APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2007, p. 4704–4706 Vol. 73, No. 14
0099-2240/07/$08.00+0 doi:10.1128/AEM.02807-06

Luciferase Detection during Stationary Phase in *Lactococcus lactis*▽

Herwig Bachmann,[1] Filipe Santos,[1,2] Michiel Kleerebezem,[1,2] and Johan E. T. van Hylckama Vlieg[1,2]*

NIZO food research, Kluyver Centre for Genomics of Industrial Fermentation, P.O. Box 20, 6710 BA Ede, The Netherlands,[1] and TI Food & Nutrition, P.O. Box 557,6700 AN Wageningen, The Netherlands[2]

Received 1 December 2006/Accepted 15 May 2007

The luminescence signal of *luxAB*-encoded bacterial luciferase strongly depends on the metabolic state of the host cell, which restricts the use of this reporter system to metabolically active bacteria. Here we show that in stationary-phase cells of *Lactococcus lactis*, detection of luciferase is significantly improved by the addition of riboflavin or flavin mononucleotide to whole-cell assay systems.

The *luxAB*-encoded luciferase of *Vibrio harveyi* is frequently used as a reporter in a variety of microorganisms (10). Simple detection and high sensitivity underlie the increasing popularity of this system (11). Luciferase catalyzes the reaction of molecular oxygen, reduced flavin mononucleotide ($FMNH_2$), and a long-chain aldehyde, yielding the corresponding carboxylic acid, flavin mononucleotide (FMN), water, and light (490 nm). Besides its use as a promoter probe, luciferase is also used for analysis of the metabolic activity of cells (13). The latter is based on the dependency of the luminescence signal on the intracellular $FMNH_2$ concentration, which is directly correlated with the metabolic activity of a cell (6). This dependency is well documented for gram-positive (7, 8, 17, 19) and gram-negative (9, 13) bacteria and is illustrated by a rapid decline in luminescence upon entry into the stationary growth phase. Here we describe an improved method for the detection of luciferase activity in stationary-phase cells of *Lactococcus lactis*.

In our studies, we used the plasmid-encoded luciferase (*luxAB*) of *V. harveyi* in the lactic acid bacterium *L. lactis* MG5267 (16). The reporter construct was generated by digesting plasmid pJIM2374 (5) with HindIII and PstI. The *luxAB* fragment was isolated, made blunt, and cloned into pCRblunt (Invitrogen, Breda, The Netherlands), yielding pNZ5512. Subsequently, the *luxAB* fragment was isolated from pNZ5512 as an EcoRV-HindIII fragment, made blunt, and ligated into PmlI-digested pNZ7125 (2), resulting in pNZ5518. The *usp45* promoter (15) was amplified from genomic DNA of *L. lactis* MG1363 with the oligonucleotides usp45REV1 (5′GAACGATCATGCCTGCAG AGTACTTGTTC) and usp45FW2 (5′CTATTACTCGAGACA CTTTTGCTC). The amplified fragment was restricted with Sau3AI and ligated into BglII-digested pNZ5518, resulting in pNZ5519, in which the *luxAB* genes are under the control of the *usp45* promoter. Furthermore, the oligonucleotides AdaptVI-1 (5′CATGGAATATCCTCCTGAATTGGGGATCCCTCGAG TTAGTTAGTGCCCGGGCTAA) and AdaptVI-2 (5′GATCTT AGCCCGGGCACTAACTAACTCGAGGGATCCCCAATTC AGGAGGATATTC) were annealed and ligated into BglII- and NcoI-digested plasmid pNZ5518, which resulted in the introduction of a SmaI restriction site (plasmid pNZ5520). Genomic DNA from *L. lactis* MG1363 was partially digested with AluI, and 0.5- to 2-kb fragments were isolated and ligated into SmaI-digested pNZ5520.

L. lactis was grown in microplates (780271 or 655180; Greiner, Alphen a/d Rijn, The Netherlands) at 30°C in rich medium M17 (12) supplemented with 0.5% lactose, 5 μg/ml chloramphenicol, and (when indicated) 10 mg/liter riboflavin (R4500; Sigma, Zwijndrecht, The Netherlands). Measurements of luminescence and optical density at 595 nm (OD_{595}) were performed by mixing 50 μl of cell suspension with 150 μl of 1.9% (wt/vol) glycerol-2-phosphate disodium salt (G6376; Sigma, Zwijndrecht, The Netherlands) in a white microplate with a transparent bottom (655095; Greiner, Alphen a/d Rijn, The Netherlands). If indicated, 10 mg/liter riboflavin or 10 mg/liter FMN (F2253; Sigma, Zwijndrecht, The Netherlands) was added to the buffer. Two minutes after the cells were mixed with the buffer, 10 μl of 0.1% nonanal (W278203; Sigma, Zwijndrecht, The Netherlands) in 40% ethanol was added to each well. Luminescence was determined at 2-min intervals over a period of 15 min after nonanal addition in a Genios microplate reader (Tecan, Zurich, Switzerland). The peak value measured for each sample was used for data analysis.

When cultivated in M17, *L. lactis* MG5267 transformed with the *luxAB* expression plasmid pNZ5519 displayed a rapid decline in the luminescence signal upon entry of the cells into the stationary phase of growth (Fig. 1). We hypothesized that FMN could represent the limiting factor in the luminescence reaction and that addition of the FMN precursor riboflavin could complement this limitation. Indeed, addition of riboflavin to either the culture medium M17 or the assay buffer leads to an up-to-100-fold increase in luminescence, enabling detection of luminescence in cells that have entered the stationary phase of growth (Fig. 1 and 2). Moreover, introduction of the *luxAB* expression plasmid pNZ5519 into riboflavin-overproducing *L. lactis* strain CB010 (3) also allowed stable luminescence signal detection in cells entering the stationary phase of growth (Fig. 1). The addition of FMN to the assay buffer had an effect similar to that of the addition of riboflavin (Fig. 2). To exclude the possibility that these observations resulted from regulatory effects on the *usp45* promoter, five alternative promoter-*luxAB* fusion constructs were analyzed under the same conditions. These clones were selected from a pNZ5520-based promoter screening library constructed in MG5267 (data not

* Corresponding author. Mailing address: NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands. Phone: 31-318659511. Fax: 31-318650400. E-mail: johan.van.hylckama.vlieg@nizo.nl.

▽ Published ahead of print on 18 May 2007.

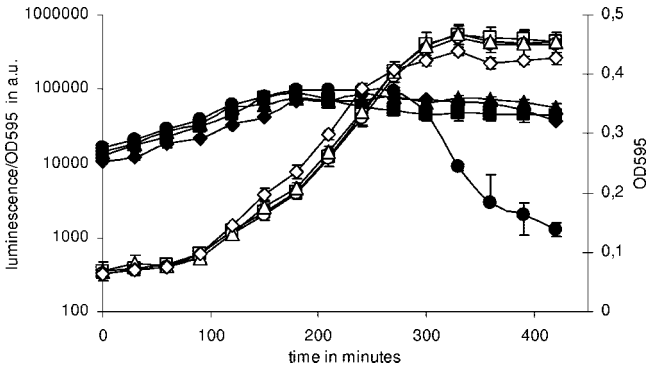

FIG. 1. Effect of riboflavin on luminescence of luciferase-expressing *L. lactis* MG5267. Growth was analyzed by monitoring OD_{595}. Filled symbols show luminescence per OD_{595} value, and open symbols show the corresponding OD_{595} measurements. Symbols: ■, MG5267(pNZ5519) grown in LM17 measured in buffer plus riboflavin; ●, MG5267(pNZ5519) grown in LM17 measured in buffer only; ▲, MG5267(pNZ5519) grown in LM17 plus riboflavin (10 mg/liter) measured in buffer only; ◆, CB010(pNZ5519) grown in LM17 measured in buffer only. Each data point represents the average of 12 biological replicates (error bars show standard deviations). a.u., arbitrary units.

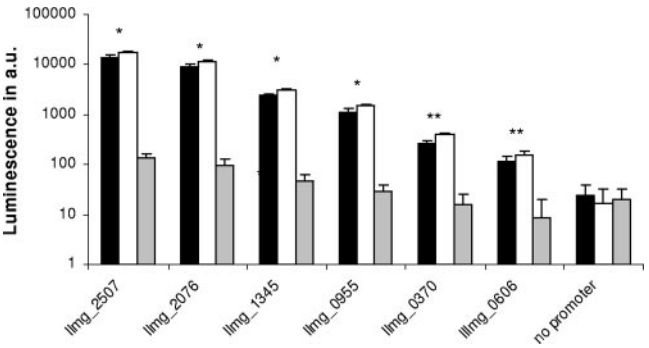

FIG. 2. Effect of addition of riboflavin or FMN on luminescence signals of stationary-phase cells of *L. lactis* MG5267 with various luciferase expression levels. Luciferase activity was measured 3.5 h after cultures entered the stationary phase. The black bars show measurements performed in buffer supplemented with 10 mg/liter riboflavin. White bars show measurements in buffer supplemented with 10 mg/liter FMN. Gray bars show measurements in buffer only. Each bar represents the average value of four biological replicates (error bars show standard deviations). *, $P < 0.001$; **, $P < 0.002$ (pairwise *t* test). The experiment was repeated four times with similar results. The names of the samples refer to the promoter sequences upstream of the luciferase genes as annotated in *L. lactis* MG1363 (18). a.u., arbitrary units.

shown). They contain random fragments of genomic *L. lactis* DNA cloned upstream of the promoterless *luxAB* gene cassette. There was a 100-fold difference in luminescence levels between the clones with the highest and lowest activity levels. For all of the constructs, luminescence in the stationary phase could be increased significantly by the addition of either riboflavin or FMN (Fig. 2). The negative control with a promoterless *luxAB* construct, pNZ5518, shows a luminescence signal comparable to background measurements, irrespective of the addition of riboflavin or FMN (Fig. 2). These results confirm that riboflavin/FMN availability is a limiting factor for the luminescence signal in *L. lactis* cells that are in the stationary phase of growth. Furthermore, they indicate that NADH for the (re)generation of the luminescence reaction cofactor, $FMNH_2$, is available in these cells.

In a different experimental setup, we supplied nonanal in a volatile form to the cultures by placing 2% nonanal diluted in mineral oil in the spaces between the wells of a covered microplate. Luminescence was measured throughout the growth curve in the wells where the cells were cultured. We ensured that neither nonanal nor oxygen was limiting the luminescence reaction in those cultures and found that despite the addition of extra riboflavin to the medium, luminescence signals in stationary phase were variable (data no shown). This finding suggests that a continuous luminescence reaction might have an effect on the metabolism of stationary-phase *L. lactis* cells.

The data presented here show that the detection of bacterial luciferase in stationary-phase *L. lactis* can be significantly improved by the addition of riboflavin or FMN. Riboflavin is known to serve as an $FMNH_2$ analogue for the luminescence reaction, but only in its reduced form (14). This excludes that the described effect is caused by transported riboflavin itself and is confirmed by our finding that luminescence in the *luxAB* negative controls was not influenced by the addition of either riboflavin or FMN. Blouin et al. reported that addition of FMN to *E. coli* cultures shortly before luminescence measurements could increase the signal, but these authors did not relate this observation to luminescence detection in stationary-phase cells (1). The phylogeny of the *L. lactis* riboflavin transporter RibU (4) suggests that our finding might also be applicable to a number of other members of the division *Firmicutes*. However, a reliable assessment of the applicability to other species requires additional experimentation. In conclusion, the detection of *luxAB*-encoded luminescence for *L. lactis* is significantly improved by the addition of riboflavin or FMN to either the culture medium or the buffer used during the luminescence assay. Furthermore, it is important to realize that a continuous luminescence reaction in *L. lactis* might influence the metabolic state of the stationary host cell.

REFERENCES

1. **Blouin, K., S. G. Walker, J. Smit, and R. Turner.** 1996. Characterization of in vivo reporter systems for gene expression and biosensor applications based on *luxAB* luciferase genes. Appl. Environ. Microbiol. **62:**2013–2021.
2. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J. Bacteriol. **186:**5721–5729.
3. **Burgess, C., M. O'Connell-Motherway, W. Sybesma, J. Hugenholtz, and D. van Sinderen.** 2004. Riboflavin production in *Lactococcus lactis*: potential for in situ production of vitamin-enriched foods. Appl. Environ. Microbiol. **70:**5769–5777.
4. **Burgess, C. M., D. J. Slotboom, E. R. Geertsma, R. H. Duurkens, B. Poolman, and D. van Sinderen.** 2006. The riboflavin transporter RibU in *Lactococcus lactis*: molecular characterization of gene expression and the transport mechanism. J. Bacteriol. **188:**2752–2760.
5. **Delorme, C., S. D. Ehrlich, and P. Renault.** 1999. Regulation of expression of the *Lactococcus lactis* histidine operon. J. Bacteriol. **181:**2026–2037.
6. **Duncan, S., L. A. Glover, K. Killham, and J. I. Prosser.** 1994. Luminescence-based detection of activity of starved and viable but nonculturable bacteria. Appl. Environ. Microbiol. **60:**1308–1316.
7. **Francis, K. P., J. Yu, C. Bellinger-Kawahara, D. Joh, M. J. Hawkinson, G. Xiao, T. F. Purchio, M. G. Caparon, M. Lipsitch, and P. R. Contag.** 2001. Visualizing pneumococcal infections in the lungs of live mice using bioluminescent *Streptococcus pneumoniae* transformed with a novel gram-positive *lux* transposon. Infect. Immun. **69:**3350–3358.
8. **Hardy, J., K. P. Francis, M. DeBoer, P. Chu, K. Gibbs, and C. H. Contag.** 2004. Extracellular replication of *Listeria monocytogenes* in the murine gall bladder. Science **303:**851–853.
9. **Koga, K., T. Harada, H. Shimizu, and K. Tanaka.** 2005. Bacterial luciferase activity and the intracellular redox pool in *Escherichia coli*. Mol. Genet. Genomics **274:**180–188.

10. **Meighen, E. A.** 1991. Molecular biology of bacterial bioluminescence. Microbiol. Rev. **55:**123–142.
11. **Roda, A., P. Pasini, M. Mirasoli, E. Michelini, and M. Guardigli.** 2004. Biotechnological applications of bioluminescence and chemiluminescence. Trends Biotechnol. **22:**295–303.
12. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29:**807–813.
13. **Unge, A., R. Tombolini, L. Molbak, and J. K. Jansson.** 1999. Simultaneous monitoring of cell number and metabolic activity of specific bacterial populations with a dual *gfp-luxAB* marker system. Appl. Environ. Microbiol. **65:**813–821.
14. **Valkova, N., R. Szittner, and E. A. Meighen.** 1999. Control of luminescence decay and flavin binding by the LuxA carboxyl-terminal regions in chimeric bacterial luciferases. Biochemistry **38:**13820–13828.
15. **van Asseldonk, M., G. Rutten, M. Oteman, R. J. Siezen, W. M. de Vos, and G. Simons.** 1990. Cloning of *usp45*, a gene encoding a secreted protein from *Lactococcus lactis* subsp. *lactis* MG1363. Gene **95:**155–160.
16. **van Rooijen, R. J., M. J. Gasson, and W. M. de Vos.** 1992. Characterization of the *Lactococcus lactis* lactose operon promoter: contribution of flanking sequences and LacR repressor to promoter activity. J. Bacteriol. **174:**2273–2280.
17. **Waterfield, N. R., R. W. Le Page, P. W. Wilson, and J. M. Wells.** 1995. The isolation of lactococcal promoters and their use in investigating bacterial luciferase synthesis in *Lactococcus lactis*. Gene **165:**9–15.
18. **Wegmann, U., M. O'Connell-Motherway, A. Zomer, G. Buist, C. Shearman, C. Canchaya, M. Ventura, A. Goesmann, M. J. Gasson, O. P. Kuipers, D. van Sinderen, and J. Kok.** 2007. Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. J. Bacteriol. **189:**3256–3270.
19. **Wiles, S., K. Ferguson, M. Stefanidou, D. B. Young, and B. D. Robertson.** 2005. Alternative luciferase for monitoring bacterial cells under adverse conditions. Appl. Environ. Microbiol. **71:**3427–3432.