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A Convenient and Robust *In Vivo* Reporter System To Monitor Gene Expression in the Human Pathogen *Helicobacter pylori*

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Thirty years of intensive research have significantly contributed to our understanding of *Helicobacter pylori* biology and pathogenesis. However, the lack of convenient genetic tools, in particular the limited effectiveness of available reporter systems, has notably limited the toolbox for fundamental and applied studies. Here, we report the construction of a bioluminescent *H. pylori* reporter system based on the *Photorhabdus luminescens luxCDABE* cassette. The system is constituted of a promoterless *lux* acceptor strain in which promoters and sequences of interest can be conveniently introduced by double homologous recombination of a suicide transformation vector. We validate the robustness of this new *lux* reporter system in noninvasive *in vivo* monitoring of dynamic transcriptional responses of inducible as well as repressible promoters and demonstrate its suitability for the implementation of genetic screens in *H. pylori*.

Helicobacter pylori is a widespread gastric pathogen that infects about half of the human population worldwide. Untreated infections can persist for decades, promoting the insurgence of severe stomach diseases, ranging from chronic gastritis to peptic ulcers and gastric carcinoma in about 1% of infected hosts (11, 15). Despite its importance as a human pathogen and the enduring interest of the scientific community in its fundamental biology, the study of *H. pylori* gene expression has been somewhat hindered by the lack of suitable genetic tools (2), in particular by the limited effectiveness of available reporter systems (4).

An ultimate reporter gene ideally would be expressed without perturbing the physiology of the recipient organism, and it would be readily detectable and quantifiable using standard laboratory instrumentation without the need to disrupt the living cell. In addition, the reporter should be highly sensitive, with low background noise, in order to permit analyte detection at low molar concentrations and, at the same time, to prove rapid enough to enable monitoring of quick response kinetics (14, 30). Finally, the signal should not perdure or stably accumulate in the cell, as this may lead to significant biases in the estimation of gene expression over time.

Of the many reporter systems tested in Gram-negative bacteria, the bioluminescent systems based on paralogues of the bacterial *luxCDABE* luciferase operons appear to best fulfill these criteria (14, 21). The luciferase activity is provided by two enzyme subunits, LuxA and LuxB, encoded by the *luxAB* cistrons, which together catalyze the oxidation of a reduced riboflavin phosphate and a long-chain fatty aldehyde, coupling the reaction with bioluminescence, e.g., emission of light in the visible range (~490 nm) (23). An enzymatic reductase complex, encoded by paralogues of the *luxCDE* cistrons, is responsible for shunting fatty acid metabolites from the central metabolism to convert them into the aldehyde substrate used by the LuxAB complex to catalyze the bioluminescence reaction *in vivo*.

In *H. pylori*, previous comparisons of reporter fusions to a *cat* cassette (providing chloramphenicol resistance), *GFP* (encoding green fluorescent protein), and a *Vibrio harveyi luxAB* operon proved the superiority of the luciferase-based system in faithfully reflecting the dynamic changes detected at the mRNA level at different time points throughout growth (24). In the latter system, fusion of the sole *luxAB* operon as a reporter imposed the external supply of the aldehyde substrate in order to catalyze the emission of bioluminescence, limiting to some extent its usefulness *in vivo* and leaving open the question of whether fusions with the whole *luxCDABE* operon would be functional in *H. pylori* as well. In addition, the *V. harveyi* luciferase has a temperature optimum below 37°C (22), which may give rise to inconsistent measures at continuous culture at 37°C or prove restrictive if expression has to be analyzed after heat shock or under conditions of metabolic stresses.

Unlike those of the marine *V. harveyi* enzyme, the *luxCDABE* gene products of the soil bacterium *Photorhabdus luminescens* retain mesophilic luciferase activity, operating at temperatures as elevated as 45°C (23). Accordingly, the *P. luminescens luxCDABE* operon has been successfully employed as a reporter system in *Campylobacter jejuni*, both to image the bacterium (18, 19) and to quantify sigma 28 promoter activity (1, 12). We therefore set out to investigate whether reporter fusions with a *luxCDABE* operon could constitute a valuable tool for the study of gene expression in *H. pylori*.

Here, we report the engineering of such a bioluminescent reporter system based on the *P. luminescens luxCDABE* cassette, and we demonstrate its usefulness in noninvasive *in vivo* monitoring of dynamic transcriptional responses, as well as its suitability for the implementation of genetic screens in *H. pylori*.

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TABLE 1 *H. pylori* strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
H. pylori		
G27	Clinical isolate; wild-type parental strain	35
G27*lux*	*vacA*::*aphA-3-luxCDABE*; G27 derivative carrying the *Campylobacter coli aphA-3* cassette and the promoterless *Photorhabdus luminescens luxCDABE* operon in the *vacA* locus; Km^r	This study
G27*lux* P_{cagP}	*vacA*::*cat*-P_{cagP} *luxCDABE*; G27*lux* derivative encompassing a 383-bp fragment of the P_{cagP} promoter cloned upstream of the *lux* operon; Cm^r	This study
G27*lux* P_{cagU}	*vacA*::*cat*-P_{cagU} *luxCDABE*; G27*lux* derivative encompassing a 360-bp fragment of the P_{cagU} promoter cloned upstream of the *lux* operon; Cm^r	This study
G27*lux* P_{pfr}	*vacA*::*cat*-P_{pfr} *luxCDABE*; G27*lux* derivative encompassing a 180-bp fragment of the P_{pfr} promoter cloned upstream of the *lux* operon; Cm^r	This study
G27*lux* $oppP_{pfr}$	*vacA*::*cat*-$oppP_{pfr}$ *luxCDABE*; G27*lux* derivative encompassing a P_{pfr} promoter cloned upstream of the *lux* operon but in divergent orientation; Cm^r	This study
G27*lux fur* P_{pfr}	*vacA*::*cat*-P_{pfr} *luxCDABE fur*::*aphA-3*; G27*lux* P_{pfr} derivative carrying a deletion of the *fur* gene; Km^r Cm^r	This study
G27*lux* P_{fecA3}	*vacA*::*cat*-P_{fecA3} *luxCDABE*; G27*lux* derivative encompassing a 182-bp fragment of the P_{fecA3} promoter and 5′UTR cloned upstream of the *lux* operon; Cm^r	This study
G27*lux* $oppP_{fecA3}$	Same as G27*lux* P_{fecA3} except for P_{fecA3} promoter and 5′UTR cloned in divergent orientation with respect to the *lux* operon; Cm^r	This study
G27*lux* P_{fecA3} SD_{lux}	*vacA*::*cat*-$P_{fecA3}SD_{lux}$ *luxCDABE*; G27*lux* derivative encompassing a 109-bp fragment of the P_{fecA3} promoter with a shortened 5′UTR devoid of the *fecA3* RBS, cloned upstream of the *lux* operon; Cm^r	This study
G27 *lux* P_{fecA3} SD_{lux}-ΔOPI_{NikR}	*vacA*::*cat*-$P_{fecA3}SD_{lux}$-ΔOPI_{NikR} *luxCDABE*; G27*lux* derivative encompassing a 66-bp fragment of the P_{fecA3} promoter missing the *fecA3* 5′UTR and the NikR OPI operator, cloned upstream of the *lux* operon; Cm^r	This study
Plasmids		
pGEM-T	TA cloning vector; Ap^r	Promega
pSB1075	Plasmid vector containing the 5.8-kb *Photorhabdus luminescens luxCDABE* operon cassette; Ap^r	34
pP_{hpn2}	pGEM-T derivative carrying a 280-bp BamHI-PstI fragment encompassing the intergenic region upstream of *hpn2* (HP1432), amplified with oligonucleotides 1431DxD (gttttggatccGCTACTACCATAAGAAAGGCTT) and 1431DxS (gttttctgcagTTCATGGTGTGCCATGATGACT); Ap^r	This study
pBS::*cat*	pBluescript KS II (Stratagene) derivative carrying a HincII *Campylobacter coli cat* cassette from pDT2548 (32) cloned into the SmaI site of the vector; Ap^r Cm^r	This study
pVC	pVAC derivative lacking the 779-bp 3′-end region of homology to *vacA*, altered with a 1,045-bp BamHI-HindIII fragment of pSB1075 encompassing the 5′ end of the *luxCDABE* cassette, including the RBS, the translation start codon, and the first 1,013 bp of the *luxC* cistron; Ap^r	This study
pVCC	pVC derivative with the *C. coli cat* cassette deriving from pBS::*cat* cloned as a Bglll-BamHI fragment in the unique BamHI site of pVC; Ap^r, Cm^r; GenBank accession number HQ207194	This study
pVCC::P_{cagP}	pVCC derivative carrying 405-bp amplicon, amplified with oligonucleotides Lux536F2 and Lux537R2, encompassing a codirectional P_{cagP} promoter upstream of *luxC*; Ap^r Cm^r	This study
pVCC::P_{cagU}	pVCC derivative carrying a 383-bp amplicon, amplified with oligonucleotides Lux530F2 and Lux531R2, encompassing a codirectional P_{cagU} promoter upstream of *luxC*; Ap^r Cm^r	This study
pVCC::P_{pfr}	pVCC derivative carrying a 180-bp fragment, amplified with oligonucleotides PpfrF and PpfrR, encompassing a codirectional P_{pfr} promoter upstream of *luxC*; Ap^r Cm^r	This study
pVCC::$oppP_{pfr}$	pVCC derivative carrying the 180-bp P_{pfr} promoter fragment, cloned in opposite direction with respect to *luxC*; Ap^r Cm^r	This study
pVCC::P_{fecA3}	pVCC derivative carrying a 182-bp fragment, amplified with oligonucleotides A3.4 and A3.5B, encompassing a codirectional P_{fecA3} promoter upstream of *luxC*; Ap^r Cm^r	This study
pVCC::$oppP_{fecA3}$	pVCC derivative carrying the P_{fecA3} promoter fragment, cloned in opposite direction with respect to *luxC*; Ap^r Cm^r	This study
pVCC::$P_{fecA3}SD_{lux}$	pVCC derivative carrying a 109-bp fragment, amplified with oligonucleotides A3.4 and A3.1B, encompassing a codirectional P_{fecA3} promoter with a shortened 5′UTR devoid of the *fecA3* RBS, cloned upstream of the *lux* operon; Ap^r Cm^r	This study
pVCC::$P_{fecA3}SD_{lux}$-ΔOPI_{NikR}	pVCC derivative carrying a 66-bp fragment, amplified with oligonucleotides A3.4 and A3.3B, encompassing a codirectional P_{fecA3} promoter missing the *fecA3* 5′UTR and the NikR OPI operator, cloned upstream of the *lux* operon; Ap^r Cm^r	This study
pVAC::km	pGEMZ derivative containing the *C. coli aphA-3* cassette flanked by upstream and downstream regions for double homologous recombination in the *vac* locus; Ap^r Km^r	9
pFur::km	pGEMZ derivative carrying the *C. coli aphA-3* cassette flanked by upstream and downstream regions for double homologous recombination in the *fur* locus; Ap^r Km^r	8

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *H. pylori* strains used are listed in Table 1. Bacteria were recovered from −80°C glycerol stocks and propagated on Columbia agar plates containing 5% horse blood (Oxoid), 0.2% cyclodextrin, and Dent's or Skirrow's antibiotic supplement. Cultures were grown at 37°C in a water-jacketed thermal incubator (9% CO_2, 91% air atmosphere with 95% humidity) or in jars using CampyGen (Oxoid) gas packs. Liquid cultures were grown in BBL brucella broth (BD) supplemented with 5% fetal calf serum (Oxoid) and Dent's or Skirrow's antibiotic supplement at 37°C with gentle agitation (125 rpm) in glass flasks or tissue culture flasks with vented caps. To monitor the kinetics of gene expression, cultures of wild-type and mutant strains were grown to mid-log phase (optical density [OD], 0.5 to ~0.7), treated with either 1 mM $FeSO_4$, 1 mM $NiSO_4$, or 100 μM 2,2-dipyridyl (all from Sigma-Aldrich), and measured at regular time intervals for the emission of bioluminescence. For growth in 96-well plates (Orange Scientific) or in an Isoplate-96 TC (optimized for luminescence reading; Perkin Elmer), single *H. pylori* colonies were picked and inoculated in single wells containing 100 μl BBL brucella broth. Plates (with loose lids) were agitated at 700 rpm with an MS3 digital shaker (IKA), placed inside the thermal incubator, and measured at regular time intervals with a multilabel reader (see below).

Colonies cultured in single wells could be recovered and expanded on Columbia and brucella agar plates up to 72 to 96 h after the initial inoculum.

H. pylori transformants (the promoterless *luxCDABE* acceptor strain and all of the promoter-containing derivatives and mutants) were obtained by double homologous recombination of the naturally competent G27 strain using 5 μg of transforming DNA as previously described (8); positive clones were selected on Columbia agar plates supplemented with kanamycin (25 μg/ml) and/or chloramphenicol (30 μg/ml) according to the resistance phenotype conferred by either the *aphA-3* (Km^r) or the *cat* (Cm^r) cassette. *Escherichia coli* DH5α cultures, used for cloning purposes, were grown according to standard procedures (27).

Generation of the promoterless *luxCDABE* acceptor strain. DNA extraction, amplification, restriction digests, and ligations were all carried out with standard molecular techniques with enzymes purchased from New England BioLabs. To construct the *H. pylori vac*::*luxCDABE* acceptor strain, a promoterless *P. luminescens luxCDABE* operon cassette was isolated as a 5.8-kb BamHI fragment from plasmid pSB1075 (33, 34) and cloned into pP_{hpn2}, a pGEM-T (Promega) derivative containing a 280-bp PCR PstI-BamHI fragment encompassing the intergenic region between *ksgA* (HP1431) and *hpn2* (HP1432), generating pP_{hpn2}-*luxCDABE*. A unique EcoRI site in pP_{hpn2}-*luxCDABE* upstream of the *luxCDABE* ribosome binding site (RBS) served to insert the *Campylobacter coli aphA-3* cassette, conferring kanamycin resistance (29). The resulting plasmid was used to recover a 7.3-kb BamHI fragment, encompassing the *aphA-3* and *luxCDABE* operons, which was cloned in the pVAC suicide vector (9), generating pVAC::*aphA-3-luxCDABE*. This plasmid includes the promoterless *lux* operon and the selectable Km^r marker in divergent orientation, flanked by regions allowing double homologous recombination in the *vacA* locus of *H. pylori* G27. After transformation (as described above), recombinant colonies of the resulting G27 *vacA*::*aphA-3-luxCDABE* strain (G27*lux* for short) (Fig. 1A) were expanded and confirmed by PCR.

Generation of the pVCC transformation vector. The pVCC suicide transformation vector was conceived to provide a convenient tool to place promoters, DNA sequences of interest, or transcriptional fusions upstream of the promoterless *luxCDABE* operon of the G27*lux* acceptor strain by means of homologous recombination and resistance cassette swapping. pVCC was constructed starting from pVAC. A 779-bp BamHI-HindIII fragment, containing the 3′ end of the *vacA* locus (the right region of homology to *vacA*), was replaced with a 1,045-bp BamHI-HindIII fragment derived from pSB1075, encompassing the 5′ end of the *luxCDABE* operon (i.e., the RBS, the translation start, and the first 1,013 bp of the *luxC* cistron), generating pVC. Eventually, a *Campylobacter coli cat* chloramphenicol resistance cassette, derived as a BglII-BamHI fragment from pBS::*cat*, was cloned into the unique BamHI site of pVC, generating pVCC (Fig. 1B). This vector bears unique BamHI, KpnI, SacI, and SnaBI sites upstream of the *lux* RBS, which can be used to clone promoters of interest through cohesive or blunt-end ligation.

Generation of the P_{cagU}, P_{cagP}, P_{pfr}, and P_{fecA3} *lux* reporter strains. The promoter regions of *cagU* (HP0531 in the 26695 strain annotation), *cagP* (HP0536), *pfr* (HP0653), and *fecA3* (HP1400) were PCR amplified from *H. pylori* G27 genomic DNA using primer pairs with either BglII or BamHI overhangs and cloned into the unique BamHI site of pVCC. Due to cohesive BglII and BamHI ends, the promoter sequence could be cloned randomly in both directions, disrupting the BamHI site on either end of the insert. This feature was used to check the orientation of the promoter. Constructs with promoters diverging in orientation with respect to the *lux* operon (oppP) were used as negative controls. The primers Lux530F2 (tgtttagatctTGGTTTGTTGGTTGCAAAAC; lowercase letters in the sequence indicate nucleotides added for cloning purposes) and Lux531R2 (taataggatccAAGAATTAAATTGAGAAATTG) were used to generate a 383-bp amplicon encompassing the P_{cagU} promoter region (360 bp) and cloned in pVCC, generating pVCC::P_{cagU}. The primers Lux536F2 (aatataggatccAAAGAAGTAGTTCAGGGCG) and Lux537R2 (ttatagatctAAATATCAATACATTTTACC) were used to amplify and clone a 405-bp fragment carrying the P_{cagP} promoter region (383 bp), generating pVCC::P_{cagP}. In addition, the primer couple PpfrF (gttttg gatccTATTGATGCCAACCC) and PpfrR (gttttagatctTGTCCCATAATT ATAGCATA) was used for amplification and cloning of the P_{pfr} promoter (180 bp), generating pVCC::P_{pfr} and pVCC::oppP$_{pfr}$. For the P_{fecA3} promoter, several constructs were created: (i) the full-length *fecA3* promoter, encompassing the −10 box, the RBS, and the start codon of *fecA*3, was amplified with oligonucleotides A3.4 (7) and A3.5B (ACTTAGATCTGC AACACAAACTC) and cloned in pVCC, generating pVCC::P_{fecA3} and pVCC::oppP$_{fecA3}$; (ii) a 3′-shortened promoter, devoid of the native *fecA3* RBS and start codon, was amplified with oligonucleotides A3.4 and A3.1B (TCACAGATCTAACGAACGCCTAT), giving rise to pVCC::$P_{fecA3}SD_{lux}$; and (iii) a mutant promoter lacking the native *fecA3* RBS and start codon, as well as the OPI NikR operator responsible for Ni^{2+}-dependent repression of P_{fecA3}, was amplified with oligonucleotides A3.4 and A3.3B (AAAAAGATCTAATTCGCAGAAT) and cloned, generating pVCC::$P_{fecA3}SD_{lux}$-ΔOPI_{NikR}. All constructs were checked for correct insertion by sequencing. pVCC derivatives containing wild-type, oppP, and mutant promoters were then used to transform the G27*lux* acceptor strain by double homologous recombination. Positive transformants carrying the *cat* cassette were selected on chloramphenicol and were sensitive to kanamycin due to swapping of the resistance cassette. The full list of *lux* reporter strains generated is summarized in Table 1. Finally, a *fur* deletion mutant, carrying the P_{pfr}-*luxCDABE* transcriptional fusion, was obtained by double homologous recombination of the G27 *vac*::*cat*-P_{pfr}-*luxCDABE* strain with the pFur::km suicide vector (8) and subsequent selection on Km^+/Cm^+ Columbia agar plates.

Luminometry. The luminescence of *lux* strains streaked on Columbia agar plates was captured through a Fluoromax Imager (Bio-Rad) with an integration time of 10 min. In the case of liquid cultures growing in flasks and treated with metal ions or chelator, samples of 0.5 to 1.0 ml were taken at regular time intervals, gently pipetted into prewarmed luminometry vials (Promega), and immediately measured in a TD-20/20 luminometer (Turner Designs) with an integration time of 60 s. Data were normalized according to the culture volume and the optical density of the culture by measuring the OD at 600 nm (OD_{600}) of the sample with a Beckman spectrophotometer. Luminescence in multiwell plates was assayed in a Victor^{3}V (1420) multilabel reader (Perkin Elmer), with the bottom trail preheated at 37°C. Plates were first shaken with a linear 2-s pulse (shaking diameter, 0.1 mm). Thereafter, the luminescence of each well was measured with an integration time of 2 s (normal aperture) in the absence of optical filters. To normalize the data, the optical density was eventually assessed by measuring for 1 s the absorbance of each well through a 595-nm-length continuous-wave lamp filter.

Primer extensions. To map the transcriptional start sites and measure the mRNA abundance of *cagU* and *cagP* transcripts, primer extension analyses were performed in triplicate on total RNA extracted at different phases of growth using radioactively labeled primers 536pe17 (AACGAT TTGTTTGTTTATGC) and 531pe4 (CTGATGCTCTGTTGTATC) hybridizing to the native *cagU* and *cagP* gene sequences, as previously described (7). Transcript abundance was measured by quantifying the *cagU* and *cagP* band intensities with a Storm PhosphorImager (Amersham-GE) using Image Quant software (Molecular Dynamics).

Data processing. Data sets were processed with Genework 2.0, Wallac, and Excel software. The luminescence and OD values of vials/wells filled with plain growth medium were used as blank controls and were subtracted from the values of the experimental samples. Three to eight independent replicates were performed for each experiment, and average values and standard deviations were calculated. The threshold of significance was set three standard deviations above the average value of the blank controls both for OD and luminescence. Experimental samples with an OD below this threshold were excluded from the analysis; samples with luminescence below the threshold were judged to be negative (null). Finally, the luminescence values, normalized according to the OD of the sample, were averaged and plotted on graphs. In the course of the blind

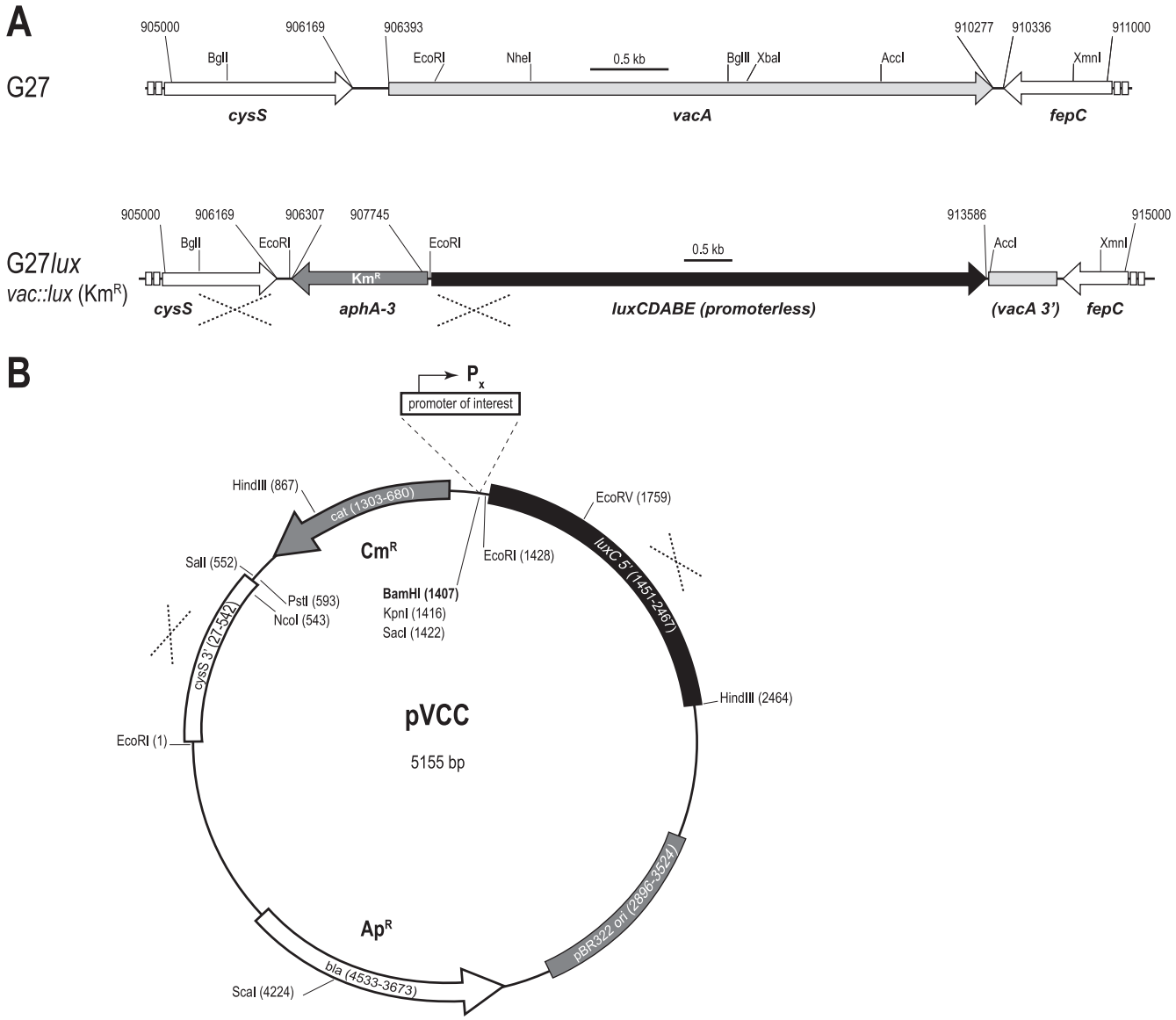

FIG 1 (A) Genomic organization of *H. pylori* G27 and the derivative G27*lux* acceptor strain. (B) Detailed map of the pVCC transformation vector (GenBank accession number HQ207194). Numeration starts from the EcoRI site upstream of the *cysS* 3′ region. Flanking regions for double homologous recombination in G27*lux* are indicated by dashed crossed lines. Unique BamHI, KpnI, SacI, and SnaBI restriction sites can be used to clone fragments of interest upstream of the *luxC* 5′ region. The *cat* promoter maps upstream of the promoter cloning site and in divergent orientation with respect to the *lux* operon to avoid bias deriving from antisense transcription.

test, the maximum value of normalized reporter luminescence, measured at regular time intervals over a period of 72 h, was used to discriminate between weak (repressed) and strong (derepressed) promoters.

Nucleotide sequence accession number. The nucleotide sequence of pVCC has been deposited in GenBank under accession number HQ207194.

RESULTS

Construction of the *H. pylori lux* reporter system. In this study, two separate elements have been used to generate an *H. pylori lux* reporter system: a G27*lux* acceptor strain and the transforming vector pVCC, both schematically represented in Fig. 1.

The first element is the G27*lux* acceptor strain (Table 1), a G27 derivative carrying a kanamycin resistance cassette upstream of a divergent, promoterless *luxCDABE* operon derived from *Photorhabdus luminescens*, all engineered in the *vacA* locus (Fig. 1A). The products of the *luxAB* genes retain a mesophilic luciferase activity (22), while the remaining *luxCDE* cistrons code for the reductase complex responsible for the biosynthesis of the aldehyde substrate, which is used by the luciferase complex to catalyze the bioluminescence reaction. By default, the G27*lux* acceptor strain lacks bioluminescent activity due to the absence of a functional promoter upstream of the *luxCDABE* operon.

The second element of the system is the pVCC suicide transformation vector, a 5,155-bp plasmid designed to conveniently introduce promoters of interest upstream of the *lux* operon by double homologous recombination in the G27*lux* acceptor strain (Table 1). It carries flanking regions with homology to the 3′ end of the *cysS* and the (promoterless) 5′ end of the *luxC* cistrons,

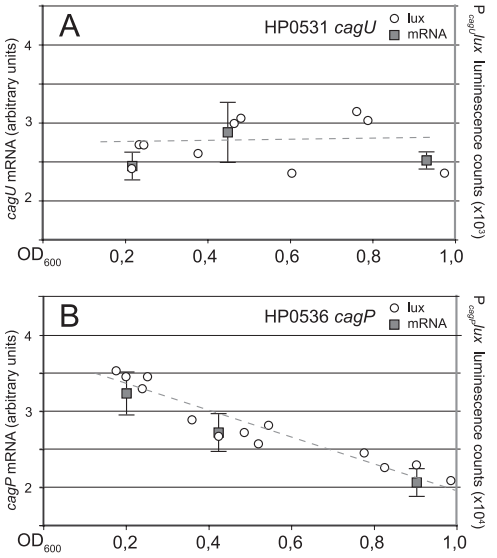

FIG 2 Linear response of the *lux* reporter system in exponential-phase and early-stationary-phase cultures. Transcript levels of *cagU* (A) and *cagP* (B) were assayed in triplicate by primer extension analysis. They are reported as arbitrary units of ^{32}P counts measured in a PhosphorImager (gray squares). Error bars indicate the standard deviations. Values of emitted luminescence of P_{cagU} and P_{cagP}::*luxCDABE* reporter fusions, measured in a multiplate reader and normalized according to the optical density (OD_{600}) of the culture, are depicted by white circles. The different trend of *cagU* and *cagP lux* fusion expression was confirmed by linear regression analysis fitted using the least-square approach (dotted line).

respectively. Between these flanking regions, pVCC encompasses a *Campylobacter coli cat* cassette, conferring selectable chloramphenicol resistance, and unique BamHI, KpnI, and SacI restriction sites in which promoters, DNA sequences, or transcriptional fusions of interest can be cloned (Fig. 1B).

Following *H. pylori* G27*lux* transformation with pVCC and a successful (double) homologous recombination event of the *cysS* and *luxC* regions, the kanamycin resistance will be lost while the new derivatives of G27*lux* will gain the ability to grow on medium supplemented with chloramphenicol as a selectable marker. Consequently, promoters cloned in pVCC upstream of *luxC* in the correct orientation accordingly will drive the expression of the *luxCDABE* operon *in vivo* to emit bioluminescence. The following can be monitored in a noninvasive and quantitative manner with a luminometer, a multiplate reader, or even with chemiluminescence imagers.

Linear response of the *lux* reporter system. The expected codon adaptation index (eCAI; 0.754) and the absence of long stretches of rare codons suggested that codon utilization of the *P. luminescens luxCDABE* cassette should not represent a problem in *H. pylori*. Thus, to first verify the response of the *lux* system in reporting promoter strength or the abundance of native transcripts in *H. pylori*, we generated transcriptional fusions of the *luxCDABE* operon with P_{cagU} and P_{cagP} promoters. These two *cag* promoters were chosen because the abundance of their respective transcripts changes according to the growth phase. The levels of *cagU* remain constant throughout growth (Fig. 2A), while the levels of *cagP* decrease markedly toward the stationary phase (Fig. 2B). Amplicons encompassing their promoter regions were cloned in pVCC (Table 1 and Materials and Methods). After transformation, positive G27*lux* derivatives, carrying the P_{cagU} and P_{cagP} promoters in the same orientation with respect to the *luxCDABE* operon, were assayed with a Victor multiplate reader. The luminescence was normalized according to the OD_{600} of the culture and compared to the levels of native *cagU* and *cagP* mRNA abundance measured through quantification of primer extension analyses at different points of the growth curve (Fig. 2). For unvarying (*cagU*; Fig. 2A) as well as for decreasing transcript levels (*cagP*; Fig. 2B), the bioluminescence emitted by the respective reporter strains faithfully reflected the trend of various levels of transcript abundance, suggesting a linear response of the *lux* reporter system in the exponential and early stationary phases. At later time points, when the cultures entered the advanced stationary phase, the emitted luminescence decreased (data not shown). This repeatedly observed phenomenon is likely attributable to the high metabolic burden associated with the expression of the *luxCDABE* reporter operon. On the other hand, no significant retardation of growth rates was observed between the parental G27 wild-type strain and the P_{cagU} and P_{cagP} *lux* derivatives (data not shown), suggesting that the expression of the *lux* operon has a modest biological cost in the exponential phase of liquid cultures.

Reporter assays with an inducible promoter. To better characterize the reporter system upon (faster) dynamic transcriptional changes, we set out to assay the response kinetics of the *pfr* promoter, driving the expression of the Pfr bacterioferritin using the *lux* reporter system. This promoter has been extensively studied in *H. pylori* and used to validate a GFP reporter system implemented on a modified endogenous low-copy-number plasmid (4). It is repressed in iron-depleted conditions by the ferric uptake regulator Fur and promptly induced in response to iron (3, 10). An amplicon of 180 bp containing the regulatory elements upstream of the *pfr* transcriptional start site was cloned in pVCC (Table 1 and Materials and Methods). After transformation, positive G27*lux* derivatives carrying the *pfr* promoter in either direct (P_{pfr}) or opposite ($oppP_{pfr}$) orientation with respect to the *luxCDABE* operon were streaked on Columbia agar plates, together with a G27*lux* derivative carrying P_{pfr} in direct orientation in a *fur* knockout background (P_{pfr} *fur*::*aphA-3*). The bioluminescence on the plates was then recorded with the charge-coupled device (CCD) camera of a gel imager (Fig. 3A). No luminescence was evident in negative controls carrying P_{pfr} in the opposite orientation ($oppP_{pfr}$) with respect to *luxCDABE*, while a dim light emission could be detected on G27*lux* derivatives carrying P_{pfr} in direct orientation. This luminescence was significantly stronger in the *fur* knockout background (*fur*::*aphA-3*) under conditions in which the P_{pfr} promoter is constitutively derepressed (10), permitting us to identify even single colonies on the plate. This desirable feature indicates that genetic screenings can be easily implemented using the *lux* reporter system in association with a strong promoter.

In parallel, we used a luminometer to quantify the luminescence of exponentially growing liquid cultures, normalized according to the optical density of the culture (Fig. 3B). The analysis reported a difference of more than four orders of magnitude between derivatives carrying P_{pfr} in direct orientation with respect to *luxCDABE* against negative controls carrying P_{pfr} in opposite orientation. These results demonstrate that in *H. pylori*, the *lux* reporter system has an intrinsically low background and is therefore suited for sensitive applications. Moreover, the reporter system readily detected the derepression of P_{pfr} in the *fur* knockout back-

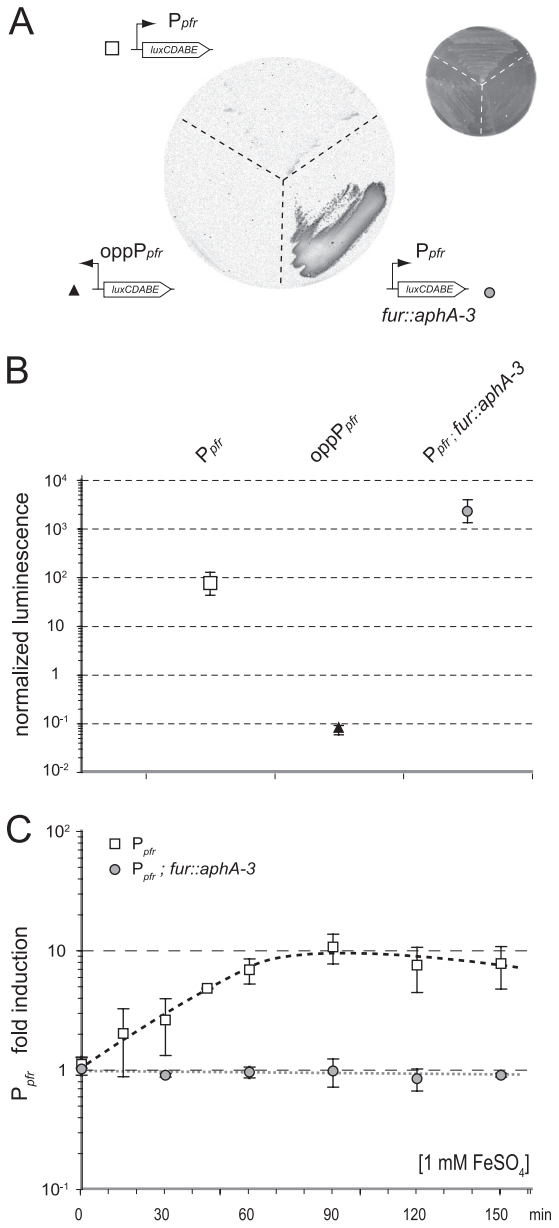

FIG 3 Reporter assays with the inducible P_{pfr} promoter. (A) Inverted dark-field image of luminescence emitted on plates by *H. pylori* reporter strains carrying the P_{pfr} promoter in either codirectional (P_{pfr}; white squares) or opposite ($oppP_{pfr}$; black triangles) direction with respect to the *luxCDABE* operon and in a wild-type or Δ*fur* (*fur*::*aphA-3*; gray circles) genetic background. Images were acquired through the CCD camera of a laboratory gel imager. The miniature shows the bright-field image of the plate. (B) Quantification of emitted luminescence (symbols are as described for panel A) measured through a luminometer and normalized according to the optical density (OD_{600}) of the cultures. (C) Iron inducibility of the P_{pfr} promoter verified through the reporter system in wild-type (white squares) and Δ*fur* (gray circles) genetic background.

ground, with normalized luminescence increasing about another order of magnitude over that of the G27*lux* P_{pfr} strain, mirroring published data on *pfr* transcript abundance in wild-type and *fur*::*aphA-3* strains (10).

Finally, we monitored by luminometry the time-response kinetics of the P_{pfr}-*luxCDABE* reporter fusion in wild-type and *fur*::*aphA-3* backgrounds after the addition of iron to exponentially growing cultures (Fig. 3C). In a wild-type background, the prompt induction of P_{pfr} is reflected in a 10-fold increase of luminescence, reaching a maximum at 60 min after iron treatment. On the contrary, in a *fur* knockout background, no induction of luminescence is detected. This is expected for the P_{pfr} promoter, as its iron-dependent regulation is directly mediated by Fur. The observed response curves accurately parallel the kinetics of the *pfr* transcript after iron treatment (10), indicating a consistent response of the reporter system.

Together, the results validate the use of the *P. luminescens luxCDABE* operon as a robust tool to monitor the dynamic responses of inducible promoters in *H. pylori*.

Reporter assays with a repressible promoter. To test whether the *lux* reporter could also be valuable to monitor the regulation of repressible promoters, we tested the system on the promoter of the *fecA3* gene, encoding a putative outer membrane ferric dicitrate transporter. This promoter is repressed in a nickel-dependent manner by binding of the NikR regulator to two adjacent operators, OPI and OPII, overlapping the transcriptional start site and the extended −10 box, respectively, with OPI being necessary and sufficient for the nickel-dependent repression (7, 13, 26). To this aim, several G27*lux* reporter strains were created (Fig. 4A and Table 1): a G27*lux* reporter carrying the full-length *fecA3* promoter, encompassing the −10 box, the RBS, and the start codon of *fecA*3 (P_{fecA3}); a control strain carrying the full-length *fecA3* promoter in opposite orientation with respect to *luxCDABE* ($oppP_{fecA3}$); a reporter carrying a 3′-shortened sequence missing the native *fecA3* RBS and start codon, in which translation starts on the heterologous *luxC* initiation sequence ($P_{fecA3}SD_{lux}$); and a P_{fecA3} SD_{lux} derivative mutant promoter lacking the OPI NikR operator responsible for Ni^{2+}-dependent repression of P_{fecA3} (P_{fecA3} SD_{lux}-ΔOPI_{NikR}). The bioluminescence of these reporter strains was then analyzed as described for the P_{pfr} reporter strains, and results are shown in Fig. 4B to D.

Streaked on plates, the negative-control strain carrying P_{fecA3} in opposite orientation ($oppP_{fecA3}$) with respect to *luxCDABE* was unable to emit detectable luminescence. On the contrary, both P_{fecA3} and P_{fecA3} SD_{lux} reporter strains showed a readily detectable signal, which is indicative of robust expression of the *lux* reporter in the absence of Ni^{2+} treatment (Fig. 4B), while an even stronger luminescence was recorded in the P_{fecA3} SD_{lux}-ΔOPI_{NikR} reporter strain.

These qualitative results were further quantified with a luminometer on exponentially growing liquid cultures. The normalized quantitative luminescence measures paralleled the qualitative observations made on plates, with more than four orders of magnitude stronger signal of strains carrying the codirectional P_{fecA3} promoter above levels for strains carrying it in divergent orientation ($oppP_{fecA3}$) with respect to the *lux* operon (Fig. 4C). Interestingly, the absence of the native *fecA3* RBS and ATG initiation codon had no significant influence on the measured levels of *lux* expression, suggesting that the Shine-Dalgarno sequence of *P. luminescens* is well recognized and supported in *H. pylori*. This feature may be convenient if the activity of different promoters or transcriptional fusions has to be compared, as it filters out the bias of dissimilar translation rates that may arise from various ribosome binding sites and/or 5′ untranslated regions (UTRs).

In addition, we recorded a 3-fold increase of luminescence in the absence of the OPI_{NikR} regulatory element, which reflects the

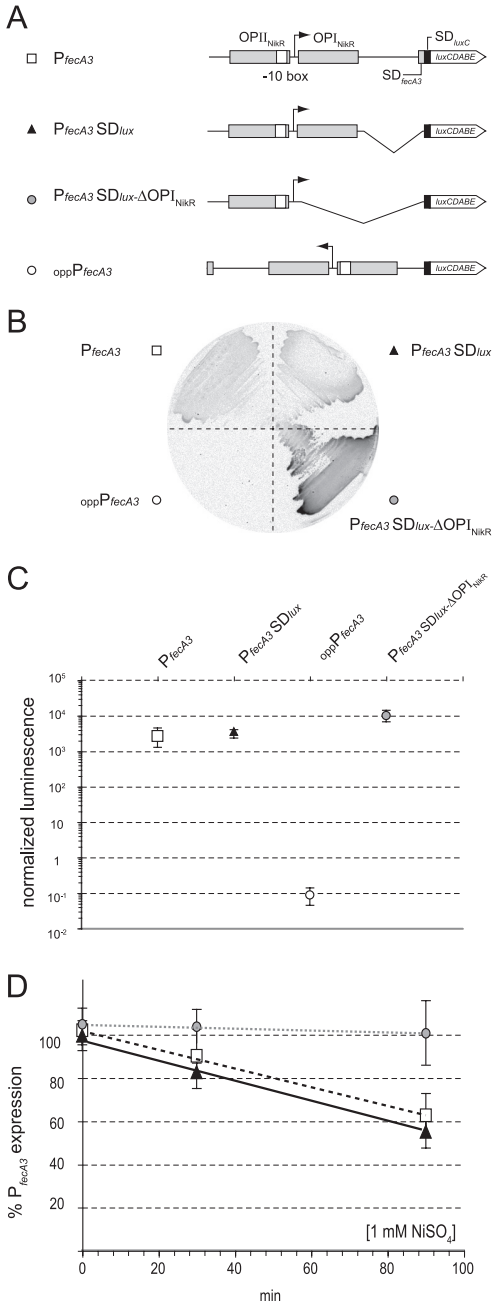

FIG 4 Reporter assays with the repressible P_{fecA3} promoter. (A) Schematic representation of the P_{fecA3} fusion constructs, showing the positions of the NikR operators, the −10 box (white box), the transcriptional start site (bent arrow), and the native *fecA3* as well as the heterologous *luxC* Shine-Dalgarno (SD) sequences. (B) Inverted dark-field image of luminescence emitted on plates by *H. pylori* reporter strains carrying the P_{fecA3} promoter in either codirectional (P_{fecA3}; white squares) or opposite (opp P_{fecA3}; white circles) direction with respect to the *luxCDABE* operon, carrying the heterologous *luxC* ribosome entry site (P_{fecA3} SD_{lux}; black triangles), and a deletion of the NikR OP-I operator important for Ni-dependent repression of *fecA3* ($P_{fecA3}SD_{lux}$-ΔOPI_{NikR}; gray circles). Images were acquired through the CCD camera of a laboratory gel imager. (C) Quantification of emitted luminescence (symbols are as described for panel B), measured through a luminometer and normalized according to the optical density (OD_{600}) of the cultures. (D) Ni^{2+}-dependent repression of the P_{fecA3} promoter verified through the reporter system with the wild-type *fecA3* promoter and 5′UTR (white squares), the SD_{lux}-substituted *fecA3* 5′UTR (black triangles), and the SD_{lux}-substituted *fecA3* 5′UTR from which the NikR operator OPI was deleted (gray circles).

constitutive derepression induced by the lack of NikR repressor binding to the promoter. Accordingly, the addition of 1 mM Ni^{2+} to the culture medium resulted in a progressive repression of the luminescence signal only in P_{fecA3} and P_{fecA3} SD_{lux}, carrying the intact nickel-responsive OPI_{NikR} element, while in its absence the *lux* reporter strain was completely insensitive to nickel (Fig. 4D). These results demonstrate that the *lux* reporter system can be used to monitor the time-response kinetics of repressible promoters, making it a very versatile instrument in the *H. pylori* molecular toolbox.

Utilization of the *lux* reporter for genetic screening. Robust *in vivo* reporter systems greatly facilitate genetic screenings, especially if positive clones can be identified and picked easily over a vast plethora of negative ones.

Strong promoters driving abundant *luxCDABE* expression permitted us to spot single luminescent colonies on plates using the CCD camera of a gel imager, a common piece of equipment in most research laboratories (Fig. 3A). However, for weak promoters the integration time needed to acquire a detectable signal had to be increased significantly. This exposed the plates to prolonged periods of suboptimal temperature and unfavorable CO_2 tension, making subsequent recovery of the positive clones demanding. Moreover, we noticed that in stationary phase, the bioluminescence emitted by *lux* expressing clones has the tendency to drop (data not shown). Thus, to optimize the *lux* reporter system for genetic screening, we implemented an alternative high-throughput method based on luminescence monitoring of single colonies growing in liquid in 96-well plates. To validate the method, a blind screen was performed.

Single colonies of G27*lux* derivatives carrying the P_{pfr} promoter in the *fur* wild-type or knockout background were individually cultured in the wells of a microtiter plate. The growth (OD) and luminescence of each clone were monitored at regular time intervals over a period of 72 h within a multilabel plate reader. Recalling the Fur-dependent repression of P_{pfr}, the operator was asked to discriminate between wild-type and *fur* knockout backgrounds based solely on the normalized reporter luminescence driven by the Fur-repressed P_{pfr} promoter. Out of 30 inoculated clones (15 G27*lux* P_{pfr} and 15 G27*lux* P_{pfr} *fur*::*aphA-3*), only one did not grow. The remaining were promptly classified in two groups according to the weak (repressed P_{pfr}) or strong (derepressed P_{pfr}) emitted luminescence (Fig. 5). Clones in the first group were judged to have a wild-type background (Fig. 5, black bars), while clones belonging to the second group were predicted by the operator to be *fur* knockouts (white bars). Notably, all clones were correctly assigned, and viable ones could be effortlessly recovered and expanded even after 72 h of culture, demonstrating that the *lux* reporter system not only may be useful to monitor transcriptional responses but also can be implemented for genetic screening in *H. pylori*.

DISCUSSION

The success of *H. pylori* as a pathogen and its impact on human health depend on the concerted expression of virulence and stress resistance factors, which are controlled by a markedly small number of transcriptional regulators organized in a shallow transcriptional regulatory network (6). Recently, the discovery in *H. pylori* of a panoply of small noncoding and antisense RNAs (28, 36) expanded the picture, pointing to an extensive posttranscriptional regulatory circuit that needs to be investigated in detail in the

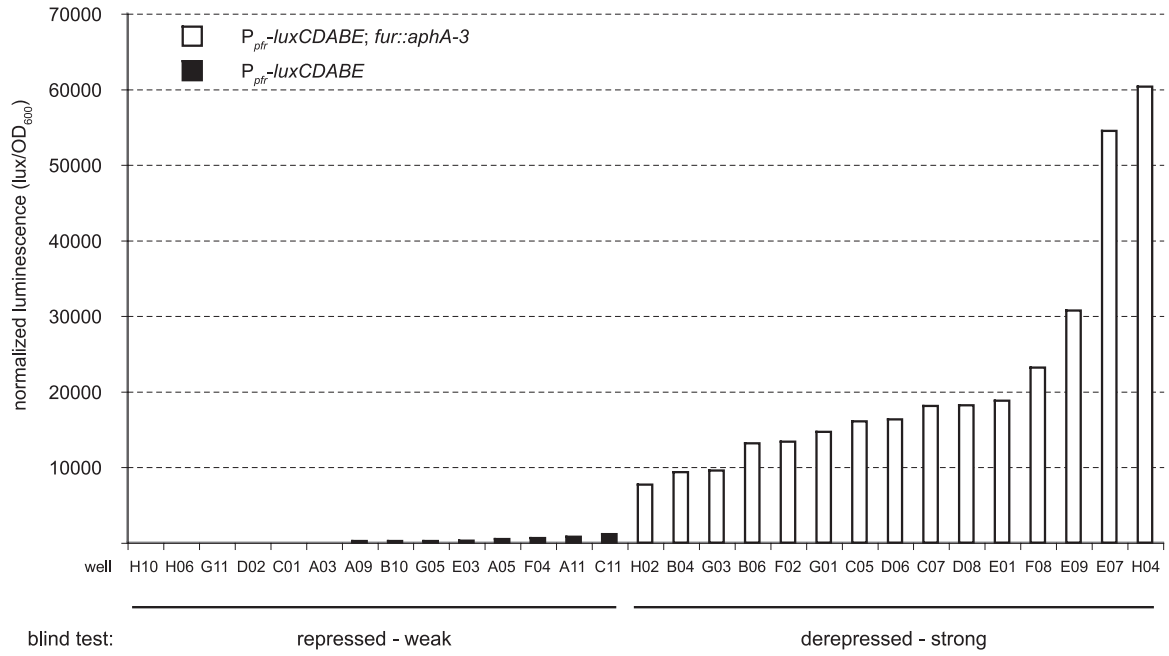

FIG 5 Genetic screening with the *lux* reporter performed in 96-well culture plates. Genotype prediction blind test of individual *H. pylori* clones expressing the P_{pfr}::*luxCDABE* promoter fusion in wild-type (black bars) and Δ*fur* (white bars) genetic backgrounds.

future. In this context, it is clear that the implementation of robust reporter systems for the *in vivo* analysis of gene expression is of pivotal importance.

Measurements of XylE or LacZ enzymatic activities have been used as heterologous reporter systems in many bacteria, and both have been implemented in *H. pylori*. XylE activity is absent from native strains, and the polypeptide encoded by the *xylE* reporter gene appears to be stable when expressed by the bacterium (17, 25). In contrast, the measurement of the β-galactosidase activity of the LacZ reporter in *H. pylori* has frequently proven critical, with low enzymatic activities detected even in the case of transcriptional fusions with the *ureA* promoter, one of the strongest promoters in *H. pylori* (31). Significant advances in the use of *lacZ* have recently been reported in the construction and tweaking of an inducible expression system to engineer conditional mutants (2). However, due to the invasive nature of the enzymatic measurement and their relatively low sensitivity, both *lacZ* and *xylE* appear to be better suited for single- or endpoint assays rather than as a workable *in vivo* resource for the analysis of weak promoters or the implementation of genetic screens.

For the latter purposes, reporter systems based on the promoterless chloramphenicol acetyltransferase *cat* cassette were developed. Fused to promoters of flagellar genes, the *cat* reporter displayed a high sensitivity. However, a major disadvantage of the *cat* reporter system is the well-known high stability of the Cat protein, which makes the system unsuited for the study of transient and dynamic changes in expression over time (24).

The same applies for fluorescent proteins, such as GFP, its cognate derivatives, or DsRed, which give slow responses, with significant rise of the signal occurring only several hours after induction (14). Another drawback is their relatively weak sensitivity, leading to detection of the signal at approximately one hundred times higher analyte concentrations than with luminescent proteins (14). Optimized GFP isoforms with very bright fluorescence and improved folding in bacteria (5) partially resolved this problem and constituted a pivotal tool for construction of reporter systems enabling the analysis of single *H. pylori* cells in culture or in contact with host cells (4, 16, 20). However, the high autofluorescence levels of *H. pylori* cells appear to compromise the use of GFP systems, especially in the advanced growth phases (16).

Here, we demonstrate that a bioluminescent reporter system based on the *P. luminescens luxCDABE* operon provides a very convenient reporter to study the kinetics of gene expression in *H. pylori*. The reporter system is constituted by a promoterless *lux* acceptor strain, deriving from of the commonly used G27 parental strain, and of a transforming vector pVCC, in which promoters of interest can be conveniently cloned. The system faithfully reported the iron-inducible Fur regulation of the *pfr* promoter (Fig. 3), as well as nickel-repressible NikR-dependent regulation of the *fecA3* promoter (Fig. 4), with very low background noise. The high signal-to-background ratio ($\geq 10^3$- to 10^4-fold difference), together with the self-sustainable expression of substrate fuelling the luciferase activity, makes the *luxCDABE* system especially suited for *in vivo* applications in which high sensitivity and continuous monitoring of the reporter output is desirable. It overcomes many of the limitations of fluorescent reporters, e.g., cellular autofluorescence, excessive stability, slow turnover of the fluorescent protein, etc., which have hampered the study of dynamic changes of *H. pylori* gene expression, especially in terms of host-pathogen interactions. For example, it is possible to use this reporter system to monitor differences in the timely activation of specific promoters upon contact with a human AGS cell line (A. Vannini and A. Danielli, data not shown).

Another advantage of the *lux* reporter over other available systems is its robustness, which allowed us to correctly assign the genotype of a mutant strain using a high-throughput screening

platform (Fig. 5). Moreover, single colonies with strong and constitutively derepressed promoters could be readily detected on plates using standard laboratory imagers equipped with a CCD camera (Fig. 3), making the selection of positive clones particularly fast and cost-effective.

Given the pervasive occurrence of antisense transcripts in *H. pylori* (28), the *lux* reporter system also has excellent features to monitor putative posttranscriptional regulation mechanisms mediated by these noncoding RNAs. Indeed, the system can be used to verify target regions and pairing cores of putative small RNAs involved in posttranscriptional regulation and also to confirm the presence of predicted transcriptional terminators (A. Vannini, data not shown).

On the other hand, several drawbacks in the adaptation of the system to *H. pylori* have been noticed. First, the bioluminescence of the reporter progressively fades when the cultures enter the advanced stationary phase. This is probably due to the high metabolic burden associated with the expression of the large *lux* operon and with the withdrawal of fatty acid metabolites from the central metabolism to synthesize the luciferase aldehyde substrate. This limits the range of workable and reproducible conditions to the exponential and early stationary phases of growth (Fig. 2). Second, we observed that low pH has a negative effect on the emission of luminescence on the promoter tested, hampering to a certain extent the usefulness of the system if acid responses have to be monitored (data not shown). Finally, another pitfall of the system is that the reporter fusion is inserted at the *vacA* locus, so that promoters are not tested at their original positions on the chromosome, while it is acknowledged that the activity of certain promoters may be influenced by the DNA context.

Nevertheless, the many desirable features of the described *lux* reporter system provide a major improvement to the available *H. pylori* toolbox. It is therefore anticipated that they will greatly help the study of kinetic responses in gene expression and implementation of genetic screens in this bacterium.

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