Expression of fluorescent proteins in bifidobacteria for analysis of host-microbe interaction

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Abstract

Bifidobacteria are an important component of the human gastrointestinal microbiota and are frequently used as probiotics. The genetic inaccessibility and lack of molecular tools commonly used in other bacteria has hampered a detailed analysis of the genetic determinants of bifidobacteria involved in their adaptation to, colonisation of, and interaction with the host. In the present study, a range of molecular tools were developed that will allow to close some of the gaps in functional analysis of bifidobacteria. A number of promoters were tested for transcriptional activity in *B. bifidum* S17 using pMDY23, a previously published promoter probe vector. The promoter of the *gap* gene (P\textsubscript{gap}) of *B. bifidum* S17 yielded the highest promoter activity among the promoters tested. Thus, this promoter and the pMDY23 backbone were used to construct a range of vectors for expression of different fluorescent proteins (FPs). Successful expression of CFP, GFP, YFP and mCherry could be shown for three strains representing three different *Bifidobacterium* sp. The red fluorescent *B. bifidum* S17 pVG-mCherry was further used to demonstrate application of fluorescent bifidobacteria for adhesion assays and detection in primary human macrophages cultured *in vitro*. Furthermore, pMGC-mCherry was cloned combining a chloramphenicol resistance marker and expression of the FP mCherry under the control of P\textsubscript{gap}. The chloramphenicol resistance marker of pMGC-mCherry was successfully used to determine gastrointestinal transit time of *B. bifidum* S17. Moreover, *B. bifidum* S17 pMGC-mCherry could be detected in faecal samples of mice after oral administration.
Introduction

Bifidobacteria are Gram-positive bacteria of the class Actinobacteria, which is highly diverse and comprises many economically and clinically relevant microorganisms such as Mycobacterium sp., Corynebacterium sp., Streptomyces sp., and others (1). Natural habitats of the members of the genus Bifidobacterium include food, sewage and oral cavities but the most important ecological niche of bifidobacteria is the intestinal tract of humans and animals (2). Some strains of bifidobacteria have been associated with beneficial effects on the health status of the host and have thus attracted considerable interest by the food and dairy industries. Some of the beneficial effects that have been claimed to be related to presence or administration of bifidobacteria are cholesterol reduction, improvement of lactose intolerance, alleviation of constipation, and immunomodulation (2–4). Two of the more promising targets for bifidobacterial treatments are amelioration of chronic intestinal inflammation (5–10) and protection against infections with enteric pathogens (11–13).

Recently, some of the mechanisms of host colonisation of bifidobacteria and their effect on the immune system have been elucidated in more detail. Bifidobacterial genomes contain up to seven gene clusters for pili (14, 15). Some of these genes were shown to be expressed under in vitro conditions and in the GIT of mice and electron and atomic force microscopy revealed the presence of pili on the surface of some of these strains (14–17). Experiments with recombinant L. lactis strains expressing two of the pilus clusters of B. bifidum PRL2010 suggest a role for adhesion to extracellular matrix proteins (16). Moreover, the importance of tad pili for colonisation of the mice was demonstrated for B. breve UCC2003 (17). Similarly, the exopolysaccharide (EPS) produced by B. breve UCC2003 confers resistance against bile and acid and improves persistence in the GIT of mice (12). Moreover, presence of EPS on the surface changes the immunogenicity of B. breve UCC2003. Higher numbers of pro-inflammatory immune cells were observed in the spleens of mice colonised with a mutant
lacking EPS compared to the wildtype (12). Of note, the EPS-producing wildtype B. breve UCC2003 was more efficient in reducing the colonisation with the murine pathogen *Citrobacter rodentium* (12). Similarly B. longum subsp. longum JCM 1217T and B. longum subsp. *infantis* 157F conferred protection against infections in a mouse model of infections with enteropathogenic *E. coli* O157 (11) and a fermented milk product containing B. *animalis* subsp. *lactis* DN-173010 reduced intestinal inflammation by inhibiting colitogenic, Gram-negative bacteria in a model of ulcerative colitis (8). In both studies, the effect of bifidobacteria could be at least partially attributed to an acidification of the intestinal pH due to acetic and lactic acid produced by bifidobacteria. Despite the progress in understanding the effect of bifidobacteria and other probiotics, the European Food Safety Authority has so far rejected all of the claims submitted for probiotic bacteria based on the fact that these claims have not been substantiated sufficiently by clinical studies in humans (18). This highlights the need for a better definition of the target groups for probiotic treatments as well as for relevant biomarkers, well-performed clinical trials and a more detailed analysis of the underlying immunological mechanisms and the populations of immune cells that mediate the observed effects. A key problem for analysis of the beneficial effects of bifidobacteria on the host is their poor genetic accessibility. While some progress has been made in improving transformation efficiencies, directed gene inactivation and transposon mutagenesis for single strains (19, 20) the vast majority of strains of interest remain difficult if not impossible to modify genetically. Moreover, the available vector systems for bifidobacteria have a rather limited host range (i.e. replicons function only in some species of the genus *Bifidobacterium* but not in others) and expression systems are poorly developed (2, 21). An indispensable prerequisite to study the effect of bifidobacteria on the immune system and its various cell populations is the ability to label bacteria. Labelled bacteria may be tracked
either *in vivo* in live animals or used to identify, isolate and analyse defined populations of immune cells that contain bacteria. Two of the methods most frequently used to label bacteria for these purposes are expression of either a luciferase or fluorescent proteins (FPs). However, both methods have only been reported for a single *B. breve* strain (22–24).

Since the discovery of the green fluorescent protein (GFP) of the jelly fish *Aequorea victoria* in 1962 (25), FPs have been isolated from a variety of (mostly marine) organisms and continuously modified and improved for a wide range of applications (26–28). Today, FPs are one of the most versatile and widely used tools for the investigation of bacterial physiology and interaction with the host. For example, FPs have been successfully used as a transcriptional reporter for expression of genes or to study protein localisation and dynamics (29–31). Furthermore, FPs are a valuable tool to monitor bacterial biofilms, to identify single, labelled cells in a complex sample or to study horizontal gene transfer (32). FPs can also be used to study virtually any aspect of the interaction of bacteria with the host both *in vitro* and *in vivo*. Change in bacterial gene expression can be monitored using FPs as transcriptional reporters, infected cells can be identified from a pool, interaction of bacteria with individual cells of the host or localisation to a subcellular compartment can be visualised *in vitro* in live or fixed cells or tissue samples or even in living animals (33–36).

In the present study, we developed a range of vectors for functional analysis of various bifidobacteria. We report for the first time expression of four fluorescent proteins in different strains of bifidobacteria and the use of these recombinant strains to image interactions with host cells. Furthermore, one of the vectors developed was successfully used to monitor host colonisation and detection of bacteria in faecal samples.
Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in supplementary Table S1. Bifidobacterium strains were cultured anaerobically in Lactobacilli MRS medium (Difco) supplemented with 0.5 g l\(^{-1}\) L-cysteine (MRSc) at 37 °C. Anaerobic conditions were achieved by cultivation using AnaeroGen sachets (Merck) in sealed jars. For cultivation of *B. bifidum* S17 harbouring plasmids, the respective medium was supplemented with antibiotics at the following concentrations: 1 µg ml\(^{-1}\) ampicillin, 5 µg ml\(^{-1}\) chloramphenicol, 2 µg ml\(^{-1}\) erythromycin, and 100 µg ml\(^{-1}\) spectinomycin unless stated otherwise.

*E. coli* DH10B was used as cloning host and was cultivated in 2× TY medium and grown to stationary phase at 37 °C with agitation. For cultivation of strains harbouring plasmids, the respective medium was supplemented with appropriate antibiotics (spectinomycin and ampicillin: 100 µg ml\(^{-1}\); chloramphenicol: 15 µg ml\(^{-1}\); erythromycin: 300 µg ml\(^{-1}\)). Sequence integrity of all cloned inserts was verified by DNA sequencing (Eurofins MWG Operon, Germany). Transformation into *B. bifidum* S17 was performed by electroporation as described previously (19).

Determination of selective antibiotic concentrations

Selective concentrations for ampicillin, chloramphenicol, erythromycin and spectinomycin were determined for each of the *Bifidobacterium* sp. strains used in this study using a microtiter plate assay. Fresh overnight (o/N) cultures were diluted in fresh MRSc containing different concentrations of antibiotic to an OD\(_{600}\) of 0.1 and distributed into individual wells of a 96 well tissue culture plate (Sarstedt AG & Co., Newton, USA). As positive controls, MRSc medium without antibiotics was inoculated to the same OD\(_{600}\). Plates were incubated under anaerobic conditions for 24 h and growth was determined by measuring the OD\(_{600}\) in a
multilabel microtiter plate reader (Infinite® M200, Tecan Group Ltd.). Experiments were performed in four technical replicates per strain and condition and three independent cultures per strain were tested. Antibiotic concentrations 10-times higher than the concentrations allowing minimal growth were considered selective.

Plasmid stability assay
Stability of plasmids in the absence of antibiotics was determined as described previously (37). Briefly, plasmid-bearing strains were cultured in MRSc without antibiotics and were diluted 1:1000 every 12 h in fresh medium for a total of 100 generations. Plasmid stability was determined every 25 generations by plating on MRSc agar without antibiotics. At least 100 colonies were randomly selected and replica-plated on MRSc agar with the appropriate antibiotic at selective concentration or MRSc agar without antibiotics and incubated at 37°C for 48 h under anaerobic conditions. Growth on both plates was verified and plasmid stability was calculated as percentage of antibiotic resistant colonies.

β-glucuronidase assay
The glucuronidase assay was performed as described elsewhere (38) with minor modifications. The assay is based on the enzymatic conversion of 4-methylumbelliferyl-β-D-glucuronide (MUG) to 4-methylumbelliferone (MU) by the β-glucuronidase GusA. MU is a direct measure of GusA activity, which in turn correlates with promoter activity. GusA activity was measured in cell free extracts of B. bifidum S17 harbouring pMDY23-derivatives with different promoters cloned upstream of the gusA reporter gene (see supplementary Materials and Methods). Recombinant strains were inoculated into 100 ml of MRSc at an OD600 of 0.1 from a fresh o/N culture and grown under anaerobic conditions at 37 °C. At the indicated timepoints, bacteria were harvested by centrifugation (4 °C, 5,000× g, 10 min).
After washing with ddH2O and GUS assay buffer (50 mM Na2HPO4 (pH 7), 1 mM EDTA, 0.1 % Triton X-100, 5 mM DTT), cells were resuspended in 1 ml GUS assay buffer and disrupted in cryo-tubes with glass beads (0.1 mm diameter; Carl Roth) at 4 °C using a RiboLyser (Hybaid) during three cycles of 30 seconds at full speed with cooling on ice for 1 min in between cycles. Lysates were centrifuged for 15 min at 14,000× g and 4 °C to remove beads and bacterial debris. Supernatants were retained as cell free extracts and protein content was measured in the supernatants using 2-D Quant kit (GE Healthcare Life Science).

GusA activity was measured in cell free extracts using 2.5 to 10 µg total protein in a final volume of 100 µl in GUS assay buffer. The reaction was started by adding 25 µl of 5 mM MUG. After an incubation time at 37 °C for 30 min, during which MUG is hydrolysed quantitatively to MU by GusA, a 10 µl aliquot was removed and mixed with 170 µl stop buffer (0.2 M NaCO3, pH 9.5) in a 96-well plate (Sarstedt AG & Co.). MU was quantified by fluorescence using Infinite® M200 multimode reader (Infinite® M200, Tecan Group Ltd.) with excitation at 388 nm and measuring fluorescence (emission) at 480 nm. As negative control, B. bifidum S17 harbouring the promoterless pMDY23 plasmid was used.

Adhesion assays

Adhesion to Caco-2 cells was determined by classical plate counting method described previously (39). Briefly, confluent monolayers of Caco-2 cells in 24 well tissue culture plates were used. At this stage approx. 1×10⁶ cells were counted per well. An o/N culture of B. bifidum S17 pVG-mCherry was washed three times in phosphate-buffered saline (PBS) and resuspended in cell culture medium (DMEM supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 1% (v/v) NEAA) and adjusted to an OD₆₀₀ of 2, which equals 2×10⁸ CFU ml⁻¹. 500 µl of this suspension were added to a well of Caco-2 cells. Following an incubation of 1 h to allow adherence, unbound bacteria were removed by three washings in cell culture
medium. Fluorescence of each well was measured in a multilabel microtiter plate reader (Infinite® M200, Tecan Group Ltd.) with excitation at 585 nm and emission at 620 nm. Then, Caco-2 cell monolayer was scraped off the bottom of the well and the 500 µl of medium containing cellular debris were quantitatively transferred to a sterile Eppendorf cup. Wells were rinsed with 500 µl of PBS and the washings were combined with the debris to give a total volume of 1 ml. The suspension was vigorously homogenised and 10-fold serial dilutions were plated on MRSc agar supplemented with 100 µg ml\(^{-1}\) spectinomycin to determine bifidobacterial cell numbers and calculate adhesion as CFU per well.

In order to allow for quantification of adherent bacteria using fluorescence, a calibration curve was established in parallel. For this purpose, bacteria were adjusted to OD\(_{600}\) = 2, 1, 0.5 or 0.25 in cell culture medium and 500 µl of these suspensions were added to separate wells of the plate containing Caco-2 cells. Following 1 h incubation, fluorescence was measured immediately without washing and CFU were determined as described above. Autofluorescence of Caco-2 cells without bacteria was measured as a background control and subtracted from all other values. For fluorescence microscopy, Caco-2 cells were fixed with 4% (w/v) PFA in PBS.

**Generation of primary human macrophages**

Primary human macrophages were generated essentially as describes previously (40). Briefly, lymphocytes were isolated from fresh buffy coats by density gradient centrifugation using lymphocyte separation medium (LSM 1077; PAA Laboratories). Monocytes were isolated from the lymphocytes pool by negative selection using Monocyte Isolation Kit II (human, Miltenyi Biotech) according to the manufacturer’s instructions. Following isolation, monocytes were cultured in Lumox® dishes (Sarstedt) for 7 days with RPMI 1640 medium containing 10% fetal calf serum, 10 mM L-glutamine, 1% (v/v) penicillin/streptomycin (PAA
Laboratories) and 1% (v/v) NEAA. For differentiation, 50 ng/ml recombinant human M-CSF (R&D Systems) were added to the dishes. After 3 days, medium was changed and growth factors were freshly added. On day 7, cells were detached by rinsing with phosphate-buffered saline (PBS; PAA Laboratories) and seeded at 1×10⁴ cells per well into 24 well plates (BD Biosciences). Prior to experiments, macrophages were grown o/N in antibiotic-free medium containing growth factors.

Macrophages were incubated with B. bifidum S17 pVG-mCherry for 2 h at a bacteria:cell ratio of 10:1. Afterwards, macrophages were washed 3 times with PBS to remove extracellular bacteria and fixed with 4% (w/v) PFA in PBS.

**Gastrointestinal transit time of B. bifidum S17 in mice and detection in faecal slurries**

All animal experiments were approved by the ethical committee for animal experimentation of the University of Ulm and the responsible legal authority at the Regierungspräsidium Tübingen. Mice were bred and kept under specific pathogen free conditions at the animal facility at the University of Ulm and were fed a standard laboratory chow and water *ad libitum.*

C57BL/6J mice (both sexes, aged 7-12 weeks; n = 6) were inoculated with a single dose of 2×10⁹ CFU of B. bifidum S17 pMGC-mCherry per animal. Faecal pellets were collected at the indicated time points (0 - 24 h after inoculation), weighed, and homogenised in 1 ml of PBS by vigorous vortexing and used as faecal slurries for fluorescence microscopy. For quantification of faecal carriage of B. bifidum S17 pMGC-mCherry, serial dilutions were prepared and plated on MRSc agar plates containing 200 µg ml⁻¹ mupirocin and 5 µg ml⁻¹ chloramphenicol. Agar plates were incubated anaerobically for 48 h at 37 °C. Bifidobacterial counts were determined as CFU g⁻¹ faeces.
Fluorescence microscopy

For fluorescence microscopy, o/N cultures of fluorescent Bifidobacterium strains were washed three times and resuspended in PBS. One drop of an appropriate dilution in PBS was placed on a microscope slide and covered with a cover slip. Macrophages and Caco-2 cells incubated with fluorescent bifidobacteria were fixed with 4% (w/v) PFA in PBS and stained for 30 min at RT in the dark with Hoechst (Invitrogen Life Technologies) diluted 1:10,000 and Phalloidin-Alexa488 (Invitrogen Life Technologies) diluted 1:500 in PBS.

Fluorescent microscopy was performed with a 100× oil immersion or a 40× objective as indicated in the figure legends on a Zeiss Axio Observer.Z1 microscope (Carl Zeiss) equipped with appropriate filter sets to detect fluorescence of Hoechst (excitation at 365 ± 10 nm, emission at 445 ± 50 nm), CFP (excitation at 436 ± 20 nm, emission at 480 ± 40 nm), GFP and Alexa488 (excitation at 470 ± 40 nm, emission at 525 ± 50 nm), YFP (excitation at 500 nm ± 25 nm, emission at 535 nm ± 30 nm), or mCherry (excitation at 545 ± 30 nm, emission at 610 ± 75 nm). Images were acquired and analysed using the software ZEN 2012 (Carl Zeiss).

Results

Identification of a suitable promoter for gene expression in bifidobacteria

As a first step towards the generation of plasmids for gene expression in bifidobacteria, five different promoters were assayed for the transcriptional activity using the previously published pMDY23 promoter probe vector (41). The range of promoters tested included the promoter regions upstream of the gap gene (BBIF_0612) encoding glyceraldehyde-3-phosphate dehydrogenase (P_{gap}) and the pk gene (BBIF_0789) for fructose-6-phosphate phosphoketolase (P_{pk}) of B. bifidum S17. Fructose-6-phosphate phosphoketolase is the key
enzyme of the bifidus shunt and was thus hypothesised to be highly expressed in bifidobacteria during growth on media containing glucose such as MRSc. The $P_{\text{gap}}$ promoter was selected based on previous reports about high transcriptional activity and successful use for protein expression in bifidobacteria (41–43). Promoter regions were retrieved from the publically available genome sequence of $B. \text{bifidum}$ S17 (Accession No. CP002220; (44). Furthermore, the synthetic promoters $P_{\text{hyper}}$, $P_{\text{help}}$ and $P_{\text{CP25}}$ were included, which were previously shown to have high transcriptional activity in a number of Gram-positive bacteria (45–47). These promoters were cloned upstream of the $\text{gusA}$ reporter gene in pMDY23 (Figure 1A), transformed into $B. \text{bifidum}$ S17 and assayed for transcriptional activity by GusA assay during growth of the recombinant strains (Figure 1B). No reporter activity could be measured for $P_{\text{CP25}}$ and $P_{\text{help}}$. $P_{\text{gap}}$ showed consistently measurable transcriptional activity except for the late stationary growth phase (i.e. after 24 h) and yielded the highest reporter activities of all promoters at any time point assayed. GusA reporter activity could also be measured for $P_{\text{p4}}$ and $P_{\text{hyper}}$ however at somewhat lower levels than $P_{\text{gap}}$ and not as consistently throughout growth. Thus, $P_{\text{gap}}$ was considered an appropriate promoter for gene expression in $B. \text{bifidum}$ S17.

**Expression of fluorescent proteins in bifidobacteria**

In order to identify a bifidobacterial vector replicon with the widest possible host range, we transformed a number of published $E. \text{coli}/Bifidobacterium$ shuttle vectors including pMDY23, pPKCm1, and pDG7 into various $Bifidobacterium$ strains. Of these plasmids pMDY23 had the broadest range of bifidobacterial hosts and was successfully and reproducibly transformed into $B. \text{adolescentis}$ NCC251, $B. \text{bifidum}$ (strains S17 and S16), $B. \text{breve}$ S27, $B. \text{longum}$ subsp. $\text{longum}$ (strains E18 and NCC2705), $B. \text{animalis}$ subsp. $\text{lactis}$ NCC362, and $B. \text{animalis}$ subsp. $\text{animalis}$ MB254, i.e. a total of 8 strains of 5 different
species (supplementary Table S2). The replicon of pMDY23 was thus chosen as the backbone of all further plasmids generated in this study. Based on pMDY23-P<sub>gap</sub>, a range of vectors for expression of different fluorescent proteins was constructed. Four different plasmids were generated essentially by replacing the gusA reporter gene in pMDY23-P<sub>gap</sub> with the genes for the fluorescent proteins CFP, GFP, YFP and mCherry (Figure 2A). Plasmids were constructed as described in the supplementary Materials and Methods. This yielded plasmids pVG-CFP, pVG-GFP, pVG-YFP and pVG-mCherry, which were successfully transformed into three strains of bifidobacteria belonging to three different species. Derivatives of <i>B. bifidum</i> S17 containing these different plasmids showed high fluorescence at the expected wavelengths and virtually all bacteria were fluorescent (Figure 2B). Moreover, the spectra of the emitted light were different enough to distinguish differently labelled <i>B. bifidum</i> S17 strains in a mix (Figure 2C). Similar results were obtained, when pVG-CFP, pVG-GFP, pVG-YFP or pVG-mCherry were transformed into <i>B. longum</i> subsp. <i>longum</i> E18 (Figure S3) or <i>B. breve</i> S27 (Figure S4). Collectively, these results indicate that these plasmids are useful to label various bifidobacterial strains of different species.

**Applications of fluorescent <i>B. bifidum</i> S17**

Red fluorescent <i>B. bifidum</i> S17 pVG-mCherry adhering to Caco-2 cells could be visualised by fluorescence microscopy (Figure 3A). In order to quantitatively assess adherent bacteria, a calibration curve was established for <i>B. bifidum</i> S17 pVG-mCherry in the presence of Caco-2 cells. This revealed a high degree of correlation of fluorescence with CFU (Figure 3B). Using this calibration curve, CFU of <i>B. bifidum</i> S17 pVG-mCherry were calculated based on fluorescence measurements and compared to actual numbers of bacteria adhering to Caco-2 cells as determined by classical plate counting (Figure 3C). This yielded almost identical
results for both methods indicating that measuring fluorescence gives results that are comparable to the classical plate counting method.

In a second approach, we sought to check if it is possible to image fluorescent bifidobacteria inside relevant host cells. For this purpose, primary human macrophages were generated ex vivo from monocytes by differentiation for 7 days in the presence of M-CSF and co-cultured with *B. bifidum* S17 pVG-mCherry. Using fluorescence microscopy, high numbers of intracellular *B. bifidum* S17 pVG-mCherry were detected (Figure 4).

**Plasmids for in vivo colonisation studies**

Initial experiments on gastrointestinal colonisation and persistence of bifidobacteria revealed that mice of the animal facility at the University of Ulm harbour an intrinsic *B. animalis* strain, which was resistant to spectinomycin at 100 µg ml⁻¹, i.e. the concentration generally used for selection of pMDY23 derivatives (data not shown). This prompted us to generate a range of vectors for in vivo tracing studies. Based on pMDY23 four plasmids with different antibiotic resistance genes and the pBluescript multiple cloning site were generated (supplementary Materials and Methods and Figure S5A). The selective concentrations to the antibiotics used were tested for a range of bifidobacterial strains frequently used in our lab. Using a 96-well microtiter plate assay, the concentrations that proved to be selective for almost all strains tested were 1, 5, 2 and 100 µg ml⁻¹ for ampicillin, chloramphenicol, erythromycin, and spectinomycin, respectively (supplementary Table S6). However, *B. animalis* subsp. *animalis* MB254 and *B. animalis* subsp. *lactis* NCC362 still showed minimal growth at 100 µg ml⁻¹ spectinomycin. These concentrations were successfully used to select clones of *B. bifidum* S17 harbouring either pMGA, pMGC, pMGE or pMGS following electroporation. All plasmids were tested for stability in *B. bifidum* S17 in the absence of antibiotic pressure. Following cultivation for
100 generations in the absence of antibiotic the percentage of resistant colonies did not drop below 100% indicating high plasmid stability. Transformation of *B. bifidum* S17 with pMGA, pMGS, or pMGE reduced growth rate and/or final OD$_{600}$ in the presence and/or absence of respective antibiotic to varying degrees (supplementary Figure S5B). No difference was observed for *B. bifidum* S17 pMGC on MRSc with or without chloramphenicol and under both conditions growth characteristics of this strain did not differ from those of the WT (supplementary Figure S5B).

Thus, *B. bifidum* S17 pMGC was selected for *in vivo* studies. To combine the advantages of antibiotic selection with expression of fluorescent proteins, pMGC-mCherry (supplementary Figure S7) was generated by cloning the $P_{gap}$-mCherry construct of pVG-mCherry into the MCS of pMGC as described in the supplementary Materials and Methods. The resulting plasmid was successfully transformed into *B. bifidum* S17 yielding *B. bifidum* S17 pMGC-mCherry, which was then used to determine gastrointestinal transit time in mice.

For this purpose, female C57BL/6J mice (n = 6) were inoculated with *B. bifidum* S17 pMGC-mCherry by a single oral dose of $2 \times 10^8$ CFU per animal. Following inoculation, the numbers of chloramphenicol-resistant bifidobacteria were enumerated in faecal pellets by plate-counting on selective agar (Figure 5A). This revealed that faecal shedding of *B. bifidum* S17 pMGC-mCherry peaked at around 5 h post inoculation when approx. $1 \times 10^8$ CFU g$^{-1}$ faeces were counted. Thereafter, counts continuously decreased until 24 h when for 4 out of 6 animals levels had dropped below the limit of detection, i.e. $1 \times 10^3$ CFU g$^{-1}$ faeces. Moreover, we were able to detect fluorescent *B. bifidum* S17 pMGC-mCherry in faecal slurries of samples collected 5 h after inoculation (Figure 5B).
Discussion

With the present study, we aimed at extending the range of available plasmid-based molecular tools for protein expression in and functional analysis of bifidobacteria. Making use of the original purpose of pMDY23 as a promoter probe vector, a number of promoters were analysed for transcriptional activity. No GusA reporter activity could be detected for the synthetic promoters P_{CP25} and P_{help}. This is somewhat surprising since both promoters were shown to drive high levels of reporter gene expression in other Gram-positive and/or Gram-negative bacteria (45, 46, 48) and P_{help} was successfully used to generate a luminescent B. breve strain (22). P_{hyper} was successfully used for recombinant protein expression in L. monocytogenes (47, 49). Similarly, reasonable transcriptional activity was observed for P_{hyper} in B. bifidum S17, however, only at early time points during growth. The promoter showing highest levels of GusA reporter activity during the first 12 h of growth was P_{gap}. This is in line with the observation that the gap promoter is highly active in B. longum NCC2705 (41). Consequently, the gap promoter of B. longum was successfully used for recombinant protein expression (42, 43). However, the absence of reporter activity at later stages of growth, i.e. in stationary phase, suggests that its use is limited to actively growing cultures.

The pMDY23 backbone was chosen for the generation of further plasmids based on successful transformation into a total of 15 strains belonging to 7 different species with 4 subspecies (supplementary Table S2 and Refs 39, 50, 51). To our knowledge this represents the widest range of bifidobacterial hosts for any E. coli/Bifidobacterium shuttle vector described so far (2, 21, 52).

Using the P_{gap} promoter and the backbone of pMDY23, a series of plasmids were constructed, which allowed expression of four different FPs in B. bifidum S17, B. breve S27 and B. longum E18. To the best of our knowledge, this represents the first report on the successful tagging of bifidobacterial strains by four different FPs. As observed previously for L.
monocytogenes (47), the excitation and emission spectra of the tagged strains were sufficiently different to distinguish four strains tagged with different constructs by fluorescence microscopy.

Adhesion to the gastrointestinal mucosa is thought to help probiotic bacteria to colonise and persist at least transiently in the gastrointestinal tract of the host (53). Thus, adhesion to intestinal epithelial cells or mucus is one of the properties frequently tested for potential probiotic strains (6, 54–56). Until now, most studies on adhesion of bifidobacteria to cultured epithelial cells have used methods involving bacteria labelled by radioisotopes or by classical plate counting (6, 39, 57–59). The results obtained in this study demonstrate that the use of fluorescent bifidobacteria in adhesion assays is a promising application yielding results comparable to those obtained with classical assays, e.g. by plate counting or using radioactively labelled bacteria. Compared to these classical methods, the use of fluorescent bacteria to determine adhesion saves consumables since no further agar plates, materials for dilutions series or expensive and elaborate scintillation equipment are required. Moreover, the results are available immediately after the measurement saving up to 48 h required for growth of colonies.

Fluorescent bifidobacteria were also successfully detected in human primary macrophages generated by ex vivo differentiation of monocytes. This demonstrates that detection of bifidobacteria inside relevant host cell populations is possible. Using similar systems, we and others have successfully visualised fluorescent strains of C. rodentium, Salmonella typhimurium, E. coli and L. monocytogenes within host tissues and identified specific host cell populations that are associated with or have phagocytosed these labelled strains (60–64). Cronin and colleagues have created an mCherry-expressing B. breve strain and were able to detect this strain in subcutaneous tumours in athymic mice by fluorescence microscopy following intravenous application (24). The same strain was tagged using a luciferase and was
imaged in the colons and tumours of live mice or in dissected GITs of colonised animals following oral or intravenous inoculation (22–24). Luminescence of this strain correlated to actual bacterial numbers as determined by plate counting (23, 24). Similarly, fluorescence has been used to image bacteria and protozoan parasites in vivo (65–68). Thus, in theory imaging of fluorescent bifidobacteria in live animals or dissected organs should be possible. However, bioluminescent reporters are by far more frequently used for in vivo imaging of bacteria due to limitations in tissue penetration of fluorescence (69).

Using the chloramphenicol resistance marker of pMGC-mCherry, we determined the gastrointestinal transit time of *B. bifidum* S17 pMGC-mCherry in mice after inoculation by a single oral dose. While these results suggest that this strain is not able to colonise mice stably in the presence of a normal microbiota, they demonstrate the general applicability of the plasmid to monitor gastrointestinal transit and colonisation of bifidobacteria. Moreover, the detection of *B. bifidum* S17 pMGC-mCherry in faecal slurries following oral administration is a third example for potential applications of fluorescent bifidobacteria. One important information that can be gathered from these experiments is that at least a portion of the administered bifidobacteria are able to transit through the GIT and are shed with faeces in a viable state.

Collectively, these results suggest that tagging bifidobacteria by expression of FPs are a valuable tool to study the interaction of potential probiotic strains with the host. Moreover, the successful expression of FPs also demonstrates the feasibility of the expression system consisting of the pMDY23 backbone and the P_{gap} promoter. Using homologous expression in bifidobacteria will help to further understand the role of single genes or proteins in host colonisation and the beneficial effects of bifidobacteria. Likewise, bifidobacteria can be further optimised by heterologous expression of factors known to confer abilities such as host colonisation or adhesion to host structures to other bacteria. Since the pMDY23 backbone
was successfully transformed in at least 7 different *Bifidobacterium sp.*, these tools will be available for a wide range of bifidobacterial strains.

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properties of recombinant bifidobacteria expressing the *Bifidobacterium bifidum*-specific lipoprotein BopA. Microb. Cell Factories **11**:80.


Legends to Figures

Figure 1: (A) Schematic representation of the PCR products of P<sub>hyper</sub>, P<sub>cp25</sub>, P<sub>help</sub>, P<sub>ps</sub>, P<sub>gap</sub> (grey bars) cloned into the unique XhoI and BglII restriction sites of pMDY23. Relevant features of the plasmids are indicated as follows: pMB1 replicon for replication in E. coli by a black bar; repAB for replication in bifidobacteria, the spectinomycin resistance gene (spc) and
the gusA reporter gene by grey arrows. (B) Analysis of the transcriptional activity of the cloned promoters in B. bifidum S17 at the indicated time points during growth in MRSc by measuring glucuronidase activities in crude extracts. Values are mean ± standard deviation of 3 technical replicates per time point and data from one representative of three independent cultures per strain are shown.

Figure 2: (A) Schematic representation of the genes encoding for different fluorescent proteins cloned into pMDY23-\(P_{\text{gap}}\). Relevant features of the plasmids are indicated as follows: pMB1 replicon for replication in E. coli by a black bar; repAB for replication in bifidobacteria and the spectinomycin resistance gene (\(\text{spc}\)) by grey arrows; \(P_{\text{gap}}\) promoter by a black arrow; genes encoding for fluorescent proteins GFP, CFP, YFP and mCherry by green, blue, yellow or red arrows, respectively. (B) Fluorescence microscopy of B. bifidum S17 transformed with pVG-CFP, pVG-GFP, pVG-YFP, or pVG-mCherry. (C) Fluorescence microscopy of a mix of the four strains shown in (B). Images were acquired using a Zeiss Axio Observer.Z1 microscope and a 100x oil immersion objective. The scale bars indicate a size of 10 µm.

Figure 3: (A) Fluorescence microscopy of adherent B. bifidum S17 pVG-mCherry on a confluent monolayer of Caco-2 cells. Nuclei of Caco-2 cells were stained with Hoechst (blue). Images were acquired using a Zeiss Axio Observer.Z1 microscope and a 40x objective. The scale bar indicates a size of 20 µm. (B) Relative fluorescence units (RFU) of B. bifidum S17 pVG-mCherry on a confluent monolayer of Caco-2 cells plotted against colony forming units (CFU) per well. The equation of the calculated linear regression as well as the Pearson’s coefficient of correlation (\(R^2\)) are indicated. (C) CFU per well of adherent B. bifidum S17 pVG-mCherry as quantified directly using classical plate counting (plated) and calculated from measured RFU using the equation of the linear regression shown in (B).
Values in (B) and (C) are mean ± standard deviation of three independent cultures with both CFU and RFU determined in technical duplicates.

Figure 4: (A) Fluorescence microscopy of *B. bifidum* S17 pVG-mCherry inside primary human macrophages. Nuclei of macrophages were stained with Hoechst (blue) and the actin cytoskeleton was stained with Phalloidin-Alexa488 (green). Images were acquired using a Zeiss Axio Observer.Z1 microscope and a 40x objective. The scale bar indicates a size of 20 µm.

Figure 5: (A) Faecal shedding of *B. bifidum* S17 pMGC-mCherry during the first 24 h after inoculation of C57BL/6J mice (*n* = 6) with a single oral dose of 2×10⁹ CFU per animal. (B) Detection of *B. bifidum* S17 pMGC-mCherry by fluorescence microscopy of faecal slurries prepared from a fresh faecal pellet sampled 5 h post inoculation. Images were acquired using a Zeiss Axio Observer.Z1 microscope and a 100x oil immersion objective. The scale bars indicate a size of 10 µm.