Rhamnose-Inducible Gene Expression in *Listeria monocytogenes*

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**Abstract**

Acid production from rhamnose is a characteristic phenotype of *Listeria monocytogenes*. We report the identification of the rhamnose transport and utilization operon located at *lmr*2846 to *lmr*2851, including the rhamnose-dependent promoter P_{rha}. Expression of reporter genes under control of P_{rha} on a single copy integration vector demonstrated its suitability for inducible gene expression in *L. monocytogenes*. Transcription initiation from P_{rha} is dose dependent, and a concentration as low as 100 μM rhamnose was found sufficient for induction. Moreover, P_{rha} is subject to glucose catabolite repression, which provides additional options for strict control of expression. Infection of human THP1 macrophages revealed that P_{rha} is repressed in intracellular *L. monocytogenes*, which is explained by the absence of rhamnose in the cytosol and possible interference by catabolite repression. The P_{rha} promoter provides a novel and useful tool for triggering gene expression in extracellular *L. monocytogenes*, whereas intracellular conditions prevent transcription from this promoter.

**Introduction**

*Listeria monocytogenes* is a non-sporulating Gram-positive rod, and the causative agent of human Listeriosis, a severe infection transmitted via contaminated food. The bacterium serves as a model organism for studies in cellular microbiology, bacterial pathogenicity and virulence, bacteriophage biology, and food safety [1,2,3,4].

*L. monocytogenes* is able to produce acid from rhamnose, whereas *L. innocua*, *L. welshimeri*, and *L. grayi* show variable rhamnose utilization, and strains of *L. ivanovii*, *L. seeligeri*, and *L. marthii* are negative [5,6]. Rhamnose is a naturally occurring L-6-deoxy hexose. It is present as a substituent of pectin in plant cell walls where it is periodically attached via α-1, 2-glycosylic linkages to galacturonic acid. In *L. monocytogenes* serovar 1/2 strains, rhamnose is not only used as a carbon source, it is also found as a decoration of the cell wall teichoic acids, and required for adsorption of A118 like bacteriophages [7].

In *Listeria*, metabolism of rhamnose results in production of 1,2-propanediol, which is further oxidized to propionate [8]. *In silico* analysis indicated that rhamnose catabolism in both *L. monocytogenes* and *L. innocua* correlates with the presence of a set of genes clustered in a rhamnose operon (*lmr*2846-*lmr*2850 and *linr*2978-*linr*2992, respectively) [9], which encodes an ABC-transporter, a rhamnulokinase, rhamnose isomerase, rhamnulose-1-phosphate aldolase, and a rhamnose epimerase. An AraC-type DNA binding transcriptional regulator (*lmr*2851 and *linr*2983, respectively) is located immediately adjacent (Fig. 1). While the function of rhamnose operon genes and products has not been experimentally confirmed in *Listeria*, the specific uptake of rhamnose and the molecular biology and transcriptional regulation of these genes and their products has been thoroughly studied in *E. coli*. Remarkably, the structure and composition of the *E. coli rha* operon differs considerably from *L. monocytogenes*. While a rhamnose epimerase is not present, *E. coli* encodes two AraC-type regulatory proteins, RhaS and RhaR, to control *rhaBAD* expression [10,11,12,13,14,15].

Gene products involved in carbohydrate transport and metabolism are often highly conserved yet specific for different prokaryotes. While *E. coli* encodes several carbohydrate transporters, e.g., the *rha-* and *ara-* operons, facilitating rhamnose, arabinose and lactose uptake, respectively, *B. subtilis* features the *xyl* operon for xylose utilization. Gene expression and synthesis of carbohydrate transporters and accessory proteins is strictly regulated, and often depends on glucose-mediated catabolite repression [16,17]. In this case, gene expression (i.e., repressor inactivation) only occurs when glucose is either not present or removed from the growth medium and replaced by the respective carbohydrate. Such tight control of promoter activity by repressor proteins can be elegantly harnessed for the design of inducible gene expression systems and vectors [18,19].

With respect to *L. monocytogenes*, inducible gene expression is a highly desirable tool to study not only its pathogenicity and virulence, but also to better understand the environmental growth properties and responses of this opportunistic pathogen. Until
now, inducible gene expression in this organism has been based upon use of the lacI repressor and isopropyl-β-D-1-thiogalactopyranoside (IPTG) as an inducer [20,21]. A disadvantage of this system is its poor stringency and often high background expression when a strong promoter is used. In fact, poor tune-ability and high level of read-through transcription can make it difficult or impossible to obtain quantitative data, or to clone or express toxic genes (L. Fieseler and M. J. Loesner, unpublished data). In contrast, positively-regulated gene expression (e.g., by an arabinose or rhamnose-dependent DNA binding AraC-type repressor) responds much slower following induction, while transcription repression is much tighter compared to negatively-regulated systems such as the lac operon [22].

Therefore, the aim of this study was to develop a tightly regulated, rhamnose-inducible gene expression system for Listeria, based on the $P_{rha}$ promoter. To monitor transcription activity, reporter genes encoding green fluorescent protein and drug resistance were placed under control of $P_{rha}$ on a single-copy integration vector. Employing both in vitro growth and intracellular infection models, we demonstrate that $P_{rha}$ enables quantitative expression of target genes, which can be modulated by the presence and concentration of rhamnose.

**Results**

**Identification of the L. monocytogenes $P_{rha}$ Promoter**

Putative -10 and -35 regions of a promoter designated as $P_{rha}$ were identified in silico immediately upstream of lmo2850, the first gene of a putative rhamnose utilization operon in L. monocytogenes (Fig. 1) [9]. In order to be able to test $P_{rha}$ for inducible gene expression, a 671 bp DNA fragment was amplified from the region upstream of the Shine-Dalgarno sequence of lmo2850 (nucleotide positions 2939946-2940597), and inserted into plasmid pPL2 to yield pLF1 (Fig. 2). Downstream of $P_{rha}$ pLF1 still features the very useful multiple cloning site from pPL2, i.e., unique EagI, NotI, SpeI, Smal, XmaI, PstI, HinfII, Sall, and KpI sites available for insertions and cloning.

**Transcription from $P_{rha}$ in Listeria is Rhamnose-dependent**

$L. monocytogenes$ is able to utilize rhamnose (50 mM) as a carbon source during growth (Fig. 3A). Cells entered log phase after approximately 2 h, entered stationary phase after approximately 7 h, featuring an optical density (OD$_{600\text{ nm}}$) of approximately 0.6. In the positive control (50 mM glucose), the culture reached a higher maximum OD$_{600\text{ nm}}$ of 1.6 after 7 h incubation, while only poor growth was observed in LB broth without additional carbon source (negative control).

To determine whether expression from $P_{rha}$ is actually rhamnose-dependent, ermC was used as a reporter. L. monocytogenes LF002 was incubated in the presence of 10 mM rhamnose; before addition of 7.5 μg/ml erythromycin to the culture. While non-induced bacteria did not multiply further, rhamnose-induced cells continued growth and reached an OD$_{600\text{ nm}}$ of 1.4 after 8 h of incubation (Fig. 3B). The positive control (L. monocytogenes LF003 with constitutive expression of ermC) featured a shorter lag phase and faster onset of growth, and reached the stationary phase after 6 h of incubation (OD$_{600\text{ nm}}$ of 1.5).

As an alternative to drug selection, the gfp gene was employed to measure expression from $P_{rha}$ in strain LF001, by monitoring green fluorescence following dose-dependent rhamnose induction. As negative control, LF004 (no promoter) was used, and LF005 (constitutive gfp expression from $P_{rha}$) served as positive control. We show that a concentration of 100 μM rhamnose was sufficient to quantitatively induce $P_{rha}$-dependent transcription in a majority of cells (Fig. 4, panels A and B). Increasing rhamnose concentration of up to 10 mM resulted in stronger fluorescence, indicating that induction is dose-dependent. Even higher rhamnose concentrations did not result in a further increase of fluorescence, indicating that a plateau was reached. Interestingly, the presence of 10 mM glucose, N-acetylglucosamine, glucosamine, galactose, mannose, or fructose in addition to 10 mM rhamnose completely repressed $P_{rha}$ activity, demonstrating that transcription of the lmo2846-lmo2850rhamnose operon is subject to a strict catabolite repression.

Interestingly, arabinose was the only sugar tested that did not affect $P_{rha}$ activity (Fig. 4C).

**$P_{rha}$ is Repressed in Intracellular L. monocytogenes**

It was interesting to determine if rhamnose would also be useful for regulating gene expression in intracellular L. monocytogenes during infection and the intracellular state. In the human intestine, rhamnose is not digested and regarded as a soluble dietary fiber material. While rhamnose as an osmolyte may affect cultured mammalian cells at higher concentrations, the amounts required for activation of $P_{rha}$ are quite low. We did not observe any negative effect on viability when THP1 macrophages [23] were exposed to 100 mM rhamnose over a period of 40 h. Cells maintained their shape and remained attached to the bottom of the cell culture flask, indistinguishable from controls without rhamnose (data not shown).

To determine transcriptional activity from $P_{rha}$ in L. monocytogenes during infection, strain LF002 that features ermC under control of $P_{rha}$ was used. We found that neither induced nor non-induced bacteria were able to multiply intracellularly in infected THP1 macrophages in presence of erythromycin (Fig. 5). Moreover, addition of rhamnose to infected macrophages did not result in ermC expression, i.e., the intracellular bacteria were unable to grow and multiply in the presence of the antibiotic. ANOVA analyses showed that the corresponding intracellular cell counts did not differ significantly from each other. In contrast, strain LF003 with constitutive expression of ermC was not affected, and significantly increased by 1.8 logs after an 8 h infection period (p<0.0001).

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**Figure 1. The L. monocytogenes rhamnose utilization operon.** Dark grey: open reading frames lmo2846-lmo2851 located on both DNA strands [9] and their functional assignments; white: $P_{rha}$ promoter, putative -10 and -35 regions are shaded; black: transcription terminators. doi:10.1371/journal.pone.0043444.g001
the absence of the drug, all strains showed identical growth responses regardless if exposed to rhamnose or not (Fig. 5).

*L. monocytogenes* strain LF006 features a chromosomal ΔprsA deletion [24] of the gene encoding the regulator PrfA, which is complemented by prfA under P_rha control on a single copy pLF6 insertion. When LF006 was pre-induced with 10 mM rhamnose prior to infection of THP1 macrophages, virulence could be fully restored (Fig. 6). Non-induced LF006 did not significantly differ in intracellular multiplication from the negative control (ΔprsA) one and two hours post infection. However, four hours post infection a slight increase in intracellular cell counts was observed (p<0.0001).

**Discussion**

Inducible gene expression systems for bacteria are important tools for biotechnology applications and basic research. Several well defined vectors are available for model organisms such as *E. coli* or *B. subtilis*. In contrast, very few systems for inducible gene expression in the opportunistic pathogen *L. monocytogenes* are available, which can be useful for tuned expression of virulence genes or genes encoding potentially toxic products. Danckz and co-workers reported an IPTG-inducible pLIV vector, where a synthetic promoter P_SPAC was used in combination with the *lacO* operator [20] and *lacL*, placed under control of the constitutive P_uni (*P_{uni}) promoter. Similar vectors have been developed by others [25]. However, negatively regulated promoters are often leaky, due to less stringent repression and feature poor repression [22]. This renders cloning of potentially lethal or toxic genes difficult or even impossible. Because transcriptional control (i.e., repression) in positively regulated promoters such as P_ara, P_xyl, or P_rha generally is much tighter, our goal was to design such a system for *Listeria*.

The P_rha promoter identified and used in this work is silent in the absence of the sugar, and can be specifically induced in a dose dependent manner by addition of 10–100 μM rhamnose to growing cells. All strains of *L. monocytogenes* can utilize rhamnose as a single carbon source; additional P_rha-binding regulatory proteins are not required. This renders application of P_rha simple and straightforward, the promoter may be fused to any gene of interest. In addition, transcription is subject to catabolite repression and the promoter is not active when glucose is present, which provides additional options for P_rha-controlled gene expression. However,
the exact mechanism of catabolite repression is not clear. It is possible that it is similar to the situation in the maltose and maltodextrin utilization gene clusters, where catabolite expression is independent of the catabolite control protein CcpA, and likely regulated by inducer exclusion [26].

During infection of macrophages, intracellular bacteria featuring P"cha"-controlled ermC expression were unable to multiply. This can be explained by the fact that rhamnose is not actively transported over the mammalian (macrophage) cytoplasmic membrane [27], which should effectively prevent induction of P"cha" in intracellular bacteria. Because rhamnose is generally not available in the cytosol of human or animal cells, P"cha" may be employed to shut down gene expression upon entry of L. monocytogenes into a host cell. Moreover, catabolite repression by intracellular glucose deposits also contribute to prevent expression from the P"cha" promoter. This feature can be useful for development of live attenuated vaccine vectors based on the intracellular lifestyle of this pathogen. The opposite approach, e.g., onset of gene expression after entry into the host cytosol has been realized using P"actA" [28]. This promoter is PrfA-dependent and specifically induced in intracellular L. monocytogenes. The aims were to kill and lyse intracellular L. monocytogenes for use as DNA delivery vehicles into eukaryotic cells, by expression of bacteriophage cell wall hydrolase βp115 under control of P"actA" [29].

The challenge in designing L. monocytogenes as a delivery vehicle for the allocation of proteins to the immune system is to attenuate the bacterium in such a way that it retains the ability to invade potential host cells, however, without causing extensive collateral damage. So far, attenuated strains often featured disruption of essential virulence factors, such as actA, milB or pfrA [30,31,32,33]. An alternative strategy is to create L. monocytogenes auxotroph mutants, such as dal and dat null strains defective in D-alanine synthesis [34]. All of these strains may have applied to stimulate specific T-cell responses in vivo. One particular pfrA defective strain (Lm-LLO-E7) was used as a vaccine against invasive carcinoma of the cervix in a clinical phase I study.

PrfA is a soluble cytosolic protein that controls transcription of key virulence factors in L. monocytogenes. We tested P"cha" to trigger pfrA expression in an infection model. The pfrA-negative phenotype could be fully complemented, but only when expression of the gene in strain LF006 was pre-induced with rhamnose prior to macrophage infection (Fig. 6). In this case, induction of pfrA resulted in an overproduction of the protein compared to the wild type, and it is reasonable to assume that the intracellular concentration of PrfA was sufficiently high to ensure its presence and function over the next few generations, i.e., cell divisions. Hence, even when rhamnose as inducer of pfrA expression is removed upon invasion and entry into host cells, the protein likely was present at a sufficient threshold concentration to activate transcription of the PrfA regulon in the daughter cells. Further experiments would be required to demonstrate that these cells progressively lose virulence after prolonged incubation, division and intracellular life. In fact, finely tuned pfrA induction from P"cha" by lower rhamnose concentrations could yield the individually desired control of a corresponding phenotype.

In these experiments, we also noted that virulence of non-induced LF006 was not as strictly attenuated as the pfrA negative control, which may suggest a low level background expression from the P"cha" promoter under these specific conditions. It is not unreasonable to assume that even very small amounts of PrfA may be sufficient to slightly elevate intracellular counts of non-induced LF006. Nevertheless, the possibility of employing P"cha" for attenuation of virulence genes in intracellular L. monocytogenes for application of the organism as live vaccine remains an attractive option.

**Materials and Methods**

**Bacterial Strains and Culture Conditions**

L. monocytogenes was cultured in half-strength Brain Heart Infusion (1/2 BHI) broth at 30° or 37°C (macrophage infection model). Chloramphenicol (10 μg/ml) or erythromycin (7.5 μg/ml) was added to the media as needed. Rhamnose or other sugars were used at different concentrations, up to a maximum of 50 mM. To determine the utilization of rhamnose by L. monocytogenes, cells were incubated in Luria-Bertani (LB) broth
substituted with 50 mM glucose and 50 mM rhamnose, respectively. *Escherichia coli* SM10 and XL1-blue MRF’ (Stratagene) were routinely cultured in LB broth at 37°C and with the addition of chloramphenicol (10 mg/ml for XL1-blue MRF’ and 20 mg/ml for SM10) when appropriate. All strains used in this study are summarized in Table 1.

### Bioinformatics

The lmo2846-lmo2850 operon in the *L. monocytogenes* genome [9] was identified and analyzed in silico (CLC Main Workbench software; CLC Bio, Aarhus, Denmark). Putative -10 and -35 regions were identified by a promoter-finding algorithm (BPROM, <http://linux1.softberry.com/berry.phtml>).

### Cloning Procedures

Plasmid pPL2 was used for cloning and single copy insertion into a tRNAArg gene via bacteriophage PSA site-specific integrase. The plasmid remains stable in the absence of drug selection, and does not cause polar effects [35].

The P_{rha} promoter region was PCR amplified using primers P_{rha-f} (5’- ATTGCCAGCTCTATCCCGTATATTTGG-3’) and P_{rha-r} (5’- AACCAGCCGACTCATTTT ATGTAAGCCGC-3’), yielding a 671 bp product. Following digestion with SacI and EagI (sites are underlined), the fragment was inserted into pPL2 to yield pLF1. The gfp gene was excised from pPL3 [36] (kindly provided by D. Higgins, Harvard Medical School, USA), using enzymes EagI and KpnI, purified by gel extraction, and inserted into the corresponding sites in pLF1 downstream of P_{rha}. The ermC gene was amplified from pLEB579 (kindly provided by T. Takala, University of Helsinki, Finland), using primers ermC-f (5’- AAAACGGCCGCCAACAAATAAAGGGTTATAATG-3’) and ermC-r (5’- AAGGTACCGAAAA ACAAGTTAAGG-GATGC-3’), digested with EagI and KpnI (sites are underlined), and cloned into pPL2. Ligation reactions were transformed into *E. coli* SM10 by electroporation, clones containing the desired inserts were identified by colony PCR, and plasmids recovered by a standard alkaline lysis method. The promoter region and inserts of interest were sequenced. Details of the plasmids and strains are listed in Table 1.

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**Figure 4. Rhamnose-inducible expression of GFP.** Panels A and B: Dose-dependent response of P_{rha}-controlled gfp expression in *L. monocytogenes* LF001 after 16 h of induction using rhamnose concentrations as indicated. Panel A, top row: fluorescence microscopy; bottom row, phase contrast microscopy. Positive and negative controls, and the effect of catabolite repression by addition of equimolar amounts of glucose and rhamnose are indicated. rha: rhamnose, glc: glucose. Panel B: quantitation of relative fluorescence (RFU) of GFP in rhamnose-induced bacteria. Positive (P_{rha}) and negative (no promoter) controls are indicated on the left. Addition of 100 µM rhamnose increased fluorescence significantly (p<0.001). Panel C: quantitative catabolite repression of P_{rha}-dependent gfp expression in the presence of rhamnose together with a second carbohydrate (indicated on the x-axis), at equimolar concentration (10 mM). Positive (P_{rha}) and negative (no promoter) controls are indicated on the right.

doi:10.1371/journal.pone.0043444.g004
E. coli SM10 carrying pLF-derived plasmids were then used for vector transformation by conjugation into L. monocytogenes 10403S, applying a mating procedure as described previously [35]. L. monocytogenes EGDe was transformed using electroporation [37]. The desired clones were selected on BHI agar containing 200 μg/ml streptomycin and/or 10 μg/ml chloramphenicol, respectively, after incubation for 2–3 days at 30°C.

Fluorescence Reporter Assays

The synthesis of GFP as a reporter for gene expression was monitored using confocal laser scanning microscopy (TCS SPE, Leica, Germany), or a fluorescence plate reader (VICTOR3-Multiplate Counter, PerkinElmer, USA). To ensure complete and correct folding of mature GFP proteins, bacteria were harvested from overnight cultures, adjusted to an OD600 nm of 1.0 per ml, and washed 3 times with phosphate buffered saline prior to analysis. For spectrophotometry, 200 μl of the suspension was transferred into a well of a black 96 well plate. Each sample was analyzed in triplicate, and the experiment was independently repeated three times. Arithmetic means and standard deviations are indicated.

Listeria Infection of Macrophages

Human THP1 macrophages [23] were cultured in RPMI 1640 medium supplemented with 20% FBS (Sigma), at 37°C in an atmosphere containing 5% CO₂. Approximately 5×10⁵ cells per well of a 24-well plate were seeded, and infected by addition of 1×10⁷ L. monocytogenes strain LF002 or LF006 (Tab. 1), for 1 h (MOI 20). For the infection, rhamnose-induced (10 mM rhamnose) or non-induced control bacteria were taken from overnight cultures incubated at 37°C, adjusted to the desired cell concentration, and washed three times in pre-warmed PBS before use. LF002 infected Macrophages were washed with prewarmed PBS and further incubated in RPMI 1640 medium containing 50 μg/ml gentamycin to inhibit extracellular bacteria. After 1 h of incubation, macrophages were lysed to determine the initial intracellular viable counts (colony forming units, cfu) of L. monocytogenes LF002. Cells were washed three times with prewarmed PBS, scraped off the bottom of each well, and resuspended and lysed by vigorous pipetting and vortexing using
Ice-cold 0.5% (v/v) Triton X-100. The lysates were diluted and plated for determination of \textit{L. monocytogenes} cfu. Then, 10 mM rhamnose was added to the remaining infected cells, e.g., (in the remaining wells of the plate), and 7.5 \(\mu\)g/ml erythromycin was added 1 h later. These cells were then also lysed using ice-cold 0.5% (v/v) Triton X-100 at different time points. The lysates were diluted and plated for determination of \textit{L. monocytogenes} cfu from samples taken at 1, 4, 8, and 12 h post infection. Each experiment was performed in triplicate, and independently repeated three times. Means and standard deviations are indicated.

\textit{L. monocytogenes} strain LF006 was used to complement the \(\Delta\text{prfA}\) mutant from a gene under Prha control (Tab 1). Macrophage infection and determination of intracellular cfu was carried out as described above, using rhamnose pre-induced and non-induced LF006 for infection. Gentamycin (50 \(\mu\)g/ml) was added to inhibit extracellular bacteria after an infection time of 1 h, but rhamnose or any other substance was not added to the infected cells. The cfu of intracellular \textit{L. monocytogenes} were determined after 1, 2 and 4 h post infection as described above.

**Statistical Analyses**

Statistical analyses were performed applying ANOVA and student’s t-test algorithms.

**Acknowledgments**

We thank Darren Higgins (Harvard University), Richard Calendar (University of California, Berkeley), Timo Takala (University of Helsinki), and Juergen Kreft (University of Würzburg) for the gift of plasmids and strains used in this study.

**Author Contributions**

Conceived and designed the experiments: LF SS MJL. Performed the experiments: LF SS JT. Analyzed the data: LF MJL. Contributed reagents/materials/analysis tools: LF MJL. Wrote the paper: LF MJL.

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