A novel tool for stable genomic reporter gene integration to analyze heterogeneity in *Photorhabdus luminescens* at the single-cell level

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Determination of reporter gene activity at the single-cell level is a prerequisite for analyzing heterogeneous gene expression in bacteria. The insect pathogenic enteric bacterium *Photorhabdus luminescens* is an excellent organism in which to study heterogeneity since it exists in two phenotypically different forms, called the primary and secondary variant. A tool for generating stable genomic integrations of reporter genes has been lacking for these bacteria, and this has hampered the acquisition of reliable data sets for promoter activities at the single-cell level. We therefore generated a plasmid tool named pPINT-mCherry for the easy and stable introduction of gene fragments upstream of an *mCherry* reporter gene followed by stable integration of the plasmid into the *P. luminescens* genome at the *rpmE/glmS* intergenic region. We demonstrate that the genomic integration of reporter genes for single-cell analysis is necessary in *P. luminescens* since plasmid-borne reporter genes mimic heterogeneity and are therefore not applicable in these bacteria, in contrast to their use in single-cell analysis in other bacteria like *Escherichia coli*.

It is well recognized that genetically identical bacteria often show a high degree of variability in homogenous environments, an observation that is referred to as phenotypic heterogeneity (1). *Photorhabdus luminescens* is an enteric bacterium that lives in mutualistic association with soil nematodes and is highly pathogenic for insects (2). The bacteria are of high biotechnological and pharmaceutical interest since they are already used as bio-insecticides in association with their nematode host (3), and they are also potentially a rich reservoir of as yet unknown natural compounds (4). *P. luminescens* exists in two pheno-

typically different forms, designated as the primary and secondary variant. Single primary cells undergo phenotypic switching and convert to secondary cells after prolonged cultivation. Primary cells exhibit phenotypic characteristics, such as the production of several extracellular enzymes, pigments, antibiotics, bioluminescence, and crystalline inclusion proteins, that are absent from the secondary variant (5,6). While both variants are equally pathogenic to insects, secondary cells are unable to support nematode growth and development (5). For these reasons, *P. luminescens* is an excellent model organism for

the study of molecular mechanisms of bacterial phenotypic heterogeneity. Here we describe a novel tool for the stable integration of promoter-reporter genes into the *P. luminescens* genome that allows for analysis of heterogeneous gene expression at the single-cell level. Our results indicate that plasmid-borne tools are not suitable for analysis of heterogeneous gene expression in these bacteria.

Materials and methods

Materials

Strains used in this study are listed in Supplementary Table S1, plasmids are

METHOD SUMMARY

The plasmid pPINT-mCherry is an easy-to-use tool for the stable integration of reporter genes at the *rpmE/glmS* site into the *Photorhabdus luminescens* genome. A specific promoter region can be cloned into the multiple cloning site (MCS) upstream of the *mCherry* gene and, after conjugation of the plasmid into *P. luminescens*, pPINT-mCherry integrates via homologous recombination at the specific single site into the genome. Correct clones can be positively selected due to the conferred antibiotic resistance. If required, the plasmid backbone and therefore the antibiotic resistance can be removed from the chromosome by the use of *sacB* negative selection.

Figure 1. The mobilizable and integrative vector pPINT-mCherry. (A) Vector map. The plasmid carries kanamycin (*npt1*) and gentamicin (*aacC1*) resistance cassettes. pPINT-mCherry integrates into the *P. luminescens* genome at the *rpmE/glmS* site via the two 600-bp long homologous flanking regions. Transcriptional as well as translational reporter genes can be generated by inserting the respective DNA into the multiple cloning site (MCS) upstream of *mCherry*. pPINT-mCherry carries the R6K origin so that it only replicates in strains carrying the λ *pir* gene. The vector is mobilizable due to the presence of the origin of transfer *oriT*. The negative selection marker *sacB* allows vector backbone removal after insertion into the chromosome. (B) Homologous sites in pPINT-mCherry used for recombination into the intergenic region of the *rpmE* and *glmS* genes in *P. luminescens*.

listed in Supplementary Table S2, and primers are listed in Supplementary Table S3. PCR was performed using the Q5 Polymerase or OneTaq Polymerase from New England Biolabs (Frankfurt, Germany). Restriction enzymes and T4 DNA ligase were also purchased from New England Biolabs. Plasmid isolations were performed with the HiYield Plasmid Mini Kit and the HiYield PCR DNA Fragment Extraction Kit was used for purification of PCR products

(Süd-Laborbedarf, Gauting, Germany). Genomic DNA was prepared using the Ultra-Clean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA).

Bacterial strains and growth conditions

P. luminescens and *E. coli* were aerobically grown in LB medium [10% (w/v) peptone, 5% (w/v) yeast extract, 10% (w/v) NaCl] at 30°C (*P. luminescens*) and 37°C (*E. coli*). For preparation of agar

plates, 1.5% (w/v) agar was added to the medium. If necessary, media were supplemented with kanamycin (50 µg/mL), ampicillin (100 µg/mL), gentamicin (15 µg/mL), and/or rifampicin (50 µg/mL). To allow the growth of the donor strain *E. coli* ST18, the medium was supplemented with 5-aminolevulinic acid (50 µg/mL) (7). Pre-cultures were grown overnight, and the following day, appropriate volumes of the pre-cultures were used for inoculation of the main cultures

Figure 2. Generation and single-cell microscopy imaging of *P. luminescens* P_{luxC}-*mCherry*, *P. luminescens* P_{antA}-*mCherry*, and *P. luminescens* P_{hexA}-*mCherry*. (A) Generation of the reporter strains. The first recombination step to integrate the reporter genes into the *P. luminescens* genome was performed by conjugation via the *E. coli* ST18 donor carrying the respective pPINT-*mCherry* plasmids. The correct insertions of the respective pPINT-*mCherry* derivatives into the *P. luminescens* chromosome were verified by PCR with one primer annealing in the reporter gene region of the plasmid (primer forward) and one primer annealing downstream of the integrated vector in the genome (primer reverse), using genomic DNA as template. When the integration occurred via the *rpmE* site, the correct insertion of the respective pPINT-*mCherry* derivative resulted in the amplification of a 1460-bp DNA fragment. C: Control using genomic DNA of wild-type *P. luminescens*. (B) Single-cell microscopy imaging of plasmid-borne and chromosomally integrated promoter-*mCherry* fusions in *P. luminescens*. *P. luminescens* P_{luxC}-*mCherry*, *P. luminescens* P_{antA}-*mCherry*, *P. luminescens* P_{hexA}-*mCherry*, and *P. luminescens* P_{less}-*mCherry* (control) harboring the respective reporter genes in the chromosome using homologous recombination via pPINT-*mCherry*, as well as *P. luminescens* pBR-Km-P_{luxC}-*mCherry*, *P. luminescens* pBR-Km-P_{antA}-*mCherry*, *P. luminescens* pBR-Km-P_{hexA}-*mCherry*, and *P. luminescens* pBR-Km-P_{less}-*mCherry* (control) were cultivated for 24 h in the presence of the appropriate antibiotics. The cells were then analyzed for promoter activities at the single-cell level by fluorescence microscopy. The exposure time is given in ms and was adjusted for each promoter depending on its strength. On the right panel the corresponding boxplots are shown. The fluorescence intensities of 500 single cells were individually measured for each experiment, resulting in 1500 cells of the respective triplicates being analyzed using the software Big Cell Brother (11). The noise values were calculated as SD/mean from 1500 cells. Representative images from one of three independently performed experiments are shown.

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to an OD_{600} of 0.05. For measurement of the fluorescence intensities of the P_{lac} -*eyfp* fusions, 0.5 mM IPTG was added to activate the *lac* promoter.

Plasmid generation

For the generation of plasmid pPINT, the *rpmE* and *glmS* homologous regions of the *P. luminescens* genome were amplified via PCR. The *rpmE* site containing the genes *rpmE* and *rpmJ* was amplified using primers FA_rpmE-SpeI_fwd and FA_rpmE-mcs_rev with genomic DNA of *P. luminescens* TT01 as template. The PCR product of the *glmS* site, containing a part of the *glmS* gene, was amplified using primers FB_glmS-mcs_fwd and FB_glmS-SalI_rev with genomic DNA of *P. luminescens* as template. Subsequently, an overlap PCR of the *rpmE* and the *glmS* site as templates was performed using primers FA_rpmE-SpeI_fwd and FB_glmS-SalI_rev. The overlap PCR product was inserted into the plasmid pNPTS-138-R6KT (8) via the restriction enzymes *SpeI* and *SalI*, resulting in the plasmid pPINT. A fragment containing a multiple cloning site, *mCherry* [template: pBR-mCherry (9)], and a gentamicin resistance cassette (template: pUC18R6KT-mini-Tn7T-Gm) was generated via PCR using the primer pairs cherry-mcs-pNPTS_fwd/cherry-mcs-pNPTS_rev and gmR-pNPTS_fwd/gmR-pNPTS_rev, respectively, followed by an overlap PCR. The overlap PCR fragment was inserted via *EcoRI* and *EagI* sites into the plasmid pPINT, resulting in the plasmid pPINT-mCherry.

For generation of plasmid pBR-Km-mCherry the kanamycin cassette [template: pNPTS-138-R6KT] was amplified via PCR using the primers Km-PstI_fwd and Km-EcoRI_rev. The resulting fragment was cut with the restriction enzymes *PstI* and *EcoRI* and ligated with the equally treated plasmid pBR-Cherry. In order to create the plasmid pBR/Km- P_{lac} -*eyfp*/ P_{hexA} -*mCherry*, P_{lac} -*eyfp* was amplified using primers Plac-eyfp-DraI_fwd and eyfp-DraI_rev with plasmid pEYFP (Takara-Clontech, Saint-Germain-en-Laye, France) as template. The PCR product was inserted into the plasmid pBR-Cherry-Km- P_{hexA} via the *DraI* restriction site. Since *P. luminescens* has no *LacI* repressor, the promoter

of the *lacZYA* operon could be used as a constitutively activated promoter without adding inducer. The promoters P_{antA} , P_{hexA} , and P_{luxC} were amplified by PCR with the primers PantA-BamHI_fwd/PantA-XmaI_rev, PhexA-BamHI_fwd/PhexA-XmaI_rev, and PluxC-BamHI_fwd/PluxC-XmaI_rev using *P. luminescens* genomic DNA as template. The PCR products were cloned into plasmid pPINT-mCherry or pBR-Km-mCherry using the restriction sites *BamHI* and *XmaI*. Restriction analysis as well as DNA sequencing (Genomics core facility, LMU Biozentrum) verified the accuracy of all generated plasmids.

Competent cells and transformations

E. coli cells were made chemically competent and transformed as described elsewhere (10). *P. luminescens* was made electrocompetent and transformed by electroporation. *P. luminescens* cells were cultivated in LB medium at 30°C up to an OD_{600} of 0.8–1.2. The cells were harvested by centrifugation at 18°C, and the cell pellet was resuspended in the same volume of 10% (v/v) glycerol and centrifuged again. Cells were then washed with 1/2 of the starting volume in 10% (v/v) glycerol and, after centrifugation, were washed again with 1/10 of the starting volume. After another centrifugation step, the cells were resuspended with 1/250 of the starting volume in 10% (v/v) glycerol. Sixty microliters of cell suspension was mixed with 100–200 ng plasmid DNA and transferred into 0.2 cm electroporation cuvettes. Electroporation was performed with a pulse of 2500 V for 4–6 ms. Cells were then mixed with 1 mL LB medium and incubated aerobically at 30°C for 1 hour. The cells were centrifuged and resuspended in 100 μ L LB. The samples were then spread on appropriate agar plates and incubated at 30°C for 2 days.

Integration of reporter genes into the *P. luminescens* genome

Integration of promoter-*mCherry* reporter genes into *P. luminescens* was performed via homologous recombination. The conjugative plasmid transfer from donor strain *E. coli* ST18 containing the required plasmid into *P. luminescens* TT01 was performed by the filter mating method (7). First, the donor and

Figure 3. Influence of pPINT-mCherry plasmid backbone on reporter gene fluorescence intensity and heterogeneity. (A) Removal of the vector backbone in strain *P. luminescens* P_{anta}-mCherry. The pPINT-mCherry vector backbone was removed by a second homologous recombination and selection of Kan^S, Gm^R, and Suc^R clones. The resulting strain was named *P. luminescens* P_{luxC}-mCherry-Kan^S. The removal of the backbone was checked via PCR using one primer binding upstream (primer forward) and one primer binding downstream (primer reverse) of the *rpmE*/*glmS* site. The PCR product for the first recombination of pPINT-P_{anta}-mCherry resulted in a band with a size of 8863 bp; for the second homologous recombination (loss of plasmid backbone), the DNA fragment was 3310 bp. No insertion at the *rpmE*/*glmS* site resulted in amplification of a DNA fragment with 1358 bp. Genomic DNA was used as template: Lane 1, *P. luminescens* P_{luxC}-mCherry/Kan^R; Lane 2, *P. luminescens* P_{luxC}-mCherry/Kan^S; and Lane 3, *P. luminescens* wild-type (control). (B) Single-cell microscopy imaging of *P. luminescens* P_{anta}-mCherry/Kan^R and *P. luminescens* P_{anta}-mCherry/Kan^S. *P. luminescens* P_{anta}-mCherry/Kan^R (remaining pPINT-mCherry plasmid backbone) and *P. luminescens* P_{anta}-mCherry/Kan^S (pPINT-mCherry plasmid backbone removed) were cultivated and analyzed for reporter gene activity by fluorescence microscopy. On the right panel, the corresponding boxplots are shown. The fluorescence intensities of 500 single cells were individually measured for each experiment, resulting in 1500 cells from the respective triplicates being analyzed using the software Big Cell Brother (11). The noise values were calculated as SD/mean from 1500 cells. Figures each represent one characteristic result out of three independently performed experiments.

the recipient strains were cultivated in LB medium, supplemented with the respective additives if required, up to an OD₆₀₀ of 0.8–1.0. The donor strain was washed 3 times in LB medium and mixed with the recipient strain in a ratio of 1:5 in a final volume 1/10 of the donor's initial culture volume. The two strains were pooled and dropped onto a nitrocellulose filter located on a LB agar plate. After incubation overnight at 30°C, the cells were resuspended in 500 µL LB, spread onto LB agar plates containing the required antibiotics, and incubated for 2 days at 30°C. Single colonies were checked for chromo-

somal integration of the plasmid via PCR (check-*rpmE*_fwd, oriT_fwd, gmR-pNPTS_fwd, check-mcherry-ins_rev, check-*glmS*_rev) using the genomic DNA of the clones as template, and subsequent DNA sequencing of the DNA fragments was performed. For removal of the vector backbone in strain *P. luminescens* P_{anta}-mCherry, the respective strain was grown for 24 h in LB medium containing gentamicin in the absence of kanamycin, and then streaked on LB agar containing 10% (w/v) sucrose and gentamicin. Clones were checked for kanamycin sensitivity by streaking on LB agar containing

kanamycin. The overall efficiency of vector backbone loss was determined to be >95%, when 50–100 individual clones were checked. The resulting strain was named *P. luminescens* P_{anta}-mCherry/Kan^S. Genomic DNA of Kan^S, Suc^R, and Gent^R clones was isolated and correct integration was verified by PCR using primers check-*rpmE*_fwd and check-*glmS*_rev annealing outside the homologous regions, and subsequent DNA sequencing was performed.

Promoter activity analysis

Promoter activities of *P. luminescens* reporter strains were investigated via

mCherry and eYFP fusions with a fluorescence microscope (Leica, Bensheim, Germany). An excitation wavelength of 546 nm and a 605 nm suppression filter with 75 nm bandwidth was used to measure mCherry. For measuring eYFP, an excitation wavelength of 460 nm and a 512 nm suppression filter with 75 nm bandwidth was used. The main cultures were inoculated at an OD₆₀₀ of 0.05 with the appropriate volume of the respective pre-cultures and were grown at 30°C for 24 h in the presence of appropriate antibiotics. Then, 7 µL of the cultures were dropped on an agarose pad [0.5% (w/v) agarose in PBS buffer, pH 7.4] located on a microscope slide and covered with a coverslip. Three independent biological experiments were performed. For each experiment, the fluorescence intensities of 500 cells were measured using the single-cell analysis tool Big Cell Brother (11).

Results and discussion

To analyze heterogeneity of gene expression at the single-cell level, a molecular tool is necessary for the stable and unique integration of reporter genes into the chromosome, but this has not yet been available for *P. luminescens*. In many Gram-negative bacteria, genomic integrations can in principle be achieved using the mini transposon Tn7, which has a unique attachment site located in the *rpmE/glmS* intergenic region (12). Although this attachment site is also present within the *P. luminescens* genome, located between the genes *rpmE* (*plu0036*) and *glmS* (*plu0037*), stable mini-Tn7 integration can be challenging. The *P. luminescens* genome appears to have multiple specific or nonspecific Tn7-attachment sites, since integration of mini-Tn7 derivatives often occurs elsewhere beyond the *rpmE/glmS* intergenic region in this organism (unpublished observations). To surmount this problem, we generated the mobilizable plasmid pPINT-mCherry (Figure 1A), which can specifically integrate via homologous recombination at the *rpmE/glmS* site of *P. luminescens* (Figure 1B). Two other known *Photorhabdus* species, *P. temperata* and *P. asymbiotica*, also contain an *rpmE/glmS* intergenic region sharing 87% and 80% identity

with the *P. luminescens rpmE/glmS* site, respectively. Since the *glmS* and *rpmE* sequences in pPINT-mCherry can be replaced using the *SpeI/PstI* and *EagI/SalI* restriction sites, respectively, pPINT-mCherry could easily be modified for functionality in other species than *P. luminescens*. The plasmid pPINT-mCherry carries the *mCherry* gene with an upstream multiple cloning site (MCS) to allow the easy insertion of promoters and/or gene fusions. pPINT-mCherry derives from the plasmid pNPTS138-R6KT (8), which contains the R6K origin of replication and thus does not replicate in *P. luminescens*. Since pPINT-mCherry is mobilizable, it can be transferred into *P. luminescens* via conjugation using the *E. coli* strain ST18 as the donor (7) and then easily be out-selected due to 5-aminolevulinic acid auxotrophy. We determined the pPINT-mCherry conjugation/recombination efficiency as $1.15 \pm 0.14 \times 10^{-6}$. Furthermore, pPINT-mCherry contains

the *sacB* gene encoding the levansucrase of *Bacillus subtilis*, allowing removal of the vector backbone from the chromosome by growing the cells in the presence of sucrose (13). For positive selection, we also inserted a gentamicin resistance cassette between the recombination sites to facilitate screening after excision of the vector backbone.

We used pPINT-mCherry for the stable integration of different promoter-reporter genes into the *P. luminescens* genome. We chose promoters from three different *P. luminescens* genes: (i) the bioluminescence *luxCDABE* operon, since bioluminescence is a characteristic feature of *P. luminescens*; (ii) the regulatory gene *hexA* as an example of a gene encoding a transcriptional regulator; and (iii) the anthraquinone synthesis operon *antABCDEF GHI*, a gene cluster responsible for secondary metabolite synthesis. As the first step, we generated *P_{luxC}-mCherry*, *P_{hexA}-mCherry*, and *P_{antA}-mCherry* fusions

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Figure 4. Single-cell microscopy imaging of *P. luminescens* and *E. coli* reporter strains carrying two different reporter genes encoded on one plasmid. *P. luminescens* TT01 and *E. coli* DH5 α λ pir were transformed with plasmid pBR/Km- P_{lac} -*eyfp*/ P_{hexA} -*mCherry*, cultivated for 24 h and analyzed for promoter activities at the single-cell level by fluorescence microscopy (red and yellow channels). The promoter of *hexA* was fused to *mCherry*, and the promoter of *lacZYA* was fused to *eyfp*. The arrows highlight cells that are not fluorescent at all. Figures each represent one characteristic result out of three independently performed experiments.

by cloning at least 350 bp upstream of the start codon of the respective genes into the pPINT-*mCherry* plasmid and then integrated these three reporters into the genome of *P. luminescens* primary cells via conjugation followed by homologous recombination. The proper insertion of the reporter genes at the *rpmE/glmS* intergenic region could be verified in all of the clones tested (Figure 2A). Subsequently, we compared the fluorescence intensities of the *mCherry* reporter strains derived either from the plasmid pBR-*mCherry* (9), which is a low-copy plasmid due to presence of the *rop* gene in combination with the pMB1 origin of replication (14), or the chromosomal integrations (Figure 2B, left panel). Although all experiments were performed in the presence of the appropriate antibiotic to avoid loss of the plasmid, all three strains harboring the plasmid-borne reporter genes showed heterogeneous distribution of promoter activity. In contrast, the chromosomal P_{luxC} -*mCherry* and P_{hexA} -*mCherry* reporters both showed a homogeneous distribution at the single-cell level. Only the P_{antA} -*mCherry* reporter strain displayed a heterogeneous distribution of reporter gene activity. The promoterless negative control (i.e., no promoter present upstream of *mCherry*) showed no fluorescence, as expected, either

when plasmid-borne or when chromosomally integrated.

We calculated the noise values of all the reporter strains in order to determine the degree of heterogeneity (Figure 2B, right panel). The maximum noise level in an apparently homogeneous population was set to 0.38, as determined for the P_{luxC} -*mCherry* chromosomal reporter. The chromosomal promoter activity of P_{hexA} with a noise value of 0.25 also displayed a homogeneous distribution of reporter gene activity. For P_{antA} , a higher noise value of 0.56 was calculated, confirming the heterogeneous distribution of P_{antA} activity. In contrast, the fluorescence noise values of all 3 plasmid-borne reporter genes were calculated to be 3- to 5-fold higher compared with that of the chromosomal integrations, which confirmed the high degree of heterogeneity at the single-cell level for all of the plasmid-encoded reporter genes used in this study.

To test if the remaining pPINT-*mCherry* vector backbone had an influence on the promoter activity, we compared the fluorescence intensities and noise values of the P_{antA} -*mCherry* reporter strain with a similar reporter strain, *P. luminescens* P_{antA} -*mCherry*/Kan^S, in which the plasmid backbone has been removed (see Supplementary Material for detail). Correct loss of the

vector backbone was checked by PCR (Figure 3A). Fluorescence intensities and noise levels were comparable for both reporter strains, revealing that the pPINT-*mCherry* plasmid backbone does not interfere with the respective promoter activity and only needs to be removed if the reporter strain must be kanamycin sensitive (Figure 3B).

To analyze whether the heterogeneous distribution of plasmid-borne promoter activities in *P. luminescens* was caused by unequal plasmid distribution, we analyzed the fluorescence of *E. coli* and *P. luminescens* harboring a reporter plasmid encoding both P_{hexA} -*mCherry* and P_{lac} -*eyfp* fusions. The *E. coli* reporter strain showed a rather homogeneous distribution of the P_{hexA} and P_{lac} activities at the single-cell level. In contrast, the *P. luminescens* reporter strain showed a heterogeneous distribution of both promoter activities. However, the two fluorophores were always present within the same cells, or the cells were completely dark (Figure 4). This suggests that the presence or absence of the fluorescent proteins in principle does not at all mark the active state of the corresponding promoter but is probably due to a non-homogeneous distribution of the plasmid among the population.

Recently, another method to perform chromosomal insertions into

the *P. luminescens* genome has been developed based on a *P. luminescens* endogenous recombinase system that is homologous to lambda-red (15), which is a useful tool for integrating promoter insertions or gene knockouts into the genome using linear DNA fragments. However, since *P. luminescens* does not grow at 37°C, it is challenging to remove the temperature-sensitive plasmid that carries the recombinase genes after the recombination event.

In summary, we have created an efficient tool for integrating (reporter) genes at a unique site in the *P. luminescens* genome. We showed that plasmids cannot be used to investigate promoter activities at the single-cell level, and thus, the stable integration of promoter-reporter genes into the genome is necessary for studying heterogeneity in *P. luminescens*. Here we used the low-copy, pBR322-derived plasmid pBR-Cherry for single-cell analysis. However, we cannot exclude the possibility that other plasmids known to be highly stable in a broad host range, such as pBBR1 derivatives (16), might also be useful tools in *Photobacterium* if high fluorescence readouts are essential when analyzing activities of weak promoters. In principle, plasmid-based reporter systems are preferred as they are timesaving compared with chromosomal integrations. Furthermore, due to their higher copy number, plasmid-encoded reporter systems often result in higher signals compared with chromosomally encoded reporter genes. However, we have shown that plasmids seem to be unequally distributed in a *P. luminescens* population, which has also been suggested previously (17). Therefore, plasmid-encoded promoter-reporter genes are sufficient in these bacteria only when analyzing reporter gene activities at the population level. The *rpmE/glmS* intergenic region that we used as the integration site is also present in the genomes of the other two known *Photobacterium* species, *P. temperata* and *P. asymbiotica*, suggesting that the pPINT-mCherry tool might be of use for performing chromosomal integrations in these organisms as well.

Author contributions

A.G. performed the experiments. R.H. coordinated the experiments. A.G. and R.H. wrote the manuscript.

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Competing interests

The authors declare no competing interests.

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