Luciferase Detection during Stationary Phase in Lactococcus lactis

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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 (FMNH2), 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). 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 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 (OD595) 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 available at print time of 18 May 2007).
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, FMNH₂, 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 not 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 FMNH₂ 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


