Use of Green Fluorescent Protein To Monitor Cell Envelope Stress in *Lactococcus lactis*^{▽§}

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Received 9 September 2009/Accepted 23 November 2009

A *Lactococcus lactis* reporter system suitable to detect cell envelope stress in high-throughput settings was developed by fusing the CesR-regulated promoter of *lmg0169* to the *gfp_{uv}* gene. A dot blot assay allowed fast detection of green fluorescent protein (GFP) fluorescence even at low production levels. Unexpectedly, this promoter was also induced by mitomycin C via CesR.

Lactococcus lactis is widely used as a starter in a large variety of fermented dairy products. The low pH due to lactic acid production and other metabolic activities contributes to the desired organoleptic properties of dairy products. Since starter performance influences greatly the success of milk fermentation, multiple studies of the responses of *L. lactis* to technologically relevant stresses have been undertaken to understand the basis of starter robustness and to improve process technology in dairy fermentations (15, 18).

In this work, we have looked for a reporter system which could be used to measure *L. lactis* cell envelope stress in high-throughput settings. Recently, the two-component system CesSR was shown to respond to cell envelope stress in *L. lactis* (7). One of the most highly upregulated genes was *lmg0169*, whose transcript was induced up to 300-fold after treatment with the cell wall-active bacteriocin Lcn972. Based on the strong response, the promoter sequence of this gene was fused to *gfp_{uv}* to develop a reporter system suitable to be used in a microtiter plate format for easy and quick handling. Green fluorescent protein (GFP) was chosen as a reporter due to its intrinsic property of fluorescing in the absence of any added cofactor or substrate, which allows “nondestructive” *in vivo* detection in living cells. GFP_{uv} is an improved GFP mutant for detection and expression in prokaryotic cells (1).

Construction of a reporter system for *L. lactis*. *L. lactis* NZ9000 (6) was used as a cloning host. The plasmids and primers used in this study are summarized in Table 1. A detailed plot of all the cloning steps, as well as the DNA sequence of the P_{*lmg0169*::gfp_{uv}} cassette, is depicted in Fig. S1 in the supplemental material. Briefly, the promoter P_{*lmg0169*} was released from pAB0169 and cloned in the high-copy-number plasmid pNZ124. The promoterless *gfp_{uv}*, obtained from pRV85, was next cloned into this intermediate plasmid to generate pNZPG. The P_{*lmg0169*::gfp_{uv}} cassette was subsequently released from pNZPG and cloned in the low-copy-number plasmid pIL252 to make pILPG. Control plasmids

pNZG and pILG without the promoter were used to measure GFP background. A standard inducing assay consisted of the addition of bacitracin at 1.0 µg/ml to exponentially growing cells at an optical density at 600 nm (OD₆₀₀) of 0.2 in M17 plus 0.5% glucose (GM17) and chloramphenicol at 5 µg/ml (pNZ124-based plasmids) or erythromycin at 5 µg/ml (pIL252-based plasmids) at 30°C. After 10 min of incubation, samples were taken to measure RNA levels. Reverse transcriptase quantitative PCR (RT-qPCR) was carried out as previously described (15) using the oligonucleotides shown in Table 1. Under inducing conditions, *gfp_{uv}* was expressed in *L. lactis* pNZPG at 22× higher levels than the control pNZG. However, when the reporter cassette was present in the low-copy-number plasmid pILPG, RNA levels were only three times higher than levels for the background (pILG). These values are lower than those reported after the induction with Lcn972, a bacteriocin that triggers the CesSR response similarly to bacitracin (7). This is likely due to a higher basal activity of the *lmg0169* promoter under noninducing conditions when cloned in a multicopy plasmid. Since the plasmid pNZPG based on pNZ124 gave the highest induction, this plasmid and its corresponding promoterless pNZG were selected.

GFP detection. Several attempts to detect GFP fluorescence with a Cary Eclipse fluorometer (Varian, Inc., Sydney, Australia) equipped with a microtiter plate adaptor were carried out. *L. lactis* pNZPG and pNZG were induced under standard conditions with 1 µg/ml of bacitracin at 30°C, and samples were taken at 1, 2, 4, 6, and 22 h after induction. Cells were harvested by centrifugation and washed in saline phosphate buffer (PBS), pH 7.3, and microtiter wells were filled with 200 µl of the bacterial suspension. The excitation and emission filters were set at 395 and 509 nm, respectively. No signal above the background was clearly recorded, even after the cells were concentrated 20-fold (data not shown). Treatment with membrane permeabilizers to increase GFP release, postincubation at 4°C, and freeze-and-thaw cycles reported to enhance GFP detection (14) also failed. Fluorescence microscopy revealed the presence of bright, discrete GFP spots inside the cells instead of a homogenous fluorescence signal as observed in *L. lactis* NZ9000/pRV85 (data not shown). These spots could be likely due to the formation of inclusion bodies. In *Escherichia coli*, fluorescence of GFP in inclusion bodies correlates with

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§ Supplemental material for this article may be found at <http://aem.asm.org/>.

[▽] Published ahead of print on 30 November 2009.

TABLE 1. Plasmids and primers used in this work

Plasmids/primers	Description ^a	Source or reference
Plasmids		
pRV85	Source of <i>gfp_{uv}</i>	4
pNZ124	High-copy-number plasmid, Cm ^r	12
pIL252	Low-copy-number plasmid, Em ^r	17
pAB0169	Source of <i>llmg0169</i> promoter (<i>P_{llmg0169}</i>)	7
pNZPG	<i>P_{llmg0169}::gfp_{uv}</i> in pNZ124	This study
pNZG	Promoterless <i>gfp_{uv}</i> in pNZ124	This study
pILPG	<i>P_{llmg0169}::gfp_{uv}</i> in pIL252	This study
pILG	Promoterless <i>gfp_{uv}</i> in pIL252	This study
Primers (5'–3')		
QRT F <i>gfp_{uv}</i>	CGAGTTTGTGTCCGAGAATG	RT-qPCR for <i>gfp</i>
QRT R <i>gfp_{uv}</i>	ATGACGGGAACATAAGACG	
QRT F <i>llmg0169</i>	TTTGTCTAGGAATAATCGTACTTGGAG	RT-qPCR for <i>llmg0169</i>
QRT R <i>llmg0169</i>	CCATCAAACGAATGACCAACAGAATG	
Tuf F	GGTAGTTGTCTGAAGAATGGAGTGTGA	RT-qPCR internal control
Tuf R	TAAACCAGGTTCAATCACTCCACACA	

^a Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant.

predicted aggregation rates: the faster the protein aggregates, the lower its fluorescence emission, and vice versa (16). In fact, lowering the temperature to 20°C increased the fluorescence readings in the induced cultures (data not shown and Table 2).

TABLE 2. Fluorescence of *L. lactis* reporter strains after induction with 0.5 µg/ml bacitracin at 20°C under several inducing conditions

Variable	Fluorescence per cell ^a		Fluorescence increment (<i>n</i> -fold)
	Promoterless <i>gfp_{uv}</i>	P _{llmg0169} :: <i>gfp_{uv}</i>	
Copy no.			
pNZ124	100.0 ± 13.4	211.4 ± 5.2	111.4 (2.1)
pIL252	90.7 ± 10.2	125.8 ± 11.7	35.1 (1.4)
Growth phase ^b			
Exponential	100.0 ± 10.5	182.4 ± 17.2	82.4 (1.8)
Stationary	90.3 ± 7.0	182.4 ± 11.9	92.1 (2.0)
Initial OD ₆₀₀ ^c			
0.2	100.0 ± 23.5	181.0 ± 20.4	81.0 (1.8)
0.5	107.5 ± 4.3	138.9 ± 6.3	31.4 (1.3)
1.0	102.1 ± 19.1	102.2 ± 13.1	0.1 (1.0)
2.0	84.8 ± 6.9	78.3 ± 6.3	−6.5 (0.9)
Induction time (h) ^c			
1	252.0 ± 14.4	322.2 ± 19.5	70.2 (1.3)
3	127.4 ± 9.6	279.5 ± 20.9	152.1 (2.2)
6	96.5 ± 8.9	252.4 ± 16.9	156.0 (2.6)
22	100.0 ± 36.8	177.4 ± 18.4	77.4 (1.8)
Temp (°C) ^{c,d}			
20	100.0 ± 8.1	248.1 ± 22.7	148.1 (2.5)
30	100.4 ± 12.1	132.5 ± 7.0	32.1 (1.3)

^a Cultures were induced for 22 h unless otherwise indicated. Fluorescence values (mean ± standard deviation of three to six measurements) were corrected by OD₆₀₀ and are shown relative to the fluorescence recorded by the promoterless *gfp* plasmid pNZG under the reference condition (bold type). With the exception of the values for the reporter plasmids based on pIL252, all the reported values refer to the plasmid pair pNZG and pNZPG.

^b Exponentially growing cells at OD₆₀₀ = 0.2 or stationary-phase cells diluted at OD₆₀₀ = 0.2 prior to induction.

^c Induction was carried out with stationary-phase cells adjusted to OD₆₀₀ = 0.2 unless indicated.

^d Induction time was 6 h.

However, it was still necessary to wash and concentrate the cells to get reliable signals, which made this method incompatible with any high-throughput settings. The inability to detect GFP directly is likely associated with a relatively low activity of the selected promoter *P_{llmg0169}*, which may not be strong enough to synthesize as much GFP as necessary. The lack of a clear fluorescent phenotype of *gfp_{uv}* cloned under the control of a strong constitutive promoter in *L. lactis* has been reported (3). Conversely, direct detection of GFP_{uv} in *L. lactis* using microtiter volumes has been shown with very strong promoters, such as the nisin A promoter *P_{nisA}* (5, 13), under nisin-inducing conditions and in modified systems that enhance promoter activity (10, 11).

Dot blot GFP detection. As an alternative method to centrifugation for concentrating cells and removing the intrinsic fluorescence of the GM17 broth, induced cultures were dot blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and fluorescence was detected by a fluorescence scanner. Exponentially growing cultures of the strains carrying the reporter plasmids were induced in microtiter plates (three to six replicates) with 0.5 µg/ml of bacitracin in a final volume of 200 µl. In preliminary inducing experiments, samples (100 µl) were removed after 22 h of incubation at 20°C. Blotting onto the membrane was performed under vacuum in a Bio-Dot microfiltration apparatus (Bio-Rad). Prior to and after sample application, 200 µl of PBS was also applied. The membranes were air dried at 30°C for 30 min in the dark and scanned with a Thypoon Trio (GE Healthcare, Uppsala, Sweden) with an excitation wavelength of 488 nm and emission filter 520BP40. Unless indicated, the fluorescence signal per cell (corrected by OD₆₀₀) was calculated by dividing each value by the average fluorescence of the strain *L. lactis* pNZG under noninducing conditions and multiplied by 100. During the development of this method, we aimed to reduce sample handling and invested time while keeping the largest increment between the fluorescence values of the induced cultures and the background signals generated by the promoterless *gfp* reporter plasmid. Several variables were examined, and the results are summarized in Table 2. As previously anticipated on

FIG. 1. Fluorescence signals recorded by the GFP dot blot assay with the reporter pNZPG (gray bars) and the promoterless *gfp* pNZG (open bars) plasmids after induction with the cell wall-active bacitracin and the DNA inhibitors mitomycin C and novobiocin in *L. lactis* NZ9000 (WT) and in the *cesR*-deficient *L. lactis* MG1363 ($\Delta cesR$).

the basis of the mRNA levels, cloning of the reporter cassette in a higher copy number gave better signals after induction. Hardly any difference was observed when we used exponentially growing cells or stationary-phase cells diluted to an OD_{600} of 0.2. Therefore, the elapsed time to reach the desired OD_{600} could be avoided. Increasing the initial cell density for induction did not result in higher fluorescence readings, likely due to a lower cell-to-inducer ratio. A similar response was observed with samples taken after 3 and 6 h postinduction, which resulted in larger increments than sampling after 22 h, likely due to the metabolic burden that GFP production may impose on the cells. Based on the lower background levels, i.e., less GFP fluorescence with the promoterless *gfp* plasmid at 6 h, this sampling time was selected for subsequent experiments. The enhanced GFP fluorescence at lower temperature, previously observed in cell suspensions, was confirmed (Table 2). A protocol was established that consisted of inducing *L. lactis* pNZG and pNZPG stationary-phase cells adjusted to an

FIG. 2. Expression ratios determined by RT-qPCR of *llmg0169* after induction of exponentially growing cultures of *L. lactis* NZ9000 (open bars) and the *cesR*-deficient *L. lactis* MG1363 (gray bars) with bacitracin (Bac), mitomycin C (MitC), and novobiocin (Nov).

OD_{600} of 0.2 for 6 h at 20°C in a final volume of 200 μl in microtiter plates. Aliquots of 100 μl were subsequently blotted, washed, and scanned. The overall procedure lasts for approximately 7 h. The use of microtiter plates makes it suitable to be implemented in a high-throughput format.

Inducers of *P_{llmg0169}* A set of several antimicrobials was assessed with the pNZG/pNZPG reporter system under the standard conditions. Well-established *CesSR* inducers (7) such as bacitracin (0 to 2 $\mu\text{g/ml}$), Lcn972 (0 to 10 arbitrary units/ml), and vancomycin (0 to 0.5 $\mu\text{g/ml}$) were used, as well as cell wall-active non-*CesSR* inducers such as nisin (0 to 0.25 $\mu\text{g/ml}$), penicillin G (0 to 0.5 $\mu\text{g/ml}$), and lysozyme (0 to 16 mg/ml). These concentration ranges were chosen so that growth, measured as OD_{600} by a Benchmark Plus microplate spectrophotometer (Bio-Rad), under the inducing conditions, was not less than 20% of the growth in the absence of antimicrobials. Bacitracin, Lcn972, and vancomycin increased fluorescence in a concentration-dependent fashion, while noninducers did not (Fig. 1; see Fig. S2 in the supplemental material). As expected, when the reporter plasmids were introduced into a *cesR*-defective *L. lactis* MG1363 strain (9), induction was lost (Fig. 1), confirming the *CesSR*-dependent regulation of *llmg0169*, previously observed by transcriptomic analysis (7), and the specificity of the fluorescent signal. Other antibiotics or antimicrobials with cell targets other than the cell wall were also tested. Tetracycline (0 to 0.13 $\mu\text{g/ml}$), H_2O_2 (0.5 mM), or NaCl (2 and 4%) did not induce *P_{llmg0169}* (Fig. S1 in the supplemental material). However, mitomycin C, which covalently binds to DNA, and the DNA gyrase inhibitor novobiocin triggered a *CesSR*-mediated induction of *P_{llmg0169}* (Fig. 1). Induction factors up to 2.4, similar to those obtained with bacitracin, were observed, and induction did not take place in *L. lactis* $\Delta cesR$.

Due to the long incubation time (6 h) required to acquire the fluorescence measurements, we proceeded to confirm whether these antimicrobials were true inducers of *llmg0169* via *CesSR* or whether fluorescence emission was due to late secondary signals generated by the antimicrobial action of these compounds. Exponentially growing ($\text{OD}_{600} = 0.2$) *L. lactis* cultures were induced in a final volume of 2 ml and incubated at 30°C for 10 min. Total RNA was obtained, and *llmg0169* RNA levels were determined by RT-qPCR as previously described (15) using specific *llmg0169* primers (Table 1). Relative expression values obtained after induction are shown in Fig. 2. Mitomycin C induced *llmg0169* expression in a fas-

ion similar to that of bacitracin. In both cases, induction was CesR dependent. The molecular mechanisms of how mitomycin C triggers the CesSR response deserve further investigation. Presumably, uncoupling chromosome replication and septum biosynthesis during cell division may be behind CesSR activation. On the other hand, novobiocin did not act as a primary inducer since, after a short exposure, similar *llmg0169* induction ratios were obtained in either the presence or absence of *cesR* (Fig. 2). Hence, this antibiotic may induce the response to cell envelope stress in *L. lactis* via CesR only after prolonged incubations, as shown by the GFP reporter system (Fig. 1). It has already been described that other molecules, such as membrane-perturbing organic acids and detergents, also behave as weak inducers of cell wall stress-responsive promoters (2, 8).

Conclusions. Microtiter-based dot blotting is an alternative method to measure GFP in low-fluorescence *L. lactis* and appropriate for high-throughput settings. The *L. lactis* strains harboring the reporter plasmids pNZPG and pNZG are suitable to monitor cell envelope stress in *L. lactis*, and counter-screening for CesR-independent induction may be performed with *L. lactis* $\Delta cesR$. Using this reporter system, mitomycin C was revealed as an additional inducer of the *L. lactis* two-component system CesSR.

This work has been funded by grant BIO2007-65061 (Ministerio de Educación y Ciencia, Spain) and grants COF08-01 and EQUIP08-01 (FICYT, Asturias, Spain).

We thank M. Zagorec (INRA, France) for supplying the plasmid pRV85 and J. Kok and J. Pinto (RuG, the Netherlands) for critical reading of the manuscript and helpful discussions.

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