Generation of Genetic Tools for Gauging Multiple-Gene Expression at the Single-Cell Level

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ABSTRACT Key microbial processes in many bacterial species are heterogeneously expressed in single cells of bacterial populations. However, the paucity of adequate molecular tools for live, real-time monitoring of multiple-gene expression at the single-cell level has limited the understanding of phenotypic heterogeneity. To investigate phenotypic heterogeneity in the ubiquitous opportunistic pathogen *Pseudomonas aeruginosa*, a genetic tool that allows gauging multiple-gene expression at the single-cell level has been generated. This tool, named pRGC, consists of a promoter-probe vector for transcriptional fusions that carries three reporter genes coding for the fluorescent proteins mCherry, green fluorescent protein (GFP), and cyan fluorescent protein (CFP). The pRGC vector has been characterized and validated via single-cell gene expression analysis of both constitutive and iron-regulated promoters, showing clear discrimination of the three fluorescence signals in single cells of a *P. aeruginosa* population without the need for image processing for spectral cross talk correction. In addition, two pRGC variants have been generated for either (i) integration of the reporter gene cassette into a single neutral site of *P. aeruginosa* chromosome that is suitable for long-term experiments in the absence of antibiotic selection or (ii) replication in bacterial genera other than *Pseudomonas*. The easy-to-use genetic tools generated in this study will allow rapid and cost-effective investigation of multiple-gene expression in populations of environmental and pathogenic bacteria, hopefully advancing the understanding of microbial phenotypic heterogeneity.

IMPORTANCE Within a bacterial population, single cells can differently express some genes, even though they are genetically identical and experience the same chemical and physical stimuli. This phenomenon, known as phenotypic heterogeneity, is mainly driven by gene expression noise and results in the emergence of bacterial subpopulations with distinct phenotypes. The analysis of gene expression at the single-cell level has shown that phenotypic heterogeneity is associated with key bacterial processes, including competence, sporulation, and persistence. In this study, new genetic tools have been generated that allow easy cloning of up to three promoters upstream of distinct fluorescent genes, making it possible to gauge multiple-gene expression at the single-cell level by fluorescence microscopy without the need for advanced image-processing procedures. A proof of concept has been provided by investigating iron uptake and iron storage gene expression in response to iron availability in *P. aeruginosa*.

KEYWORDS *Pseudomonas aeruginosa*, plasmids, gene expression, biosensor, transcriptional fusions, single cells, genetic tools, fluorescence genes, iron, siderophores, iron regulation

Monoclonal bacterial populations are commonly considered groups of genetically identical cells in which all members of the population should mechanistically show the same gene expression profile when thriving in the same environment.

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However, several studies in the last 20 years partly changed this concept. It is now acknowledged that genetically identical cells of a bacterial population can differ from each other with regard to gene expression, even in the absence of appreciable environmental differences among cells (1, 2). This phenomenon, known as phenotypic heterogeneity, has been observed for several bacterial processes, such as persistence, competence, the onset of spore formation, cell-to-cell communication, carbon source utilization, and others (3–7). Phenotypic diversity among individual cells could confer adaptive benefits to bacterial populations; it could increase population fitness in fluctuating environments (bet-hedging strategy) and favor the division of labor among phenotypically different members of the bacterial population (2, 8, 9).

Traditional gene expression analyses based on *lacZ* or *lux* reporter gene fusions and/or real-time quantitative PCR (RT-qPCR) provide information on the average promoter activity of the bulk bacterial population. The advent of reporter systems based on the expression of fluorescent proteins coupled with fluorescence microscopy or flow cytometry analyses has paved the way to the study of gene expression at the single-cell level, enabling the detection of phenotypic heterogeneity. In these reporter systems, the promoter of a gene of interest drives the expression of a fluorescent protein. By monitoring the fluorescent signal in individual bacterial cells, it is possible to determine if the tested promoter is active in all the cells of the population or if subpopulations coexist in which the promoter shows different activation levels. Most of the reporter systems developed with this aim allow the expression of a single fluorescent protein, while systems for the expression of two or more fluorescent proteins in the same cell have seldom been employed (10–15). Even if reporter systems based on several fluorescent proteins can provide valuable information on the expression of multiple genes at the single-cell level, their use is often limited by the broad excitation and emission spectra of the fluorescent reporters, which result in spectral cross talk that hinders clear discrimination of specific fluorescent signals unless complex image-processing procedures are used (15, 16). As a result, the study of bacterial phenotypes involving multiple genes that have to be monitored simultaneously in the same cells is still challenging.

Pseudomonas aeruginosa is a paradigmatic example of a bacterium in which complex phenotypes depend on the tightly regulated expression of multiple genes. *P. aeruginosa* is a Gram-negative, versatile environmental bacterium able to infect multiple hosts, such as humans, other mammals, insects, and plants (17, 18). The remarkable adaptability of this bacterium to different environments mainly relies on its broad metabolic potential and ability to fine-tune gene expression in response to environmental stimuli via the integration of multiple signaling pathways and global regulatory networks (17, 19). Nevertheless, with the exception of a few studies reporting heterogeneity in the expression of the type III secretion system, secreted proteases, and exopolysaccharide genes (20–22), phenotypic heterogeneity in *P. aeruginosa* is still poorly investigated, probably due to the paucity of suitable and easy-to-use molecular tools for multiple-gene expression analysis at the single-cell level in this bacterium.

In this study, a user-friendly set of both episomal and integrative plasmids for simultaneously gauging the activity of multiple *P. aeruginosa* promoters at the single-cell level is described. These genetic tools are based on a reporter cassette containing the *mCherry*, *gfp*, and *cfp* genes, coding for mCherry (red), GFP (green), and CFP (cyan) fluorescent proteins, respectively. Three different promoter regions can be cloned with conventional restriction enzymes upstream of each reporter gene, generating transcriptional fusions that allow real-time monitoring of the activity of up to three promoters at the single-cell level by fluorescence microscopy imaging in the red, green, and cyan emission channels. The modular architecture of the episomal plasmid generated for *P. aeruginosa* studies allowed easy genetic manipulation, resulting in a broad-host-range vector that can be used in bacterial genera other than *Pseudomonas*.

RESULTS

Generation of the pRGC plasmid. The pRGC plasmid was constructed by combining DNA fragments via molecular cloning and mutagenesis steps (see Fig. S1 in the supplemental material). First, a scaffold plasmid, named pMLR, was generated by combining functional elements from two vectors: (i) the pUCP18 plasmid (23), providing the pRO1600 origin of replication for *Pseudomonas* spp. (24) and the associated replication protein, and (ii) the pVRL1 plasmid (25), providing the *aacC1* gene for gentamicin (Gm) resistance, the ColE1-like origin for replication in *E. coli*, and the *lacZα* gene with the multiple cloning site (MCS). The replication of pMLR in *P. aeruginosa* cells was verified by transformation and selection on LB agar plates supplemented with 100 μg/ml Gm.

Prior to cloning the fluorescent genes in pMLR, the 231-bp region encompassing the CAP binding site, the *lac* promoter (*Plac*), and the first 8 codons of *lacZα* was deleted from pMLR, thereby generating the pMLRΔ*lac* plasmid. This mutagenesis step was necessary to prevent the transcription of downstream reporter genes from the *Plac* promoter and the formation of a chimeric protein composed of LacZα and the cloned insert. Finally, the *mCherry*, *gfp*, and *cfp* reporter genes were cloned sequentially in the MCS of pMLRΔ*lac*. The resulting pRGC (red, green, cyan) plasmid (6,376 bp) allows the cloning of one promoter upstream of the *mCherry* gene by using the unique restriction sites EcoRI, Sall, XhoI, and ApaI and of two distinct promoters (or one bidirectional promoter) between the *gfp* and *cfp* genes by using the unique restriction sites NotI, XbaI, SpeI, BamHI, and XmaI or SmaI (Fig. 1A).

To avoid translational readthrough effects and maximize the translation efficiency of the reporter genes, three translational stop codons in all reading frames and a strong ribosome binding site (RBS) (25) were inserted in the DNA region between the restriction sites for promoter cloning and the translational start codons of the reporter genes (an example is given in Fig. 1B).

Reporter genes are arranged in pRGC to minimize transcriptional interference; the *cfp* and *gfp* coding sequences have opposite orientations, while a unidirectional *t₀* transcriptional terminator is present downstream of the *gfp* gene, upstream of the *mCherry* coding sequence (Fig. 1A).

pRGC copy number and stability. The pRGC copy number in *P. aeruginosa* PAO1 was 8.4 ± 1.4 copies per chromosome unit, as determined by means of quantitative PCR analysis. Thus, pRGC can be classified as a low-copy-number plasmid (26).

Plasmid stability was assessed after one or two serial passages of *P. aeruginosa* PAO1 (pRGC) in LB for 24 h without antibiotic selection and then plating on LB agar plates with or without Gm. Plasmid stability at 24 h (passage 1; ca. 10 generations) and 48 h (passage 2; ca. 20 generations) was expressed as the N_{Gm}/N_0 ratio, where N_{Gm} and N_0 are the number of CFU grown on LB agar plates supplemented with and without Gm, respectively. The N_{Gm}/N_0 values were 0.61 ± 0.24 at 24 h and 0.49 ± 0.31 at 48 h, indicating that about 40% or 50% of the *P. aeruginosa* cells lost the plasmid after 10 or 20 generations in the absence of antibiotic selection, respectively.

Functional analysis of pRGC for simultaneous gauging of three fluorescence signals at the single-cell level. The potential of pRGC in the simultaneous monitoring of three different promoters strictly depends on the avoidance of signal interference between reporter gene products; the emission signals resulting from the excitation of mCherry, GFP, and CFP should avoid bleed-through effects to be detected as distinct fluorescent channels. Moreover, the expression of each reporter gene should not alter the expression of the other reporter genes. Functional analyses on pRGC have been performed to verify these assumptions.

Briefly, three pRGC variants carrying the constitutive *P_{km}* promoter upstream of the *mCherry*, *gfp*, or *cfp* reporter genes were generated, and excitation/emission spectra of *P. aeruginosa* strains carrying the resulting pRGC-*P_{km}::mCherry*, pRGC-*P_{km}::gfp*, or pRGC-*P_{km}::cfp* plasmid were determined. As shown in Fig. S2, the three fluorescent proteins are functionally expressed in *P. aeruginosa*, and their excitation/emission profiles are coherent with the theoretical mCherry, GFP, and CFP spectra (27–29). Although excitation and emission spectra of mCherry, GFP, and CFP (Fig. 2A and B)

FIG 1 Physical maps of the episomal and integrative plasmids generated in this study. Relevant features of pRGC (A), mini-RGC (C), and pRGC-BHR (D) are illustrated in different colors. Sites for unique cutter restriction enzymes are indicated. Genes are reported on scale over the total length of the vectors. Plasmid physical maps were obtained with Snapgene software (GSL Biotech). (B) Sequence details of the transcriptional fusion between the *Pkm* promoter and the *mCherry* gene in the pRGC-*Pkm::mCherry* vector. The Shine-Dalgarno sequence (SD) is located 6 nucleotides upstream of the *mCherry* start codon.

partially overlap, emission spectrum ranges devoid of signal interference can be determined for the three reporters (corresponding to the selected red, green, and cyan areas of the emission spectra in Fig. 2B), making it possible to define suitable excitation wavelengths (λ_{ex}) and emission (λ_{em}) ranges for confocal microscopy imaging: mCherry, λ_{ex} of 543 nm, λ_{em} ranging from 620 nm to 740 nm; GFP, λ_{ex} of 488 nm, λ_{em} ranging from 548 nm to 555 nm; CFP, λ_{ex} of 458 nm, λ_{em} ranging from 468 nm to 480 nm. As shown in Fig. 2C, this setup allows clear discrimination of the three fluorescence signals in a mixed population of *P. aeruginosa* PAO1 cells expressing mCherry, GFP, or CFP via the pRGC-*Pkm::mCherry*, pRGC-*Pkm::gfp*, or pRGC-*Pkm::cfp* plasmid, respectively.

The possibilities that the expression of one fluorescent protein could interfere with the expression of the other fluorescent proteins in the same cell and that the expression of multiple fluorescent proteins could impact *P. aeruginosa* growth were also investigated. In detail, the fluorescence signals emitted by *P. aeruginosa* PAO1 cells carrying pRGC-derivative plasmids for constitutive (*Pkm*-dependent) expression of one, two, or three fluorescent proteins were verified by confocal microscopy imaging. A comparison of fluorescence emission from the tested strains upon excitation with lasers at predefined wavelengths (543 nm for mCherry; 488 nm for GFP; 458 nm for CFP) revealed no signal interference and a good signal-to-noise ratio for the three fluorescence proteins (Fig. 3), suggesting that pRGC is a suitable vector for simultaneously gauging the activity of three promoters in single cells of *P. aeruginosa*. Notably, growth

FIG 2 Spectral and microscopy analyses of *P. aeruginosa* carrying pRGC variants for mCherry, GFP, or CFP expression. (A and B) Excitation (A) and emission (B) spectra of *P. aeruginosa* PAO1 cells carrying the pRGC-*Pkm::mCherry*, pRGC *Pkm::gfp*, or pRGC *Pkm::cfp* plasmid. The red, green, and blue vertical dashed lines in panel A and the regions shaded in red, green, and blue in panel B indicate laser excitation wavelengths and the emission ranges used in this study for confocal microscopy imaging of mCherry, GFP, and CFP, respectively. (C) Confocal microscopy images of a mixed population of *P. aeruginosa* cells carrying the pRGC-*Pkm::mCherry*, pRGC-*Pkm::gfp*, or pRGC-*Pkm::cfp* plasmid. From left to right, bright field, phase contrast; λ_{ex} 543, red fluorescence channel imaged with laser excitation at 543 nm, showing *P. aeruginosa* PAO1 cells carrying the pRGC-*Pkm::mCherry* plasmid; λ_{ex} 488, green fluorescence channel imaged with laser excitation at 488 nm, showing *P. aeruginosa* PAO1 cells carrying the pRGC-*Pkm::gfp* plasmid; λ_{ex} 458, cyan fluorescence channel imaged with laser excitation at 458 nm, showing *P. aeruginosa* PAO1 cells carrying the pRGC-*Pkm::cfp* plasmid; merge, overlap of phase contrast and fluorescence images.

was not or only marginally affected in cells carrying the pRGC-derivative plasmids for the *Pkm*-driven expression of the fluorescent proteins compared to the cells carrying the empty pRGC vector (Fig. S3).

Exploitation of pRGC for the analysis of *P. aeruginosa* iron-regulated promoters. In *P. aeruginosa*, the expression of the *pchE* and *pvdA* genes, implicated in the biosynthesis of the pyochelin and pyoverdine siderophores, respectively, is derepressed under iron starvation (30–32), while the *bfrB* gene, coding for a heme-binding bacterioferritin, is induced by the presence of iron (33–35).

To exploit the potential of pRGC in the simultaneous monitoring of the activity of multiple *P. aeruginosa* promoters in response to iron levels at the single-cell level, the promoter regions of *pchE*, *pvdA*, and *bfrB* were cloned in pRGC upstream of the *mCherry*, *gfp*, and *cfp* reporter genes, respectively. *P. aeruginosa* cells carrying the resulting pRGC-Fe plasmid were cultured in M9 minimal medium in the presence of different FeCl_3 concentrations, ranging from 0 to $12 \mu\text{M}$. After 7 h of incubation at 37°C , pyoverdine production was quantified in the cell-free supernatants of the corresponding cultures, and aliquots of the cultures were analyzed by confocal microscopy imaging. The cultures grown in M9 without iron addition and in M9 supplemented with $0.75 \mu\text{M}$ FeCl_3 produced high levels of pyoverdine (Fig. S4A), denoting iron starvation conditions. Conversely, $3 \mu\text{M}$ FeCl_3 significantly decreased pyoverdine levels, and pyoverdine production was abrogated in the presence of $12 \mu\text{M}$ FeCl_3 (Fig. S4A). Accordingly, as shown in the enlargements of representative fields imaged by confocal microscopy in Fig. 4A, the red and cyan fluorescent signals recorded in single cells of cultures grown in M9 without iron addition or in M9 supplemented with $0.75 \mu\text{M}$ FeCl_3 revealed high activation of the *pchE* and *pvdA* promoters, while low-level green signals, indicating *PbfrB* activity, were detectable in only a few cells

FIG 3 *P. aeruginosa* cells expressing different combinations of fluorescent proteins. Confocal microscopy imaging of *P. aeruginosa* PAO1 cells carrying different pRGC variants in which the *Pkm* promoter drives the expression of one, two, or three fluorescent reporter genes, as indicated above the panels. Each field was sequentially imaged with lasers at 543 nm (λ_{ex} 543; mCherry), 488 nm (λ_{ex} 488; GFP), and 458 nm (λ_{ex} 458; CFP). All panels are merges of phase contrast and fluorescent channel images.

under these conditions. Higher FeCl_3 concentrations (i.e., 3 and 12 μM) clearly reduced *PpchE* and *PpvdA* activity while promoting *PbfrB* activation. These data are in line with the quantification of *PpchE*, *PpvdA*, and *PbfrB* activity in bulk populations (Fig. S4B).

Automatic counting of cells expressing mCherry, GFP, and/or CFP in multiple fields (>2,500 cells per condition) revealed some heterogeneity in the activation of the tested promoters (Fig. 4B and C). Indeed, while almost all cells (>94%) expressed both *PpchE* and *PpvdA* under iron starvation conditions (i.e., 0 and 0.75 μM FeCl_3), *PpchE* and/or *PpvdA* were active in 88.9% and 6.4% of cells in M9 supplemented with 3 μM FeCl_3 , respectively, while the fraction of cells expressing *PpchE* and/or *PpvdA* dropped to 9.5% and 0.3% in the presence of 12 μM FeCl_3 (Fig. 4B). Hence, while both *PpchE* and *PpvdA* promoters are active under conditions of iron starvation, increased iron availability seems to exert a stronger repressive effect on *PpvdA* than on *PpchE*, with 92.7% and 96.4% of the *PpchE*-expressing cells showing no activation of *PpvdA* for FeCl_3 concentrations of 3 and 12 μM , respectively. This observation is in line with previous reports showing that *pvd* genes are more sensitive to the repression mediated by the ferric uptake regulator (Fur)- Fe^{2+} complex than *pch* genes (36).

Regarding *PbfrB* activity, the fraction of cells expressing GFP was 8.3, 36.6, 92.5, and 99.4% in M9 supplemented with 0, 0.75, 3, and 12 μM FeCl_3 , respectively (Fig. 4B). Notably, the fraction of cells grown in M9 and in M9 supplemented with 0.75 μM FeCl_3 that activated *PbfrB* also displayed the activation of *PpchE*, *PpvdA*, or both (Fig. 4C), indicating that a significant fraction of cells has a detectable (albeit low) level of *bfrB* expression under iron starvation and that, under these conditions, a subpopulation of cells expressing both iron-repressible and iron-inducible genes exists. Therefore, distinct subpopulations of cells heterogeneously expressing the *pvdA*, *pchE*, and *bfrB* genes in response to various iron levels coexist in *P. aeruginosa* cultures (Fig. 4C).

Overall, the above-described data provide preliminary evidence that pRGC is a suitable genetic tool for studying the expression of multiple genes and their regulation in response to environmental stimuli in single cells of *P. aeruginosa*.

Generation of integrative and broad-host-range variants of pRGC. Taking advantage of the modular architecture of pRGC, plasmid derivatives were generated for

FIG 4 Simultaneous detection of the activity of three iron-regulated promoters in *P. aeruginosa* cells. (A) Confocal microscopy imaging of *P. aeruginosa* PAO1 cells carrying the pRGC-Fe plasmid grown in M9 alone or supplemented with the $FeCl_3$ concentrations indicated on the left. Each field was sequentially imaged with lasers at 543 nm (λ_{ex} 543; mCherry), 488 nm (λ_{ex} 488; GFP), and 458 nm (λ_{ex} 458; CFP). Representative enlargements of phase contrast bright-field and merge images are also shown. (B and C) Histograms showing the percentage of *P. aeruginosa* PAO1 (pRGC-Fe) cells that emit fluorescent signals in the cyan, red, and/or green channels as a consequence of P_{pvdA} , P_{pchE} , and P_{bfrB} activation, respectively. The cell count was performed on multiple fields for each $FeCl_3$ concentration ($>2,500$ cells for each condition).

single-copy chromosomal integration in *P. aeruginosa* and for replication in bacterial genera other than *Pseudomonas*.

To generate a stable, single-copy reporter system, the DNA fragment of pRGC carrying the *mCherry*, *gfp*, and *cfp* genes was cloned in the MCS of the integrative plasmid

mini-CTX1. This vector allows single-copy integrase-mediated insertion of exogenous DNA fragments in the *attB* neutral site of *P. aeruginosa* chromosome (37). The resulting mini-RGC plasmid (Fig. 1C) has been validated by generating mini-RGC derivatives in which the *Pkm* constitutive promoter was alternatively cloned upstream of the three reporter genes *mCherry*, *gfp*, and *cfp*. The resulting plasmids were introduced in *P. aeruginosa* PAO1 cells by conjugation, allowing plasmid integration in the *attB* neutral site of the chromosome. As observed for pRGC, confocal microscopy imaging of cells carrying mini-RGC-*Pkm::mCherry*, mini-RGC-*Pkm::gfp*, or mini-RGC *Pkm::cfp* showed clear fluorescent signal detection and no overlap of the three fluorescent protein emission channels (Fig. 5A) despite the lower reporter gene dosage due to single-copy chromosomal integration. The stability of the integrative mini-RGC-*Pkm* derivatives allowed the detection of discrete subpopulations of *P. aeruginosa* PAO1 cells constitutively expressing mCherry, GFP, or CFP in mixed flow cell biofilms cultured for at least 3 days in the absence of antibiotic selection (Fig. 5B). In contrast, fluorescent cells were only sporadically detected in flow cell biofilms formed by *P. aeruginosa* PAO1 carrying pRGC-*Pkm* variants already after 24 h of incubation without antibiotic selection (data not shown). This confirms that the integrative mini-RGC variant represents a suitable tool for long-term expression studies.

A limitation to the use of pRGC consists of the relatively narrow host range of this plasmid; the ColE1-like origin of replication of pRGC ensures its replication in *Enterobacteriaceae* (38), while the pRO1600 origin allows replication in *Pseudomonas* spp. (39). To broaden the pRGC host range, a plasmid variant has been generated by replacing the pRO1600 origin of replication with a DNA fragment of pME6032 (40) encompassing the pVS1 origin of replication, the pVS1 *repA* gene coding for the replication protein RepA, and the *staA* gene coding for the stability protein StaA. The ability of the resulting construct, named pRGC-BHR (broad host range), to replicate in *Acinetobacter baumannii* ATCC 19606^T, *Agrobacterium tumefaciens* C58C1, *Burkholderia dolosa* LMG 18943^T, *Enterobacter aerogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC 27736, and *P. aeruginosa* PAO1 was verified by plasmid electroporation and selection on LB agar plates supplemented with Gm. Moreover, pRGC-BHR functionality was assessed by generating a pRGC-BHR variant containing the *Pkm* promoter (previously verified to drive constitutive expression in *P. aeruginosa*) upstream of each one of the reporter genes *mCherry*, *gfp*, and *cfp*. The resulting pRGC-BHR-*Pkm::3FP* plasmid caused simultaneous emission of fluorescent signals in the red, green, and cyan channels in *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *E. aerogenes*, and *A. tumefaciens* cells (Fig. 5C). Both GFP and CFP were functionally expressed in *B. dolosa*, while no fluorescent signals in the red channel were detected in single cells of this bacterium (Fig. 5C). In *A. baumannii*, mCherry and GFP were expressed at low levels, while no CFP signal was detectable. With the same settings, no fluorescent signals were detected in the tested strains carrying the pRGC-BHR empty vector (data not shown).

The copy number of pRGC-BHR in *P. aeruginosa* PAO1 was 3.9 ± 1.7 copies per chromosome unit, indicating that pRGC-BHR can be classified as a low-copy-number plasmid. Plasmid stability evaluation, performed as described for pRGC, revealed that pRGC-BHR has N_{gm}/N_0 ratios of 0.92 ± 0.21 and 0.88 ± 0.05 in *P. aeruginosa* PAO1 after 10 and 20 generations, respectively, in the absence of antibiotic selection. This indicates that, at least in *P. aeruginosa*, pRGC-BHR is more stable than pRGC.

DISCUSSION

Fundamental insights about bacterial behaviors have been gained from the analysis of bulk populations. However, bulk studies provide information about the mean behavior of the whole bacterial population and do not allow phenotypic differences between individual cells to be detected. Single-cell studies can provide novel insights into bacterial physiology, including adaptive responses to stimuli derived from the environment. Gene expression analysis at the single-cell level is particularly relevant for versatile microorganisms, such as *P. aeruginosa*, which integrates a number of

FIG 5 Functional validation of mini-RGC and pRGC-BHR. (A and B) Confocal microscopy imaging of a mixed coculture of *P. aeruginosa* PAO1 alternatively carrying mini-RGC-Pkm::mCherry, mini-RGC-Pkm::gfp, or mini-RGC-Pkm::cfp (A) and a flow cell biofilm formed by the same mixed coculture as those for panel A (B). (C) Cultures of the indicated bacterial strains carrying the pRGC-BHR-Pkm::3FP plasmid. Representative images of at least three independent experiments are shown. From left to right for panels A and C, bright field, phase contrast; $\lambda_{\text{ex}} 543$, red fluorescence channel imaged with laser excitation at 543 nm, showing *P. aeruginosa* PAO1 cells carrying mini-RGC-Pkm::mCherry; $\lambda_{\text{ex}} 488$, green fluorescence channel imaged with laser excitation at 488 nm, showing *P. aeruginosa* PAO1 cells carrying mini-RGC-Pkm::gfp; $\lambda_{\text{ex}} 458$, cyan fluorescence channel imaged with laser excitation at 458 nm, showing *P. aeruginosa* PAO1 cells carrying mini-RGC-Pkm::cfp; merge, superimposition of phase contrast and fluorescence images. Images from left to right for panel B are the same as those for panels A and C, but the bright field is not shown.

environmental signals through multiple regulatory networks for optimal tuning of gene expression (17, 19).

A growing number of studies have highlighted extensive heterogeneity for key bacterial phenotypic traits, as reviewed in reference 2. Most of these studies made use of fluorescent proteins as reporters. The heterogeneity of single-gene expression is quite simple to study through reporter systems based on one fluorescent protein, while other experimental approaches can be used to study bacterial processes relying on the expression of multiple genes at the single-cell level, e.g., single-cell transcriptomics and proteomics (41, 42). Despite the latter approaches providing valuable information on the overall gene expression profile of single cells, their application to bacteria is limited by the small number of mRNA molecules and proteins per cell, by their cost, and by time-consuming sample preparation, which complicates time course analysis of gene expression (43). On the contrary, the use of fluorescent reporters for single-cell studies is an inexpensive method that does not require cell lysis, so it allows an easier real-time tracking of promoter activity in living cells. However, coupling more than two fluorescent proteins as reporters can result in spectral cross talk, preventing the applicability of this approach to multiple-gene expression analysis in single cells (16).

Most of the studies that succeeded in developing biosensors based on dual or multiple fluorescent proteins have been performed in *Escherichia coli* (44–47), and plasmids for the simultaneous investigation of multiple promoters at the single-cell level also have been described for other bacteria. As an example, Schlüter and colleagues developed a triple reporter cassette for translational fusions based on the expression of the Cerulean, mCherry, and mVenus fluorescent proteins that has been exploited for the single-cell-level analysis of *Sinorhizobium meliloti* phenotypes related to quorum sensing (QS) activation and exopolysaccharide production (12).

Recently, a plasmid based on four reporter genes for different fluorescent proteins (i.e., Turbo YFP, mTag BFP2, mNEON Green, and E2-Orange) was developed for *P. aeruginosa* (14). This plasmid, named pSEVA-QSGEF, has been used to evaluate the impact of salt stress on the activity of QS-related promoters in bulk population via synchronous fluorescent spectroscopy, a technique that guarantees fluorescence signal separation but that has not been employed to gauge the expression of multiple genes in single cells. Similarly, by means of a synthetic biology approach, Han and coworkers investigated the activity of multiple QS systems in *P. aeruginosa* by using a new genetic tool for transcriptional fusions of four different promoters with genes coding for the fluorescent proteins sfGFP, mScarlet1, CyOFP1, and Venus (15). This synthetic plasmid was functional for the analysis of multiple promoters at the single-cell level, but an *ad hoc*-developed algorithm was required to minimize spectral cross talk of the different fluorescent signals. Moreover, promoter cloning upstream of the reporter genes in this plasmid is based on the Gibson assembly strategy (48) and cannot be performed with traditional cloning techniques. Overall, although the genetic tool developed by Han and coworkers is endowed with promising traits, technical hurdles could limit its exploitation by many laboratories.

In this scenario, our work reports the generation of user-friendly genetic tools that can easily be exploited for simultaneous analysis of gene expression directed by up to three promoters in single cells of *P. aeruginosa* and other proteobacteria via fluorescence microscopy without the need for image-processing procedures for spectral cross talk correction.

As expected, the use of pRGC revealed overlapping regions between mCherry and GFP spectra and between GFP and CFP spectra. However, our data demonstrate that problems related to spectral cross talk can be solved by adjusting the confocal microscope setup, in particular the emission bandwidth for fluorescent signal sequential acquisition. Indeed, this approach allowed us to clearly discriminate the three fluorescent channels without the need for unmixing algorithms. Notably, a good emission signal was maintained despite the narrow bandwidth applied to image the fluorescent

proteins when their expression was driven by both the *P_{km}* constitutive promoter and the *P. aeruginosa* iron-regulated promoters *P_{pchE}*, *P_{pvdA}*, and *P_{bfrB}*, likely due to the good quantum yield of the fluorescent reporters (49).

The *P_{pchE}* and *P_{pvdA}* promoters drive the expression of the *pchEFG* operon and *pvdA* gene required for the biosynthesis of the endogenous *P. aeruginosa* siderophores pyochelin and pyoverdine, respectively (30, 31). In the presence of sufficient intracellular iron, the Fur-Fe²⁺ complex directly represses the transcription of the *pchR* and *pvdS* genes, coding for the transcriptional regulator PchR and the alternative sigma factor PvdS, required for *P_{pchE}* and *P_{pvdA}* activation, respectively (34, 50–52). On the contrary, the Fur-Fe²⁺ complex positively regulates the expression of the iron storage bacterioferritin gene *bfrB* via an uncharacterized and PrrF1/PrrF2-independent mechanism (34, 51, 53). Our results on *P_{pchE}*-, *P_{pvdA}*-, and *P_{bfrB}*-driven reporter gene expression in response to different extracellular levels of iron are in line with available literature on bulk *P. aeruginosa* populations. However, single-cell expression analysis of these promoters highlighted some heterogeneity in the response to iron availability, with coexisting subsets of cells expressing the *P_{pchE}* and *P_{pvdA}* iron-repressed promoter and/or the *P_{bfrB}* iron-activated promoter in different combinations, depending on iron concentration in the medium. Interestingly, higher heterogeneity in the expression of the tested iron uptake and iron storage genes was observed for iron concentrations in the low micromolar range (i.e., 0.75 and 3 μ M), a condition in which most cells of the population showed pyochelin gene induction, concomitant with a heterogeneous transition toward the repression of pyoverdine genes and activation of the bacterioferritin gene. The repression of the high-affinity siderophore pyoverdine at iron concentrations in which active transport mediated by pyochelin is still necessary could be a means to prevent toxicity caused by high intracellular iron (51), as also outlined by concomitant induction of the iron storage bacterioferritin gene. It also should be considered that many regulatory systems are involved in the fine regulation of the *P. aeruginosa* iron uptake systems in response to metabolic and environmental cues (e.g., nutrients, oxygen, quorum-sensing signal molecules, c-di-GMP) (54), possibly contributing to the observed heterogeneity. These preliminary observations require further investigation and will hopefully inspire new studies in *P. aeruginosa* aimed at unraveling the activation state of various processes involved in iron scavenging and metabolism at the single-cell level. In this context, pRGC could be used to investigate if different environmental conditions promote the differentiation of *P. aeruginosa* cells in subpopulations adopting distinct iron acquisition strategies, such as siderophore or heme uptake systems (55).

Promoter cloning upstream of the pRGC reporter genes is feasible thanks to the presence of several unique restriction sites. Since the *gfp* and *cfp* genes are located next to each other in divergent orientations, these reporter genes could also be exploited for the analysis of divergently oriented promoters. In addition, pRGC has a modular architecture, with each functional element (e.g., fluorescent reporter genes and regions that ensure plasmid replication in *E. coli* and *P. aeruginosa*) being defined by upstream and downstream unique restriction sites, facilitating the construction of pRGC variants by the replacement of its components.

A major limitation of pRGC is the low stability in *P. aeruginosa* cells without antibiotic selection. Moreover, while multicopy vectors for reporter fusions facilitate the study of weak promoters, their use could not faithfully reflect the physiological behavior of the tested promoters due to the altered DNA-binding site(s)/transcriptional regulator(s) ratio and can result in apparent heterogeneity in gene expression due to nonhomogeneous plasmid distribution among cells. To avoid artifacts due to cell-to-cell variation in plasmid copy number and to overcome problems related to the low stability of pRGC in the absence of antibiotic selection, the mini-RGC plasmid for single-copy chromosomal integration has been generated. In this case, the three fluorescent signals were clearly discernible from each other and disclosed a good signal-to-noise ratio in spite of the lower gene dosage (the reporter

genes were present in one copy per cell). The mini-RGC plasmid does not require antibiotic selection, so it can be exploited for long-term experiments in which antibiotics supply is not advisable, such as continuous cultures, *in vivo* experiments, and biofilm formation assays.

To extend the use of the RGC cassette in bacterial species other than *Pseudomonas* spp., the pRGC-BHR vector was generated by replacing the pUCP18-derived origin of replication of pRGC with the pME6032-derived pVS1 origin. According to the literature, the pVS1 origin enables plasmid replication in bacterial species belonging to the genera *Acinetobacter*, *Aeromonas*, *Agrobacterium*, *Burkholderia*, *Chromobacterium*, *Comamonas*, *Rhizobium*, *Shewanella*, *Sinorhizobium*, and *Xanthomonas*, in addition to *Pseudomonas* spp. (40, 56–61). In the present study, pRGC-BHR replication has been verified in *Acinetobacter*, *Agrobacterium*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Escherichia*, and *Pseudomonas*; however, not all tested strains showed functional expression of the fluorescent proteins when carrying the pRGC-BHR derivative plasmid pRGC-BHR-*Pkm::3FP*. The lack of CFP and *mCherry* expression in *A. baumannii* and *B. dolosa*, respectively, highlights possible codon usage-related issues (25). Moreover, the possibility that low *mCherry* and GFP expression in *A. baumannii* could be due to low *Pkm* promoter activity in this bacterium cannot be ruled out at this stage. Overall, the usability of pRGC-BHR in proteobacteria other than *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *E. aerogenes*, and *A. tumefaciens* requires further investigation.

The pRGC-BHR vector was maintained in most *P. aeruginosa* cells for at least 48 h without antibiotic selection, possibly due to the presence of the stability (*sta*) region derived from pME6032 (56), thereby overcoming a major drawback of pRGC.

Both pRGC and pRGC-BHR can be classified as low-copy-number plasmids. pRGC-BHR was present in 4 copies per cell, consistent with a previous study on the pVS1 origin of replication (56). The pRGC copy number (about 8 copies per cell) is lower than that of other plasmids containing the pRO1600 origin of replication, e.g., pUCP18 and pUCP19 (10 to 25 copies per cell) (23). While plasmid size can affect the copy number, such differences also could be due to the different techniques used for plasmid copy number estimation. It should be considered that in this study, plasmid copy number and stability in the absence of antibiotic selection were determined for the pRGC and pRGC-BHR empty vectors, but the expression level of the reporter genes upon cloning of multiple promoters could affect these parameters.

Overall, this work provides a set of well-characterized and easy-to-use genetic tools for the simultaneous single-cell analysis of up to three different promoters in *Pseudomonas* spp. and potentially in other proteobacteria. Gene expression analysis was performed by fluorescence microscopy in this study, but it could feasibly be extended to flow cytometry and eventually coupled with cell sorting. In particular, flow cytometry would be valuable to obtain quantitative data of fluorescent signals at the single-cell level. Microfluidic devices could also be exploited to perform single-cell studies of bacterial populations grown under tightly controlled conditions (62).

Possible phenotypic differences in single cells of bacterial populations can be observed not only in planktonic cultures but also when cells are organized in densely aggregated and three-dimensional structured populations, such as biofilms or swarming colonies. In particular, biofilm composition unavoidably implies the formation of gradients of nutrients and other cues, possibly resulting in the phenotypic differentiation of bacterial subpopulations (63). In this context, the tools developed in this work could help to study multiple-gene expression not only in single cells of planktonic populations but also in distinct cell clusters within bacterial biofilms.

MATERIALS AND METHODS

Bacterial strains and culture media. The bacterial strains used in this study are listed in Table 1. Bacteria were routinely grown at 37°C in Luria-Bertani (LB) broth under aerated conditions or on LB agar plates (64). *P. aeruginosa* PAO1, carrying the pRGC-Fe plasmid, was grown in M9 minimal medium (64) supplemented with 20 mM glucose as a carbon source and FeCl₃ (Sigma-Aldrich) at the concentrations

indicated in the text. When required, gentamicin (Gm; 10 µg/ml for *Enterobacter aerogenes*, *Escherichia coli*, and *Klebsiella pneumoniae*; 30 µg/ml for *Agrobacterium tumefaciens*; 100 µg/ml for *Acinetobacter baumannii* and *P. aeruginosa*; 600 µg/ml for *Burkholderia dolosa*) or tetracycline (Tc; 200 µg/ml for *P. aeruginosa*; 20 µg/ml for *E. coli*) was added to the medium.

DNA manipulation. Oligonucleotides used in this study are listed in Table 2. Plasmid DNA preparation, restriction enzyme digestions, agarose gel electrophoresis, and ligations were performed using standard methods (64). The transformation of *A. baumannii*, *A. tumefaciens*, *B. dolosa*, *E. aerogenes*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa* was carried out by electroporation (65–69). The presence of pRGC-BHR-Pkm::3FP in the transformed strains was confirmed by PCR and by plasmid extraction and restriction analyses. Plasmid DNA was purified from bacterial cultures using NucleoSpin plasmid (Macherey-Nagel) according to the manufacturer's instructions. PCR amplifications were performed using Thermo Scientific Phusion high-fidelity DNA polymerase. FastDigest restriction enzymes were purchased from Thermo Fisher Scientific. The ligation of DNA fragments was performed with the T4 DNA ligase (Promega). The mini-RGC plasmid and its derivatives were transferred from *E. coli* S17.1λ *pir* donor strains to *P. aeruginosa* PAO1 by conjugation (64).

Plasmid construction. The 1,606-bp fragment required for plasmid replication in *Pseudomonas* spp. (24) was amplified by PCR from pUCP18 (23) with primers pRO1600_FW-pRO1600_RV; the 2,625-bp fragment corresponding to the *aacC1* gene, the ColE1-like replication origin, and the *lacZα* gene was amplified by PCR from pVRL1 (25) with primers Scaffold_FW-Scaffold_RV. The resulting amplicons were digested with *NsiI* and ligated to generate the intermediate pMLR construct (see Fig. S1A in the supplemental material). Subsequently, the 231-bp region encompassing the CAP binding site, the *Plac* promoter, and the first codons of *lacZα* was deleted from pMLR using the Q5 site-directed mutagenesis kit with primers Δ*lac*_FW-Δ*lac*_RV, generating pMLRΔ*lac* (Fig. S1B). The *mCherry* gene was PCR amplified from the pmCherry plasmid (Clontech) using primers *mCherry*_FW-*mCherry*_RV and cloned into pMLRΔ*lac* via *Apal*-*KpnI* digestion, generating pMLR-*mCherry* (Fig. S1C). A DNA fragment encompassing the *gfpmut3b* gene (here referred to as *gfp*) and the *t₀* transcriptional terminator was PCR amplified from the pVRL2-*gfp* plasmid (25) using primers *gfp*_FW-*gfp*_RV and cloned into pMLR-*mCherry* via *SmaI*-*EcoRI* digestion, generating pMLR-*mCherry-gfp* (Fig. S1D). Finally, the *ecfp* gene (here referred to as *cfp*) was PCR amplified from the miniTn7-*cfp* plasmid (70) using primers *cfp*_FW-*cfp*_RV and cloned into pMLR-*mCherry-gfp* with restriction enzymes *NotI*-*SacI*, resulting in the pRGC plasmid (Fig. S1E).

The mini-RGC plasmid was generated by cloning the RGC cassette encompassing the *mCherry*, *gfp*, and *cfp* genes, excised from pRGC by *KpnI*-*SacI* restriction, into the integrative plasmid mini-CTX1 (37).

To generate the pRGC-BHR plasmid, the 4,022-bp fragment encompassing the pVS1 origin of replication, the pVS1 *repA* gene, and the *staA* gene was PCR amplified from pME6032 (40) with primers Ori-pVS1_FW and Ori-pVS1_RV and ligated to pRGC via *NsiI* restriction in place of the pUCP18-derived origin of replication.

To generate pRGC, mini-RGC, and pRGC-BHR variants for constitutive expression of *mCherry*, *gfp*, and *cfp*, the 38-bp promoter sequence of the kanamycin resistance gene (*Pkm*; 5'-CCGGAATTGCCAGCTGGGCGCCCTCTGGTAAGGTTGG-3') was selected (71, 72). Double-strand DNA fragments encompassing *Pkm* were obtained by oligonucleotide annealing; the 5' and 3' ends of each oligonucleotide were designed to obtain, after annealing of the oligonucleotide pairs, three *Pkm*-containing DNA fragments with distinct overhangs ready for ligation with plasmids digested with specific restriction enzymes. In detail, the annealing of the oligonucleotide pairs *Pkm*::*mCherry*_FW/*Pkm*::*mCherry*_RV, *Pkm*::*gfp*_FW/*Pkm*::*gfp*_RV, and *Pkm*::*cfp*_FW/*Pkm*::*cfp*_RV generated 5' and 3' ends allowing cloning of the *Pkm* promoter upstream of the *mCherry*, *gfp*, and *cfp* genes in plasmids digested with *XhoI*-*Apal*, *BamHI*-*SmaI*, and *XbaI*-*NotI*, respectively. For the annealing procedure, 100 ng of each FW and RV oligonucleotide was boiled for 5 min in annealing buffer (145 mM Tris-HCl, pH 7.6, 14.5 mM MgCl₂, 23 mM dithiothreitol). The samples then were incubated at 65°C for 20 min, 42°C for 20 min, 37°C for 20 min, and room temperature for 20 min.

To generate the pRGC-Fe plasmid, the promoter region of the *pchE* gene (*PpchE*) was PCR amplified from the *P. aeruginosa* PAO1 chromosome with primers *PpchE*_FW and *PpchE*_RV and cloned into the pRGC plasmid upstream of the *mCherry* gene via *XhoI*-*Apal* restriction, resulting in the pRGC-*PpchE*::*mCherry* vector. The promoter region of the *bfrB* gene (*PbfrB*) was PCR amplified from the *P. aeruginosa* PAO1 chromosome with primers *PbfrB*_FW and *PbfrB*_RV and cloned into the pRGC-*PpchE*::*mCherry* plasmid upstream of the *gfp* gene via *BamHI*-*SmaI* restriction, resulting in the pRGC-*PpchE*::*mCherry-PbfrB*::*gfp* vector. Finally, the promoter region of the *pvdA* gene (*PpvdA*) was PCR amplified from the *P. aeruginosa* PAO1 chromosome with primers *PpvdA*_FW and *PpvdA*_RV and cloned into the pRGC-*PpchE*::*mCherry-PbfrB*::*gfp* plasmid upstream of the *cfp* gene via *XbaI*-*NotI* restriction, resulting in the pRGC-Fe vector.

Determination of fluorescent protein spectra. Fluorescence excitation and emission spectra of *mCherry*, GFP, and CFP were recorded from cultures of *P. aeruginosa* PAO1 alternatively carrying the pRGC-*Pkm*::*mCherry*, pRGC-*Pkm*::*gfp*, or pRGC-*Pkm*::*cfp* plasmid. As a control, *P. aeruginosa* PAO1 cells carrying the empty vector pRGC were used. Two hundred microliters of overnight cultures of each strain was washed twice with sterile saline and dispensed in a 96-well microtiter plate, and fluorescence spectra were recorded by means of a Tecan Spark 10M microplate reader. Excitation spectra at emission wavelengths of 660 nm for *mCherry*, 580 nm for GFP, and 510 nm for CFP were recorded from 450 to 620 nm for *mCherry*, from 350 to 540 nm for GFP, and from 350 to 470 nm for CFP. Emission spectra at excitation wavelengths of 510 nm for *mCherry*, 430 nm for GFP, and 370 nm for CFP were recorded from 560 to 750 nm for *mCherry*, from 475 to 750 nm for GFP, and from 420 to 750 nm for CFP. Fluorescence

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
Strains		
<i>E. coli</i> DH5 α	Cloning strain	76
<i>E. coli</i> S17.1 λ pir	Conjugative strain for suicide plasmids	77
<i>P. aeruginosa</i> PAO1	ATCC 15692 type strain	ATCC
<i>A. baumannii</i> ATCC 19606 ^T	Type strain	78
<i>A. tumefaciens</i> C58C1	Derivative of C58, cured for pTiC58	79
<i>B. dolosa</i> LMG 18943 ^T	Type strain	80
<i>E. aerogenes</i> ATCC 13048	Type strain	ATCC
<i>K. pneumoniae</i> ATCC 27736	Type strain	ATCC
Plasmids		
pVRL1	<i>E. coli</i> - <i>Acinetobacter</i> sp. shuttle vector used as a source of the region required for plasmid replication in <i>E. coli</i> , <i>aacC1</i> resistance gene and MCS region; Gm ^r	25
pUCP18	<i>E. coli</i> - <i>Pseudomonas</i> sp. shuttle expression vector used as source of the region required for plasmid replication in <i>Pseudomonas</i> ; Cb ^r	23
mini-CTX1	Suicide vector for site-specific integration in <i>P. aeruginosa</i> chromosome; Tc ^r	37
pME6032	IPTG-inducible shuttle expression vector used as source of broad-host-range origin of replication; Tc ^r	40
miniTn7- <i>cfp</i>	Vector used as source of <i>cfp</i> ; Ap ^r , Cm ^r , Gm ^r	70
pVRL2- <i>gfp</i>	Vector used as source of <i>gfp</i> ; Gm ^r	25
pmCherry	Vector used as source of <i>mCherry</i> ; Ap ^r	Clontech
pMLR	<i>E. coli</i> - <i>Pseudomonas</i> sp. shuttle expression vector; Gm ^r	This study
pMLR Δ <i>lac</i>	<i>E. coli</i> - <i>Pseudomonas</i> sp. shuttle expression vector carrying a deletion in the <i>lac</i> promoter region; Gm ^r	This study
pMLR- <i>mCherry</i>	<i>E. coli</i> - <i>Pseudomonas</i> sp. promoter probe shuttle vector for transcriptional fusion carrying <i>mCherry</i> promoterless gene; Gm ^r	This study
pMLR- <i>mCherry-gfp</i>	<i>E. coli</i> - <i>Pseudomonas</i> sp. promoter probe shuttle vector for transcriptional fusion carrying <i>mCherry</i> and <i>gfp</i> promoterless genes; Gm ^r	This study
pRGC	<i>E. coli</i> - <i>Pseudomonas</i> sp. promoter probe shuttle vector for transcriptional fusion carrying <i>mCherry</i> <i>gfp</i> and <i>cfp</i> promoterless genes; Gm ^r	This study
pRGC- <i>Pkm::mCherry</i>	pRGC plasmid for the <i>Pkm</i> -driven constitutive expression of <i>mCherry</i> ; Gm ^r	This study
pRGC- <i>Pkm::gfp</i>	pRGC plasmid for the <i>Pkm</i> -driven constitutive expression of <i>gfp</i> ; Gm ^r	This study
pRGC- <i>Pkm::cfp</i>	pRGC plasmid for the <i>Pkm</i> -driven constitutive expression of <i>cfp</i> ; Gm ^r	This study
pRGC- <i>Pkm::mCherry-Pkm::gfp</i>	pRGC plasmid for the <i>Pkm</i> -driven constitutive expression of <i>mCherry</i> and <i>gfp</i> ; Gm ^r	This study
pRGC- <i>Pkm::mCherry-Pkm::cfp</i>	pRGC plasmid for the <i>Pkm</i> -driven constitutive expression of <i>mCherry</i> and <i>cfp</i> ; Gm ^r	This study
pRGC- <i>Pkm::gfp-Pkm::cfp</i>	pRGC plasmid for the <i>Pkm</i> -driven constitutive expression of <i>gfp</i> and <i>cfp</i> ; Gm ^r	This study
pRGC- <i>Pkm::3FP</i>	pRGC plasmid for the <i>Pkm</i> -driven constitutive expression of <i>mCherry</i> , <i>gfp</i> and <i>cfp</i> ; Gm ^r	This study
pRGC-Fe	pRGC plasmid in which <i>Ppche</i> , <i>PpbfB</i> , and <i>PpvdA</i> iron-regulated promoters drive the expression of <i>mCherry</i> , <i>gfp</i> , and <i>cfp</i> , respectively; Gm ^r	This study
mini-RGC	Promoter-probe suicide vector for site-specific integration of the RGC reporter cassette in <i>P. aeruginosa</i> chromosome; Tc ^r	This study
mini-RGC- <i>Pkm::mCherry</i>	Mini-RGC plasmid for the <i>Pkm</i> -driven constitutive expression of <i>mCherry</i> ; Tc ^r	This study
mini-RGC- <i>Pkm::gfp</i>	Mini-RGC plasmid for the <i>Pkm</i> -driven constitutive expression of <i>gfp</i> ; Tc ^r	This study
mini-RGC- <i>Pkm::cfp</i>	Mini-RGC plasmid for the <i>Pkm</i> -driven constitutive expression of <i>cfp</i> ; Tc ^r	This study
pRGC-BHR	Broad-host-range promoter probe shuttle vector for transcription fusions carrying <i>mCherry</i> , <i>gfp</i> and <i>cfp</i> promoterless genes; Gm ^r	This study
pRGC-BHR- <i>Pkm::3FP</i>	pRGC-BHR plasmid for the <i>Pkm</i> -driven constitutive expression of <i>mCherry</i> , <i>gfp</i> and <i>cfp</i> ; Gm ^r	This study

^aGm^r, gentamicin resistance; Tc^r, tetracycline resistance; Cb^r, carbenicillin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; IPTG, isopropyl- β -D-thiogalactopyranoside.

emission values were expressed as relative fluorescence units (RFU). RFU values were normalized to the corresponding cell density (optical density at 600 nm [OD₆₀₀]) of the sample, and the contribution of *P. aeruginosa* autofluorescence was removed by subtracting the RFU values recorded for *P. aeruginosa* PAO1 cells carrying the empty vector pRGC. For each excitation and emission spectra, the maximum value of RFU was considered 100%.

Single-cell fluorescence analysis via laser-scanning confocal microscopy imaging. For microscopy imaging, 5 μ l of bacterial cultures were spotted on a microscope glass slide overlaid with 0.5% (wt/vol) agarose. Fluorescent microscopy analyses were performed with a Leica SP5 confocal laser-scanning microscope equipped with a 63 $\times$ oil immersion objective. The LasX software (Leica) was used for image acquisition. For each sample, multiple phase contrast and fluorescence images were acquired with the following excitation and emission parameters: mCherry, λ_{ex} of 543 nm, λ_{em} ranging from 620 nm to 740 nm; GFP, λ_{ex} of 488 nm, λ_{em} ranging from 548 nm to 555 nm; CFP, λ_{ex} of 458 nm, λ_{em} ranging from 468 nm to 480 nm.

TABLE 2 Oligonucleotides used in this study

Oligonucleotide	Sequence ^a	Restriction site
Scaffold_FW	CCAATGCATCAGCAGCAACAGCAGCAG	Nsil
Scaffold_RV	CCAATGCATGTCTACGGCATCCTCCAG	Nsil
pRO1600_FW	CCAATGCATGGATCTAGGTGAAGATCCTTT	Nsil
pRO1600_RV	CCAATGCATGTTACCCAACCTAATCGCCTT	Nsil
Δlac _FW	TGGGCGCTCTTCCGCTTC	
Δlac _RV	GCAATTAACCCCTCACTAAAGGGAAC	
mCherry_FW	TATGGGCGCTAAGTAAGTAAAAAGAGGAGAAATTAAGCATGGTGAGCAAGGGCGAG	Apal
mCherry_RV	CCGGGTACCCCTCACTTGATACAGCTCGTCC	KpnI
gfp_FW	TCCCCCGGGTAAGTAAGTAATTAAGAGGAGAAATTAAGCATG	SmaI
gfp_RV	CCGGAATTCCTCCTGAAAAATCTCGCCAAG	EcoRI
cfp_FW	TATGCGGCCGCTAACTAACTAAAGAGGAGAAATTAAGCATGGTGAGCAAGGGCGAGG	NotI
cfp_RV	AGGGAGCTCTTACTTGATACAGCTCGTCCAT	SacI
Pkm::mCherry_FW	TGAGCCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGGGCC	XhoI
Pkm::mCherry_RV	CCCAACCTTACCAGAGGGCGCCCAAGCTGGCAATTCGGC	Apal
Pkm::gfp_FW	GATCCCCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGCC	BamHI
Pkm::gfp_RV	GGGCCAACCTTACCAGAGGGCGCCCAAGCTGGCAATTCGGC	SmaI
Pkm::cfp_FW	CTAGACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGC	XbaI
Pkm::cfp_RV	GGCCGCCCAACCTTACCAGAGGGCGCCCAAGCTGGCAATTCGGGT	NotI
PpchE_FW	CCGCTCGAGCTCCCCAGCGAGATCCG	XhoI
PpchE_RV	CCGGGGCCCGCATCTGAGGTCGGTTGCC	Apal
PbfrB_FW	CGCGGATCCCGTTCTGTCGCTGCCTGAAAA	BamHI
PbfrB_RV	TCCCCCGGGTCTGATGGGTGTGATTCTG	SmaI
PpvdA_FW	TGCTCTAGACCCATTGCGAGATCGCCGA	XbaI
PpvdA_RV	ATAAGAATGCGGCCGCTTGGCCCCCTTTCAAGCGG	NotI
Ori-pVS1_FW	CCAATGCATCATGTGCGACAAGTCCTAAG	Nsil
Ori-pVS1_RV	CCAATGCATACCTATCTCAGCGATCTGTC	Nsil
aacC1_FW	GATCTATATCTATGATCTCGC	
aacC1_RV	GATCACATAAGCACCAAGCG	
pqsC_FW	GCTCGCAGCTACGAAACG	
pqsC_RV	CGAGGAAGCCGAACAGATC	

^aRestriction sites are underlined in the primer sequences.

Growth curves of *P. aeruginosa* strains carrying pRGC variants. Cultures of *P. aeruginosa* carrying the empty vector pRGC, pRGC-Pkm::mCherry, pRGC-Pkm::gfp, pRGC-Pkm::cfp, or pRGC-Pkm::3FP were incubated overnight at 37°C with shaking in LB supplemented with 100 μ g/ml Gm. After overnight growth, cultures were washed with fresh LB and diluted to an OD₆₀₀ of 0.05 in LB supplemented with 100 μ g/ml Gm. Two hundred microliters of each culture was dispensed in a 96-well microtiter plate. The OD₆₀₀ of the cultures was determined every 2 h with the Tecan Spark 10M.

Biofilm assays. Biofilm formation on a glass surface was investigated in a flow cell system, as previously described (73). Briefly, flow cells (Technical University of Denmark [DTU]) were inoculated with a 1:1:1 mixture of *P. aeruginosa* PAO1 cultures alternatively carrying the pRGC-Pkm::mCherry, pRGC-Pkm::gfp, or pRGC-Pkm::cfp plasmid. Stationary-phase cultures were diluted 1:100 in 1% (vol/vol) tryptic soy broth (TSB) (64) before injection in the flow cell. Flow of fresh 1% (vol/vol) TSB (170 μ l/min) was initiated after 1 h of incubation at room temperature. Biofilms were imaged after 3 days of incubation at 25°C with a Leica SP5 confocal laser scanning microscope. The LasX software was used for image acquisition. Multiple fluorescence images were acquired with lasers at an λ_{ex} of 543 nm (for mCherry), λ_{ex} of 488 nm (for GFP), and λ_{ex} of 458 nm (for CFP). Multidimensional images were processed with the open-source image processing software ImageJ.

Pyoverdine quantification. Pyoverdine production was measured as previously described (74). Briefly, overnight cultures of *P. aeruginosa* pRGC-Fe grown in the M9 minimal medium supplemented with 100 μ g/ml Gm were washed twice with fresh M9 and diluted to an OD₆₀₀ of 0.05 in M9 supplemented with different FeCl₃ concentrations (ranging from 0 to 12 μ M). After 7 h of incubation at 37°C with shaking, pyoverdine was measured in the resulting cultures as the A₄₀₅ of culture supernatants appropriately diluted in 100 mM Tris-HCl (pH 8.0), and this value was normalized to the cell density of bacterial cultures (OD₆₀₀).

Determination of plasmid stability and plasmid copy number. The pRGC and pRGC-BHR plasmids were introduced in *P. aeruginosa* PAO1 cells for stability assays. Plasmid-harboring strains were grown for 18 h in LB supplemented with 100 μ g/ml Gm, diluted 1:1,000 in LB without antibiotic, and cultured for an additional 24 h at 37°C. The resulting cultures were again diluted 1:1,000 in LB without antibiotic before incubation for an additional 24 h at 37°C. Numbers of CFU were determined each 24 h both on LB agar plates without antibiotics (N_0) and on LB agar plates supplemented with 100 μ g/ml Gm (N_{Gm}). Plasmid stability was determined as the N_{Gm}/N_0 ratio.

The copy number of pRGC and pRGC-BHR plasmids in *P. aeruginosa* PAO1 was determined by RT-qPCR, as previously described (75), with minor modifications. Two primer pairs for the amplification of

the plasmid Gm resistance gene (*aacC1*) and of the chromosomal *pqsC* gene (GenBank accession no. [AAG04387.1](#)) were designed. Total DNA from *P. aeruginosa* PAO1 cultures alone or harboring the plasmids was purified using the Wizard genomic DNA purification kit (Promega) by following the manufacturer's instructions. The pRGC and pRGC-BHR plasmids were purified with NucleoSpin plasmid (Macherey-Nagel) from *P. aeruginosa* PAO1 cells by following the manufacturer's instructions. The amount of DNA in each sample was quantified by spectrophotometric analysis and adjusted to a concentration of 25 ng/μl. RT-qPCR was performed using the AriaMX real-time PCR system (Agilent Technologies) with software version 1.0. Reactions were performed in 20 μl, containing 1× iTaq universal SYBR green supermix (Bio-Rad), 0.4 μM each primer, and 2 μl of diluted template DNA (final DNA amount ranging from 0.19 ng to 50 ng per sample). Separate reaction mixtures were prepared for the detection of chromosomal or plasmid-specific amplicons for each template DNA concentration. The thermal cycling protocol was initial denaturation of 4 min at 95°C, followed by 40 cycles of 15 s at 95°C and 45 s at 60°C. Cycle threshold (C_T) values were determined after automatic adjustment of the baseline. Standard curves ($R^2 \geq 0.99$) from five independent samples containing PAO1 chromosomal DNA (chromosome alone) or plasmid alone were generated placing the log value of the amount of template DNA (determined according to dilution) on the x axis and average C_T on the y axis. These standard curves were used to extrapolate the copy number of pRGC and pRGC-BHR in samples containing both chromosomal and plasmid DNA.

Data availability. The full-length pRGC, mini-RGC, and pRGC-BHR sequences have been deposited in the GenBank database under the accession numbers [MW148406](#), [MW148404](#), and [MW148405](#), respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4 MB.

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REFERENCES

- Dubnau D, Losick R. 2006. Bistability in bacteria. *Mol Microbiol* 61:564–572. <https://doi.org/10.1111/j.1365-2958.2006.05249.x>.
- Ackermann M. 2015. A functional perspective on phenotypic heterogeneity in microorganisms. *Nat Rev Microbiol* 13:497–508. <https://doi.org/10.1038/nrmicro3491>.
- Avery SV. 2005. Cell individuality: the bistability of competence development. *Trends Microbiol* 13:459–462. <https://doi.org/10.1016/j.tim.2005.08.006>.
- Veening JW, Stewart EJ, Berngruber TW, Taddei F, Kuipers OP, Hamoen LW. 2008. Bet-hedging and epigenetic inheritance in bacterial cell development. *Proc Natl Acad Sci U S A* 105:4393–4398. <https://doi.org/10.1073/pnas.0700463105>.
- Lewis K. 2010. Persister cells. *Annu Rev Microbiol* 64:357–372. <https://doi.org/10.1146/annurev.micro.112408.134306>.
- Grote J, Kryciak D, Streit WR. 2015. Phenotypic heterogeneity, a phenomenon that may explain why quorum sensing does not always result in truly homogenous cell behavior. *Appl Environ Microbiol* 81:5280–5289. <https://doi.org/10.1128/AEM.00900-15>.
- Schreiber F, Ackermann M. 2020. Environmental drivers of metabolic heterogeneity in clonal microbial populations. *Curr Opin Biotechnol* 62:202–211. <https://doi.org/10.1016/j.copbio.2019.11.018>.
- Veening JW, Smits WK, Kuipers OP. 2008. Bistability, epigenetics, and bet-hedging in bacteria. *Annu Rev Microbiol* 62:193–210. <https://doi.org/10.1146/annurev.micro.62.081307.163002>.
- West SA, Cooper GA. 2016. Division of labour in microorganisms: an evolutionary perspective. *Nat Rev Microbiol* 14:716–723. <https://doi.org/10.1038/nrmicro.2016.111>.
- Fagerlund A, Dubois T, Økstad OA, Verplaetse E, Gilois N, Bennaceur I, Perchat S, Gominet M, Aymerich S, Kolstø AB, Lereclus D, Gohar M. 2014. SinR controls enterotoxin expression in *Bacillus thuringiensis* biofilms. *PLoS One* 9:e87532. <https://doi.org/10.1371/journal.pone.0087532>.
- Solopova A, van Gestel J, Weissing FJ, Bachmann H, Teusink B, Kok J, Kuipers OP. 2014. Bet-hedging during bacterial diauxic shift. *Proc Natl Acad Sci U S A* 111:7427–7432. <https://doi.org/10.1073/pnas.1320063111>.
- Schlüter JP, Czuppon P, Schauer O, Pfaffelhuber P, McIntosh M, Becker A. 2015. Classification of phenotypic subpopulations in isogenic bacterial cultures by triple promoter probing at single cell level. *J Biotechnol* 198:3–14. <https://doi.org/10.1016/j.jbiotec.2015.01.021>.
- Bervoets I, Charlier D. 2018. A novel and versatile dual fluorescent reporter tool for the study of gene expression and regulation in multi- and single-copy number. *Gene* 642:474–482. <https://doi.org/10.1016/j.gene.2017.11.061>.

14. Zhang C, Parrello D, Brown PJB, Wall JD, Hu Z. 2018. A novel whole-cell biosensor of *Pseudomonas aeruginosa* to monitor the expression of quorum sensing genes. *Appl Microbiol Biotechnol* 102:6023–6038. <https://doi.org/10.1007/s00253-018-9044-z>.
15. Han J, Xia A, Huang Y, Ni L, Chen W, Jin Z, Yang S, Jin F. 2019. Simultaneous visualization of multiple gene expression in single cells using an engineered multicolor reporter toolbox and approach of spectral crosstalk correction. *ACS Synth Biol* 8:2536–2546. <https://doi.org/10.1021/acssynbio.9b00223>.
16. Shaner NC, Steinbach PA, Tsien RY. 2005. A guide to choosing fluorescent proteins. *Nat Methods* 2:905–909. <https://doi.org/10.1038/nmeth819>.
17. Moradali MF, Ghods S, Rehm BH. 2017. *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. *Front Cell Infect Microbiol* 7:39. <https://doi.org/10.3389/fcimb.2017.00039>.
18. Crone S, Vives-Flórez M, Kvich L, Saunders AM, Malone M, Nicolaisen MH, Martínez-García E, Rojas-Acosta C, Catalina Gomez-Puerto M, Calum H, Whiteley M, Kolter R, Bjarnsholt T. 2020. The environmental occurrence of *Pseudomonas aeruginosa*. *APMIS* 128:220–231. <https://doi.org/10.1111/apm.13010>.
19. Balasubramanian D, Schnepel L, Kumari H, Mathee K. 2013. A dynamic and intricate regulatory network determines *Pseudomonas aeruginosa* virulence. *Nucleic Acids Res* 41:1–20. <https://doi.org/10.1093/nar/gks1039>.
20. Rietsch A, Mekalanos JJ. 2006. Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*. *Mol Microbiol* 59:807–820. <https://doi.org/10.1111/j.1365-2958.2005.04990.x>.
21. Turner KH, Vallet-Gely I, Dove SL. 2009. Epigenetic control of virulence gene expression in *Pseudomonas aeruginosa* by a LysR-type transcription regulator. *PLoS Genet* 5:e1000779. <https://doi.org/10.1371/journal.pgen.1000779>.
22. Armbruster CR, Lee CK, Parker-Gilham J, de Anda J, Xia A, Zhao K, Murakami K, Tseng BS, Hoffman LR, Jin F, Harwood CS, Wong GC, Parsek MR. 2019. Heterogeneity in surface sensing suggests a division of labor in *Pseudomonas aeruginosa* populations. *Elife* 8:e45084. <https://doi.org/10.7554/eLife.45084>.
23. Schweizer HP. 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* 97:109–121. [https://doi.org/10.1016/0378-1119\(91\)90016-5](https://doi.org/10.1016/0378-1119(91)90016-5).
24. West SE, Schweizer HP, Dall C, Sample AK, Runyen-Janecky LJ. 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* 148:81–86. [https://doi.org/10.1016/0378-1119\(94\)90237-2](https://doi.org/10.1016/0378-1119(94)90237-2).
25. Lucidi M, Runci F, Rampioni G, Frangipani E, Leoni L, Visca P. 2018. New shuttle vectors for gene cloning and expression in multidrug-resistant *Acinetobacter* species. *Antimicrob Agents Chemother* 62:e02480-17. <https://doi.org/10.1128/AAC.02480-17>.
26. Mayer MP. 1995. A new set of useful cloning and expression vectors derived from pBlueScript. *Gene* 163:41–46. [https://doi.org/10.1016/0378-1119\(95\)00389-n](https://doi.org/10.1016/0378-1119(95)00389-n).
27. Heim R, Prasher DC, Tsien RY. 1994. Wavelength mutations and posttranslational autooxidation of green fluorescent protein. *Proc Natl Acad Sci U S A* 91:12501–12504. <https://doi.org/10.1073/pnas.91.26.12501>.
28. Cormack BP, Valdivia RH, Falkow S. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173:33–38. [https://doi.org/10.1016/0378-1119\(95\)00685-0](https://doi.org/10.1016/0378-1119(95)00685-0).
29. Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol* 22:1567–1572. <https://doi.org/10.1038/nbt1037>.
30. Visca P, Ciervo A, Orsi N. 1994. Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdine biosynthetic enzyme L-ornithine N5-oxygenase in *Pseudomonas aeruginosa*. *J Bacteriol* 176:1128–1140. <https://doi.org/10.1128/jb.176.4.1128-1140.1994>.
31. Reimann C, Serino L, Beyeler M, Haa D. 1998. Dihydroaeruginosic acid synthetase and pyochelin synthetase, products of the *pchEF* genes, are induced by extracellular pyochelin in *Pseudomonas aeruginosa*. *Microbiology* 144:3135–3148. <https://doi.org/10.1099/00221287-144-11-3135>.
32. Ochsner UA, Wilderman PJ, Vasil AI, Vasil ML. 2002. GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. *Mol Microbiol* 45:1277–1287. <https://doi.org/10.1046/j.1365-2958.2002.03084.x>.
33. Ma JF, Ochsner UA, Klotz MG, Nanayakkara VK, Howell ML, Johnson Z, Posey JE, Vasil ML, Monaco JJ, Hassett DJ. 1999. Bacterioferritin A modulates catalase A (KatA) activity and resistance to hydrogen peroxide in *Pseudomonas aeruginosa*. *J Bacteriol* 181:3730–3742. <https://doi.org/10.1128/JB.181.12.3730-3742.1999>.
34. Palma M, Worgall S, Quadri LE. 2003. Transcriptome analysis of the *Pseudomonas aeruginosa* response to iron. *Arch Microbiol* 180:374–379. <https://doi.org/10.1007/s00203-003-0602-z>.
35. Weeratunga SK, Lovell S, Yao H, Battaile KP, Fischer CJ, Gee CE, Rivera M. 2010. Structural studies of bacterioferritin B from *Pseudomonas aeruginosa* suggest a gating mechanism for iron uptake via the ferroxidase center. *Biochemistry* 49:1160–1175. <https://doi.org/10.1021/bi9015204>.
36. Pasqua M, Visaggio D, Lo Sciuto A, Genah S, Banin E, Visca P, Imperi F. 2017. Ferric uptake regulator Fur is conditionally essential in *Pseudomonas aeruginosa*. *J Bacteriol* 199:e00472–17. <https://doi.org/10.1128/JB.00472-17>.
37. Hoang TT, Kutchma AJ, Becher A, Schweizer HP. 2000. Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid* 43:59–72. <https://doi.org/10.1006/plas.1999.1441>.
38. Kues U, Stahl U. 1989. Replication of plasmids in Gram-negative bacteria. *Microbiol Rev* 53:491–516. <https://doi.org/10.1128/MR.53.4.491-516.1989>.
39. Olsen RH, DeBusscher G, McCombie WR. 1982. Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. *J Bacteriol* 150:60–69. <https://doi.org/10.1128/JB.150.1.60-69.1982>.
40. Heeb S, Itoh Y, Nishijyo T, Schnider U, Keel C, Wade J, Walsh U, O'Gara F, Haas D. 2000. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in Gram-negative, plant-associated bacteria. *Mol Plant Microbe Interact* 13:232–237. <https://doi.org/10.1094/MPMI.2000.13.2.232>.
41. Newman JR, Ghaemmaghami S, Ihmels J, Breslow DK, Noble M, DeRisi JL, Weissman JS. 2006. Single-cell proteomic analysis of *S. cerevisiae* reveals the architecture of biological noise. *Nature* 441:840–846. <https://doi.org/10.1038/nature04785>.
42. Sorek R, Cossart P. 2010. Prokaryotic transcriptomics: a new view on regulation, physiology and pathogenicity. *Nat Rev Genet* 11:9–16. <https://doi.org/10.1038/nrg2695>.
43. Heinemann M, Zenobi R. 2011. Single cell metabolomics. *Curr Opin Biotechnol* 22:26–31. <https://doi.org/10.1016/j.copbio.2010.09.008>.
44. Pedraza JM, van Oudenaarden A. 2005. Noise propagation in gene networks. *Science* 307:1965–1969. <https://doi.org/10.1126/science.1109090>.
45. Dunlop MJ, Cox RS, III, Levine JH, Murray RM, Elowitz MB. 2008. Regulatory activity revealed by dynamic correlations in gene expression noise. *Nat Genet* 40:1493–1498. <https://doi.org/10.1038/ng.281>.
46. Cox RS, III, Dunlop MJ, Elowitz MB. 2010. A synthetic three-color scaffold for monitoring genetic regulation and noise. *J Biol Eng* 4:10. <https://doi.org/10.1186/1754-1611-4-10>.
47. Ravikumar S, Ganesh I, Yoo I-K, Hong SH. 2012. Construction of a bacterial biosensor for zinc and copper and its application to the development of multifunctional heavy metal adsorption bacteria. *Process Biochem* 47:758–765. <https://doi.org/10.1016/j.procbio.2012.02.007>.
48. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison IIC, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6:343–345. <https://doi.org/10.1038/nmeth.1318>.
49. Rodriguez EA, Campbell RE, Lin JY, Lin MZ, Miyawaki A, Palmer AE, Shu X, Zhang J, Tsien RY. 2017. The growing and glowing toolbox of fluorescent and photoactive proteins. *Trends Biochem Sci* 42:111–129. <https://doi.org/10.1016/j.tibs.2016.09.010>.
50. Leoni L, Ciervo A, Orsi N, Visca P. 1996. Iron-regulated transcription of the *pvdA* gene in *Pseudomonas aeruginosa*: effect of Fur and PvdS on promoter activity. *J Bacteriol* 178:2299–2313. <https://doi.org/10.1128/jb.178.8.2299-2313.1996>.
51. Vasil ML, Ochsner UA. 1999. The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol Microbiol* 34:399–413. <https://doi.org/10.1046/j.1365-2958.1999.01586.x>.
52. Leoni L, Orsi N, de Lorenzo V, Visca P. 2000. Functional analysis of PvdS, an iron starvation sigma factor of *Pseudomonas aeruginosa*. *J Bacteriol* 182:1481–1491. <https://doi.org/10.1128/jb.182.6.1481-1491.2000>.
53. Wilderman PJ, Sowa NA, FitzGerald DJ, FitzGerald PC, Gottesman S, Ochsner UA, Vasil ML. 2004. Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. *Proc Natl Acad Sci U S A* 101:9792–9797. <https://doi.org/10.1073/pnas.0403423101>.
54. Llamas MA, Imperi F, Visca P, Lamont IL. 2014. Cell-surface signaling in *Pseudomonas*: stress responses, iron transport, and pathogenicity. *FEMS Microbiol Rev* 38:569–597. <https://doi.org/10.1111/1574-6976.12078>.
55. Huang W, Wilks A. 2017. Extracellular heme uptake and the challenge of bacterial cell membranes. *Annu Rev Biochem* 86:799–823. <https://doi.org/10.1146/annurev-biochem-060815-014214>.

56. Itoh Y, Watson JM, Haas D, Leisinger T. 1984. Genetic and molecular characterization of the *Pseudomonas* plasmid pVS1. *Plasmid* 11:206–220. [https://doi.org/10.1016/0147-619x\(84\)90027-1](https://doi.org/10.1016/0147-619x(84)90027-1).
57. Van den Eede G, Deblaere R, Goethals K, Van Montagu M, Holsters M. 1992. Broad host range and promoter selection vectors for bacteria that interact with plants. *Mol Plant Microbe Interact* 5:228–234. <https://doi.org/10.1094/mpmi-5-228>.
58. Miller WG, Leveau JH, Lindow SE. 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Mol Plant Microbe Interact* 13:1243–1250. <https://doi.org/10.1094/MPMI.2000.13.11.1243>.
59. Stuurman N, Pacios Bras C, Schlaman HR, Wijffes AH, Bloemberg G, Spaik HP. 2000. Use of green fluorescent protein color variants expressed on stable broad-host-range vectors to visualize rhizobia interacting with plants. *Mol Plant Microbe Interact* 13:1163–1169. <https://doi.org/10.1094/MPMI.2000.13.11.1163>.
60. Lagendijk EL, Validov S, Lamers GE, de Weert S, Bloemberg GV. 2010. Genetic tools for tagging Gram-negative bacteria with mCherry for visualization *in vitro* and in natural habitats, biofilm and pathogenicity studies. *FEMS Microbiol Lett* 305:81–90. <https://doi.org/10.1111/j.1574-6968.2010.01916.x>.
61. Wilton R, Ahrendt AJ, Shinde S, Sholto-Douglas DJ, Johnson JL, Brennan MB, Kemner KM. 2017. A new suite of plasmid vectors for fluorescence-based imaging of root colonizing pseudomonads. *Front Plant Sci* 8:2242. <https://doi.org/10.3389/fpls.2017.02242>.
62. Weaver WM, Tseng P, Kunze A, Masaali M, Chung AJ, Dudani JS, Kittur H, Kulkarni RP, Di Carlo D. 2014. Advances in high-throughput single-cell microtechnologies. *Curr Opin Biotechnol* 25:114–123. <https://doi.org/10.1016/j.copbio.2013.09.005>.
63. van Gestel J, Vlamakis H, Kolter R. 2015. Division of labor in biofilms: the ecology of cell differentiation. *Microbiol Spectr* 3:MB-0002-2014. <https://doi.org/10.1128/microbiolspec.MB-0002-2014>.
64. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
65. Wirth R, Friesenegger A, Fiedler S. 1989. Transformation of various species of Gram-negative bacteria belonging to 11 different genera by electroporation. *Mol Gen Genet* 216:175–177. <https://doi.org/10.1007/BF00332248>.
66. Farinha MA, Kropinski AM. 1990. High efficiency electroporation of *Pseudomonas aeruginosa* using frozen cell suspensions. *FEMS Microbiol Lett* 58:221–225. [https://doi.org/10.1016/S0378-1097\(05\)80043-6](https://doi.org/10.1016/S0378-1097(05)80043-6).
67. Weigel D, Glazebrook J. 2006. Transformation of *Agrobacterium* using electroporation. *CSH Protoc* 2006:pd.b.prot4665. <https://doi.org/10.1101/pdb.prot4665>.
68. Dubarry N, Du W, Lane D, Pasta F. 2010. Improved electrotransformation and decreased antibiotic resistance of the cystic fibrosis pathogen *Burkholderia cenocepacia* strain J2315. *Appl Environ Microbiol* 76:1095–1102. <https://doi.org/10.1128/AEM.02123-09>.
69. Lucidi M, Visaggio D, Prencipe E, Imperi F, Rampioni G, Cincotti G, Leoni L, Visca P. 2019. New shuttle vectors for real-time gene expression analysis in multidrug-resistant *Acinetobacter* species: *in vitro* and *in vivo* responses to environmental stressors. *Appl Environ Microbiol* 85:e01334-19. <https://doi.org/10.1128/AEM.01334-19>.
70. Lamberts L, Sternberg C, Molin S. 2004. Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. *Environ Microbiol* 6:726–732. <https://doi.org/10.1111/j.1462-2920.2004.00605.x>.
71. Stapleton MJ, Jagger KS, Warren RL. 1984. Transposon mutagenesis of *Pseudomonas aeruginosa* exoprotease genes. *J Bacteriol* 157:7–12. <https://doi.org/10.1128/JB.157.1.7-12.1984>.
72. Beck E, Ludwig G, Auerswald EA, Reiss B, Schaller H. 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene* 19:327–336. [https://doi.org/10.1016/0378-1119\(82\)90023-3](https://doi.org/10.1016/0378-1119(82)90023-3).
73. Sternberg C, Tolker-Nielsen T. 2006. Growing and analyzing biofilms in flow cells. *Curr Protoc Microbiol* Chapter 1:Unit 1B.2. <https://doi.org/10.1002/9780471729259.mc01b02s00>.
74. Rampioni G, Bertani I, Pillai CR, Venturi V, Zennaro E, Leoni L. 2012. Functional characterization of the quorum sensing regulator RsaL in the plant-beneficial strain *Pseudomonas putida* WCS358. *Appl Environ Microbiol* 78:726–734. <https://doi.org/10.1128/AEM.06442-11>.
75. Lee C, Kim J, Shin SG, Hwang S. 2006. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. *J Biotechnol* 123:273–280. <https://doi.org/10.1016/j.jbiotec.2005.11.014>.
76. Grant SG, Jessee J, Bloom FR, Hanahan D. 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. *Proc Natl Acad Sci U S A* 87:4645–4649. <https://doi.org/10.1073/pnas.87.12.4645>.
77. Simon R, Priefer U, Pühler A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Nat Biotechnol* 1:784–791. <https://doi.org/10.1038/nbt1183-784>.
78. Janssen P, Maquelin K, Coopman R, Tjernberg I, Bouvet P, Kersters K, Dijkshoorn L. 1997. Discrimination of *Acinetobacter* genomic species by AFLP fingerprinting. *Int J Syst Bacteriol* 47:1179–1187. <https://doi.org/10.1099/00207713-47-4-1179>.
79. Deblaere R, Bytebier B, De Greve H, Deboeck F, Schell J, Van Montagu M, Leemans J. 1985. Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. *Nucleic Acids Res* 13:4777–4788. <https://doi.org/10.1093/nar/13.13.4777>.
80. Coenye T, LiPuma JJ, Henry D, Hoste B, Vandemeulebroecke K, Gillis M, Speert DP, Vandamme P. 2001. *Burkholderia cepacia* genomovar VI, a new member of the *Burkholderia cepacia* complex isolated from cystic fibrosis patients. *Int J Syst Evol Microbiol* 51:271–279. <https://doi.org/10.1099/00207713-51-2-271>.