ABSTRACT

Many food safety-related studies require the tracking of inoculated food-borne pathogens to monitor their fate in food complex environments. In the current study, we demonstrate the potential of plasmids containing the fluorescence protein gene *evoglow-Pp1* (Evocatal, Dusseldorf, Germany) as a real-time reporter system for *Listeria* strains. This anaerobic fluorescent protein provides an easily detectable phenotype of microorganisms for food safety studies. This work is the first to report a reliable method to identify fluorescently labeled *Listeria* strains in food ecosystems.

Key words: *Listeria*, anaerobic fluorescent protein, food safety studies

Short Communication

The incidence of food-borne infections has markedly increased over the last years, with nearly a quarter of the population at higher risk of illness today (Newell et al., 2010). The major food-borne pathogens are *Salmonella* spp., *Escherichia coli* O157:H7, *Campylobacter* spp., and *Listeria monocytogenes* (Warriner and Narmar, 2009; Cook et al., 2012; Lianou and Koutsoumanis, 2013). *Listeria monocytogenes* causes listeriosis in high-risk groups including pregnant women, neonates, and immunocompromised individuals (Farber and Peterkin, 1991). *Listeria monocytogenes* is a psychrotrophic microorganism widely distributed in the natural environment that can colonize food-processing facilities. The microorganism has the ability to persist in food-processing areas and equipment and contaminate processed food (Walker et al., 1990). *Listeria monocytogenes* has been isolated from milk and dairy products, which are one of the many sources of food-borne listeriosis (EFSA, 2015).

Different methodologies have been developed to detect multiple pathogens through rapid, simple, and economical tests (Bhagwat, 2003; Kawasaki et al., 2005). Interest is increasing in the understanding of mechanisms that bacteria use to interact with food microbiota or to survive, persist, or colonize the gastrointestinal tract. Thus, a need exists for studies on the behavior of pathogenic bacteria in their natural habitats, including foods or the gastrointestinal tract.

To study the survival of these pathogenic bacteria, the use of precise and sensitive molecular tools is indispensable. For this purpose, fluorescent proteins have been exploited as a stable marker system with an easily detectable phenotype to track bacteria in complex ecosystems. Fluorescent reporter proteins are valuable noninvasive molecular tools for the *in vivo* real-time imaging of living cells and tissues as well as for *in vitro* fluorescence labeling (Chudakov et al., 2005). In the present report, we describe the implementation of an anaerobic fluorescent protein as marker for *Listeria innocua* as a surrogate for *L. monocytogenes* in dairy foods.

Listeria innocua CECT 410 was cultured at 37°C in brain heart infusion (BHI; Difco Laboratories, Detroit, MI, either broth or agar); BHI supplemented with chloramphenicol (5 µg/mL; Sigma-Aldrich, St. Louis, MO) was used as the selective medium for labeled *Listeria* clones after transformation. *Lactococcus lactis* MG1363 used as a host microorganism was provided by Udo Wegmann (Institute of Food Research, Norwich, UK) and grown at 30°C in M17 medium (Scharlau Chemie SA, Barcelona, Spain) supplemented with 5 g/L of glucose (GM17). This medium supplemented with chloramphenicol (5 µg/mL; Sigma-Aldrich) was used as the selective medium for transformed *Lc. lactis* MG1363.

For the construction of *Listeria* marked with the anaerobic fluorescence protein gene (*evoglow-Pp1*; Evocatal, Dusseldorf, Germany), elongation factor Tu promoter from pNZ:Tu-aFP (Landete et al., 2015) was replaced by different promoters from *Lactobacillus casei* BL23. The promoters chosen were those that precede

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the following genes: elongation factor G (EF-G), elongation factor P (EF-P) and initiation factor IF-2 (IF-2). The DNA manipulation procedures were as described in Green and Sambrook (2012). The pNZ:Tu-aFP was digested with restriction endonucleases (*Bgl*II and *Nco*I) to remove the elongation factor Tu promoter. The different promoters were amplified by PCR using DNA of *Lb. casei* BL23 as the template following a standard protocol and using the oligonucleotides F-PromP/R-PromP, F-PromG/R-PromG, and F-PromIF-2/R-PromIF-2 (Table 1); afterward, they were digested with restriction enzymes (*Bgl*II and *Nco*I). The PCR fragments and digested plasmid were ligated by ligase enzyme (T4 DNA ligase, Invitrogen, Carlsbad, CA) at 16°C overnight. The ligation mixtures with the resulting plasmids pNZ:P-aFP, pNZ:G-aFP, and pNZ:IF2-aFP were introduced into the *Lc. lactis* strain MG1363 by electroporation. Transformants were selected by resistance to chloramphenicol (Landete et al., 2014a) and checked by restriction mapping and sequencing of the inserted fragment. Subsequently, these 3 plasmids and pNZ:Tu-aFP (Landete et al., 2015) were isolated and used to transform *L. innocua* (Table 1). Competent cells of *L. innocua* were prepared according to Landete et al. (2014a) using BHI for growing, and ampicillin (10 µg/mL; Sigma-Aldrich) was added during the incubation to increase the transformation frequencies. *Listeria* containing the plasmids were selected by resistance to chloramphenicol.

The generation time of each *Listeria* strain was calculated using the equation proposed by Smits and Riemann (1988). The stability of the constructs was assayed by growing the *L. innocua* strains harboring the plasmids in nonselective broth for approximately 100 generations and plating on nonselective agar plates. Antibiotic-resistance maintenance was monitored by the transference of the resulting colonies onto antibiotic-containing agar plates.

The difference in fluorescence intensity between the different *Listeria* strains harboring the plasmids with the *evoglow-Pp1* gene was measured by a fluorimeter. *Listeria* strains harboring the plasmids pNZ:Tu-aFP, pNZ:P-aFP, pNZ:G-aFP, and pNZ:IF2-aFP were grown in the BHI broth in aerobic and anaerobic conditions without agitation. Fluorescence of cells (0.2 mL from each culture at 0.5 optical density) was measured after sedimentation by centrifugation (4,000 × *g*, 5 min, 4°C) and resuspended in PBS. Fluorescence was measured on a Tecan GENios microplate reader (Tecan, Salzburg, Austria) by excitation at 410 nm with a slit set at 2.5, and detection of emission at 500 nm with a slit set at 10. As background, the same strains harboring pNZ8048 plasmids were used, and their values of fluorescence were subtracted from those obtained from cells harboring plasmids with the anaerobic *evoglow-Pp1* gene. Experiments were performed in triplicate.

To test the detection of marked *L. innocua* in a complex microbiota environment such as cheese, *L. innocua*

Table 1. Bacterial strains and plasmids used in this work

Item	Relevant phenotype or genotype ¹	Source
Strain		
MG1363	<i>Lactococcus lactis</i> ssp. <i>cremoris</i> strain, plasmid-free derivative of NCDO712	Gasson (1983)
CECT 410	<i>Listeria innocua</i>	CECT ²
BL23	<i>Lactobacillus casei</i> BL23	B. M. Chassy (University of Illinois, Urbana, IL)
<i>L. innocua</i> pNZ:TuR-aFP	<i>L. innocua</i> contained pNZ:Tu-aFP	This work
<i>L. innocua</i> pNZ:P-aFP	<i>L. innocua</i> contained pNZ:P-aFP	This work
<i>L. innocua</i> pNZ:G-aFP	<i>L. innocua</i> contained pNZ:G-aFP	This work
<i>L. innocua</i> pNZ:IF2-aFP	<i>L. innocua</i> contained pNZ:IF2-aFP	This work
Plasmid		
pNZ:Tu-aFP	pNZ8048 with promoter of elongation factor Tu of <i>Lactobacillus reuteri</i> CECT925 replaced Pnis	Landete et al. (2015)
pNZ:P-aFP	pNZ8048 with promoter of elongation factor P of <i>Lb. casei</i> BL23	This work
pNZ:G-aFP	pNZ8048 with promoter of elongation factor G of <i>Lb. casei</i> BL23	This work
pNZ:IF-2-aFP	pNZ8048 with promoter of initiation factor IF-2 of <i>Lb. casei</i> BL23	This work
Primer		
Forward-P.BL23	TTTTAGATCTGAGTTCACTCTGCGATATTAGTAAC	
Reverse-P.BL23	TTTTCATGGTTTGTTCTCCTTCATTCGC	
Forward-G.BL23	TTTTAGATCTGATCGAAGTTCGTCCGGATCGTCG	
Reverse-G.BL23	TTTTCATGGGGCGTATTGCTCCTCTCTTGTT	
Forward-IF2.BL23	TTTTAGATCTTTGGGTTTGCCCGCCGCGCCAGTC	
Reverse-IF2.BL23	TTTTCATGGCTTACGCTCCCTGCAATTTAGGAG	

¹Restriction sites used for cloning are underlined.

²Spanish Type Culture Collection.

Figure 1. Fluorescence mean values and SD obtained from each promoter under aerobic (gray) and anaerobic (black) conditions. EF-Tu = elongation factor Tu; EF-P = elongation factor P; EF-G = elongation factor G; IF-2 = initiation factor 2.

carrying pNZ:Tu-aFP was inoculated at approximately 8 to 9 log cfu/mL into a semihard cheese homogenate (1:10 in sterile 0.1% peptone water). Cheese homogenates with strains containing pNZ8048 plasmid served as the controls. Ten-fold dilutions were cultured in BHI plates and incubated for 36 h at 37°C in aerobic conditions. Fluorescent colonies were detected by ChemiDoc MP apparatus (Bio-Rad, Hercules, CA) and the presence of pNZ:Tu-aFP was checked by means of PCR. Moreover, fluorescent cells from cheese homogenates were detected by fluorescence microscopy and flow cytometry according to Landete et al. (2015).

The transformed *L. innocua* with the highest level of fluorescence expression was *L. innocua* pNZ:P-aFP followed by *L. innocua* pNZ:Tu-aFP. *Listeria innocua* pNZ:G-aFP showed an intermediate level of expression, and *L. innocua* pNZ:IF2.aFP showed a low level of expression (Figure 1). The fluorescence intensity was similar in aerobic and anaerobic conditions, demonstrating that it was not affected by the presence or absence of oxygen. Between 97 and 99% of *L. innocua* transformed with pNZ:G-aFP, pNZ:P-aFP, pNZ:IF-2-aFP, and pNZ:Tu-aFP retained the plasmids after 100 generations of growth under nonselective pressure. The plasmid pNZ8048 containing *evoglow-Pp1* has been reported to be very stable in lactic bacteria and bifidobacteria (Landete et al., 2014a, 2015); this stability was maintained in *Listeria*. The analyses of generation time (tg) demonstrated that the transformed *L. innocua* strains and the parent strain had a similar growth (tg = 0.98 h).

Listeria containing pNZ:Tu-aFP was easily detected in cheese homogenates by cell counting methods, by plating in aerobic conditions, and by visualization of direct fluorescence by fluorescence microscopy and flow cytometry in anaerobic conditions. Figure 2 shows that the fluorescent colonies of *Listeria* containing pNZ:Tu-aFP could be differentiated from other nonmarked microorganisms from the cheese. The presence of pNZ:Tu-aFP was confirmed in fluorescent colonies by PCR. No fluorescent cells were observed in the control without labeled *Listeria*. The fluorescence signal was improved

Figure 2. *Listeria innocua* containing pNZ:Tu-aFP are discriminated from their nonfluorescent counterparts against a background of cheese microbiota by a ChemiDoc MP imager (Bio-Rad, Hercules, CA). A: White epi illumination, standard filter; B: Alexa488 blue epi illumination, 530/28 filter.

when the colonies were maintained at 4°C overnight. The signal persisted even after 10 d under refrigeration.

In the present report, we describe the implementation of an anaerobic fluorescent protein (*evoglow-Pp1*) as marker for *L. innocua* and as a surrogate for *L. monocytogenes* in dairy foods safety studies. Furthermore, this reporter system could be used as a marker for pathogenic bacteria to monitor their fate throughout the manufacture and ripening of dairy foods as cheese, or when administered in vivo to analyze their interactions within gastrointestinal ecosystems.

Green fluorescence protein-labeled bacteria have been used in monitoring either single cells or a cell population in survival studies of *E. coli* in aquatic environments (Leff and Leff, 1996) and in investigating the survival and spatial location of *Salmonella* in alfalfa sprouts (Gandhi et al., 2001). However, several potential problems, such as the influence of pH on the fluorescence and the presence or absence of oxygen, can be encountered when using labeled bacteria with classical fluorescence protein (Landete et al., 2015). The use of *evoglow-Pp1* avoids the environmental condition restriction related to oxygen requirements in the development of the fluorophore or poor fluorescence at low pH. *Listeria* is a facultative anaerobe, so the use of a fluorescent protein working in the absence of oxygen is a useful strategy for its detection. These new oxygen-independent fluorescence proteins based on photosensory flavoproteins, named flavin mononucleotide-based fluorescent proteins, have been recently developed and overcome the potential problems caused by oxygen and pH limitations reported in previous fluorescence proteins (Drepper et al., 2007). In this sense, *evoglow-Pp1* were used in probiotic *Lactococcus*, *Lactobacillus*, *Enterococcus* (Landete et al., 2015), and *Bifidobacterium* (Landete et al., 2014b) to track the behavior of this probiotic species in food and fecal environments under both aerobic and anaerobic conditions.

According to the quantitative results, the strains with the greatest fluorescence emission were *L. innocua* pNZ:P-aFP and *L. innocua* pNZ:Tu-aFP (Figure 1); the elongation factor P and Tu promoters used in the expression cassettes are strong promoters and have been shown to be functional in a range of bacterial genera. This result was further verified by flow cytometry as well as image capture through ChemiDoc MP imaging system (Bio-Rad) and fluorescence microscopy (data not shown). In contrast, constructions with *gfpuv* (Clontech Laboratories, Takara, Shiga, Japan) could not be visualized directly from cheese homogenate through flow cytometer or fluorescence microscopy because *gfpuv* needs aeration to emit fluorescence, as shown in previous work (Landete et al., 2014a, 2015).

The pNZ:Tu-aFP and pNZ:P-aFP plasmids provide a stable real-time and noninvasive reporter system for *Listeria* strains that do not disturb the growth of the parent strain and emit a fluorescent signal strong enough to be detected by several techniques.

Our work is the first to report a reliable method for tracking fluorescently labeled *Listeria* strains in a food environment. We describe the implementation of fluorescent proteins as markers for *L. innocua* and as a surrogate for *L. monocytogenes* in a dairy food for safety studies and demonstrate that the anaerobic fluorescent protein provides an easily detectable phenotype. This strategy might be useful for challenge tests.

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