ABSTRACT: The pyochelin (PCH) siderophore produced by the pathogenic bacterium Pseudomonas aeruginosa is an important virulence factor, acting as a growth promoter during infection. While strong evidence exists for PCH production in vivo, PCH quantification in biological samples is problematic due to analytical complexity, requiring extraction from large volumes and time-consuming purification steps. Here, the construction of a bioluminescent whole cell-based biosensor, which allows rapid, sensitive, and single-step PCH quantification in biological samples, is reported. The biosensor was engineered by fusing the promoter of the PCH biosynthetic gene pchE to the luxCDABE operon, and the resulting construct was inserted into the chromosome of the ΔpvdAΔpchDΔfpvA siderophore-null P. aeruginosa mutant. A bioassay was setup in a 96-well microplate format, enabling the contemporary screening of several samples in a few hours. A linear response was observed for up to 40 nM PCH, with a lower detection limit of 1.64 ± 0.26 nM PCH. Different parameters were considered to calibrate the biosensor, and a detailed step-by-step operation protocol, including troubleshooting specific problems that can arise during sample preparation, was established to achieve rapid, sensitive, and specific PCH quantification in both P. aeruginosa culture supernatants and biological samples. The biosensor was implemented as a screening tool to detect PCH-producing P. aeruginosa strains on a solid medium.

KEYWORDS: bioluminescence, biosensor, luciferase, pyochelin, Pseudomonas aeruginosa, siderophore
implicated in PCH biosynthesis and uptake are repressed by PchR in the absence of cytoplasmic PCH, while they are induced when PchR is activated upon PCH-(FeIII) binding. Since an excess of cytoplasmic iron is toxic for bacterial cells, all P. aeruginosa iron acquisition systems are repressed under iron-replete conditions by the binding of the ferric uptake regulator protein (Fur) to the promoter regions of PVD and PCH biosynthesis and uptake genes. This highly specific regulatory circuitry ensures PCH production when (i) cells are iron starved (hence, Fur repression is relieved) and (ii) PCH is effective in feeding the cell with iron (hence, PchR binds cytoplasmic PCH-Fe(III), activates PCH biosynthesis, and transport genes).

PCH has a major impact on P. aeruginosa pathogenesis, as it contributes to overcoming the iron starvation response of the host during bacterial infection. Since P. aeruginosa cells with iron and contributes to pathogenicity in a mouse model of lung infection. Indirect evidence of in vivo expression of PCH biosynthesis genes was inferred from transcriptional profiling of P. aeruginosa during septicemia, urinary tract, lung, and wound infections, and the PCH transport gene pfpA was identified among the most highly expressed virulence genes in vivo. However, in vivo detection of PCH is problematic, and PCH production during P. aeruginosa infection was occasionally documented in CF sputum samples, which showed significant induction of pch genes. In vitro studies suggest that PCH is also responsible for secondary pathogenic effects on host tissues through the generation of hydroxyl radicals in combination with pyocyanin, which is an extracellular phenazine compound produced by P. aeruginosa.

The paucity of information about PCH production levels during P. aeruginosa infection is due to the complexity of available methods to quantify this siderophore in biological samples. At present, PCH quantification methods rely on solvent extraction followed by concentration of the extract and PCH purification and detection by thin-layer chromatography, spectrofluorimetry, or HPLC. These methods are time-consuming, require handling of hazardous solvents and specialized equipment for PCH extraction and quantification, respectively, and unavoidably cause non-negligible loss of material, which could ultimately result in the underestimation of the PCH concentration.

In this study, we report the construction of a bioluminescent whole cell-based biosensor for the rapid, specific, and single-step quantification of PCH, overcoming the numerous drawbacks of current PCH detection methods. The sensitivity, range of linear response, and specificity of the biosensor were experimentally determined. The biosensor was developed in the 96-well microtiter plate format and successfully used for PCH quantification in P. aeruginosa culture supernatants and biological samples, then adapted to the agar plate format for the qualitative screening of PCH-producing P. aeruginosa clinical isolates.

**EXPERIMENTAL SECTION**

**Bacterial Strains and Growth Media.** Bacterial strains and plasmids used in this study are listed in Table S1. Escherichia coli and P. aeruginosa were grown in Luria-Bertani broth (LB) and LB agar plates. The P. aeruginosa biosensor strain ΔpvdΔpchΔpfpA PchE::lux is freely available to the scientific community and can be provided upon request to the corresponding authors. When required, antibiotics were used at the following concentrations: ampicillin (Ap, 100 μg/mL), tetracycline (Tc, 12.5 μg/mL), and gentamicin (Gm, 10 μg/mL) for E. coli; Gm (200 μg/mL), carbenicillin (Cb, 250 μg/mL), and Tc (100 μg/mL) for P. aeruginosa. P. aeruginosa strains were also grown in the iron-poor casamino acid (DCAA) medium and in casamino acid (CAA) agarose plates (10 g/L CAA, Difco; 15 g/L Certified Molecular Biology Agarose, Bio-Rad).

**PVD Extraction and Quantification.** PVD produced by P. aeruginosa strains was quantified after 24 h growth at 37 °C in DCAA by measuring the absorbance at 405 nm of culture supernatants appropriately diluted in 0.1 M Tris–HCl, pH 8.0. Values were normalized to the cell density of the bacterial culture (OD600), as previously described. PVD was purified as previously described. Briefly, P. aeruginosa ΔpchD was grown in DCAA for 24 h. The culture supernatant was purified by filtration through a Sep-Pak C18 Vac-Cartridge 3 cc (Waters). The filtered culture supernatant containing PVD was loaded and washed with double-distilled water to remove unwanted components. PVD was then eluted with 50% (vol/vol) methanol, evaporated to dryness in a desiccator, and dissolved in a small volume of double distilled water. The PVD concentration was determined by spectrophotometric measurement of the apo form at OD405 (ε = 1.4 × 10^4 M⁻¹ cm⁻¹).

**PCH Extraction and Quantification.** PCH was extracted from P. aeruginosa ΔpvdA by the ethyl acetate extraction of acidified 36 h-old culture supernatants in DCAA. Briefly, the supernatant was adjusted to pH 1.5–2.0 with 1 N HCl and extracted with 1 volume of ethyl acetate. After evaporation of the organic phase, the dry residue was suspended in 100 μL of methanol. PCH extracts were purified by an automated reverse-phase chromatographer equipped with a UV–vis detector (BIOTAGE Isola One Flash Chromatography System, RP-C18 column, gradient water/methanol). The amount of apo-PCH was determined by spectrophotometric measurement at OD320 (ε = 4400 M⁻¹ cm⁻¹). A 40 mM of stock solution of PCH was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C until used. The ethyl acetate extract of PAO1, ΔpvdA, ΔpchD, and P. aeruginosa TR1 was also applied to a silica gel G (60P254) thin-layer chromatography (TLC) plate (Merck) using acetone:methanol:0.2 M acetic acid (5:2:1) as the mobile phase. PCH from TLC plates was qualitatively characterized by (i) yellow-green fluorescence emission under UV light and (ii) iron-binding capacity when sprayed with 0.1 M FeCl₃ in 0.1 M HCl resulting in red-brown spots.

**Chemical Synthesis of PCH and Enantio-PCH.** The two enantiomers N-methyl-L-cysteine and N-methyl-D-cysteine, which are required in the final step of PCH and enantio-PCH chemical synthesis, were prepared according to literature procedures starting from commercially available L- or D-cysteine. In this synthetic procedure (Figure S1), cysteine enantiomers were separately reacted with trityl alcohol in TFA to protect the thiol group as N-protected cysteine or its enantiomer. The simultaneous condensation of aldehyde with the N-protected L-o or D-cysteine or its enantiomer carbamate that, after N-methylation with methyl iodide, gave the fully protected S-trityl, N-Boc, N-methyl-L-cysteine or n-cysteine. The simultaneous deprotection of amino and thiol groups (Boc and trityl cleavage, respectively) gave the final N-methyl-L- or D-cysteine. PCH and enantio-PCH were then synthesized using the literature protocol over four steps (Figure S2). Commercially available 2-hydroxybenzoinitrile was condensed with L-cysteine providing the thiazolidine intermediate. The treatment of the thiazolidine intermediate with N-methyl-N-methyl hydroxylamine in the presence of condensing agents afforded the corresponding Weinreb amide, and the subsequent reduction with lithium aluminum hydride (LAH) gave aldehyde as a racemic mixture. The final condensation of aldehyde with the previously synthesized N-methyl-L-cysteine or its enantiomer N-methyl-D-cysteine followed by spontaneous cyclization gave PCH and enantio-PCH, respectively, as a mixture of four diastereomers (Figure S2).

**Generation of Plasmids and Reporter Strains.** Unmarked in-frame deletion mutants in pvdA and pchR genes were constructed by suicide plasmid insertion mutagenesis. E. coli was used for recombinant DNA manipulations. The constructs for mutagenesis were generated by directional cloning into the pDM4 or pME3087 vector (Table S1) of two DNA fragments of ~600 bp.
encompassing the regions upstream and downstream of the sequence to be deleted. Fragments were amplified by PCR, digested with the appropriate restriction enzymes, and cloned into pDM4 or pME3087, generating the derivative vectors pDM4P4fpvA and pME3087PchR (Table S1). PCR primers and restriction enzymes used for cloning of PCR products are listed in Table S2. All constructs were verified by DNA sequencing. Deletion vectors were conjugaely transferred from E. coli S17.1λpir into the P. aeruginosa suitable deletion mutants (Table S1). The in-frame deletion mutations were obtained by recombination, as previously described. All the deletion events were verified by PCR using primers flanking the deleted region and ampiclon sequencing. For the generation of mini-CTX PchE::lux insertion, a DNA fragment encompassing the pchE promoter was amplified using the primers listed in Table S2 from the P. aeruginosa PAO1 chromosome. The amplicon was digested with BamHI-HindIII and ligated in the mini-CTX-lux plasmid. The resulting mini-CTX PAO1 chromosome. The amplicon was digested with BamHI-HindIII and ligated in the mini-CTX-lux plasmid. The resulting mini-CTX PchE::lux was transferred from E. coli S17.1λpir into P. aeruginosa strains by conjugation. Excision of the mini-CTX plasmid was achieved by Flp-mediated recombination via pFLP2, as previously described.

Biosensor Response to PCH and Other Iron-Binding Compounds. To test the biosensor response to PCH, P. aeruginosa PAO1 ΔpvdAΔpchDAfpvA PchE::lux was grown for 16 h at 37 °C in DCAA supplemented with 1 μM FeCl3. To exclude any iron carryover, the cells were washed with saline prior to being suspended in DCAA. The biosensor strain PAO1 ΔpvdAΔpchDAfpvA PchE::lux was inoculated at final OD600 of 1, 0.5, and 0.25 into black, clear-bottom 96-wells microtiter plates (Greiner) in the presence of an increasing concentration of purified PCH. Different inoculum volumes (200, 100, and 50 μL) were tested in the assay, OD600 and light count per second (LCPS) were monitored every 15 min in a Tecan Spark 10 M multilabel plate reader (Tecan, Männedorf, Switzerland) for up to 6 h at 25 °C. Once the optimal biosensor assay condition is defined (OD600 = 0.25; final volume 50 μL per well), these parameters were applied in all subsequent assays. The limit of detection (LOD) and the limit of quantification (LOQ) of the PCH biosensor were determined according to the equations: LOD = 3 × (SD/S) and LOQ = 10 × (SD/S),51,52 where SD is the standard deviation of the blank value (# = 10 replicates) and S is the slope of the calibration curve (i.e., the sensitivity). The biosensor response was also assessed in the presence of chemically synthesized PCH and endoto-PCH, purified PVD, sodium salicylate (Sigma), deferoxamine (Desferal; Novartis), deferiprone (3-hydroxy-1,2-dimethylpyridin-4(1H)-one; Sigma-Aldrich), and FeCl3 (Sigma) at the indicated concentrations.

Biological Fluids and CF Sputa. Sputum and first-morning urinary specimens were collected from 10 healthy donors who tested culture-negative for P. aeruginosa. Each fluid was pooled, centrifuged (13,000g, 10 min, 4 °C), and sterilized through a 0.20 μm filter. Sterile artificial tears (Irilens 0.4%) were purchased from Montefarmaco (Italy). In addition, sputa from eight anonymous CF strains and biological fluids were collected, centrifuged, and sterilized through a Millipore membrane (pore size 0.45 μm, Sarstedt), and stored at −20 °C until the assay.

PCH Quantification in P. aeruginosa Culture Supernatants and Biological Fluids. PCH detection in culture supernatants of P. aeruginosa strains and biological fluids (including CF sputa) was performed using the P. aeruginosa ΔpvdAΔpchDAfpvA PchE::lux biosensor. P. aeruginosa strains (listed in Table S1) were grown for 24 h at 37 °C in DCAA. The culture supernatants were collected, filtered through a Millipore membrane (pore size 0.45 μm, Sarstedt), and stored at −20 °C until use. Five microliters of appropriate dilutions of culture supernatants or biological fluids were added to 45 μL of DCAA inoculated with the biosensor strain (final OD600 = 0.25). Microtiter plates were incubated at 25 °C, and OD600 and LCPS were measured after 3.5 h using a Tecan Spark 10 M multilabel plate reader. A calibration curve was generated with purified PCH at known concentrations (5–320 nM) and used to calculate the concentration of PCH in each sample.

Detection of PCH and Siderophores on Agar Plates. Single colonies of different P. aeruginosa strains (listed in Table S1) were cultured for 8 h in LB at 37 °C. Bacteria were washed in saline, diluted to OD600 = 0.1, and 5 μL of each bacterial suspension was spotted onto casamino acid (CAA) agarose plate (5 g/L CAA and 15 g/L agarose). After 24 h incubation at 37 °C, PCD production was detected under UV light, and cells were killed through exposure to chloroform vapors for 15 min. Plates were then subsequently overlaid with CAA soft agarose (3 g/L casamino acids, Difco; 7.5 g/L agarose Bio-Rad) containing the biosensor strain (OD600 = 0.5). After incubation at 37 °C for 1 h, plates were visualized with a ChemiDoc XR+ Imaging System (Bio-Rad), using a 3 min exposure time.

RESULTS AND DISCUSSION

Design and Construction of a PCH-Responsive Whole Cell-Based Biosensor. To generate a biosensor capable of quantifying PCH at the nanomolar level, the luxCDABE operon, encoding the luciferase enzyme from the bacterium Photorhabdus luminescens, was chosen as the reporter system, being characterized by a high signal/noise ratio and not requiring supplementation with an exogenous substrate for signal (blue photon) emission.51,52 A transcriptional fusion between the PchR-dependent PchE promoter and the luxCDABE operon was generated and integrated in the attB neutral chromosomal site in the P. aeruginosa ΔpvdAΔpchDAfpvA triple mutant (Figure 1). This recipient strain is a siderophore null mutant, i.e., impaired in the synthesis of both PVD and PCH, and is also unable to uptake PVD due to the absence of the FpvA receptor. The inability to produce siderophores prevents PpchE auto-induction by endogenously synthesized PCH. Moreover, the lack of the PVD transporter FpvA prevents the entrance of exogenous PVD into the biosensor cell, thus avoiding the repression of the PchE::lux fusion by Fe(III) delivered through the Fe(III)-PchR pathway. However, the ΔpvdAΔpchDAfpvA mutant still produces both FptA and FptX transporters, so it has no defect in PCH uptake from the extracellular milieu. Consequently, we predicted (i) the bioluminescence emission of the whole-cell biosensor to be dependent on the levels of exogenously added PCH, which is transported intracellularly
and serves as inducer of the \textit{PpchE::luxCDABE} promoter-probe gene fusion, and (ii) the bioluminescence emission to be proportional to the PCH concentration (Figure 1).

**Experimental Setup for the Use of the Bioluminescent Whole Cell-Based Biosensor.** The expression of \textit{pch} (PCH biosynthesis) genes and, consequently, of the \textit{PpchE::luxCDABE} reporter fusion is repressed by Fur under iron replete conditions. This is because the \textit{PpchE} promoter contains a Fur-Fe(II)-binding region, which abrogates the transcriptional activity under conditions of iron availability.22

In order to prevent Fur-Fe(II)-mediated repression of the biosensor, the iron-poor medium DCAA \footnote{The DCAA medium (DCAA, Difco) was used because it contains no iron in its composition.} was chosen for setting the operational conditions of the bioluminescent biosensor. Although iron scarcity is essential for studying the biosensor response to PCH, too severe iron limitation imposed by DCAA (≤0.5 \(\mu\)M) \footnote{The concentration of iron ions was measured using an atomic absorption spectrophotometer.} would suppress the growth of a siderophore-null \textit{P. aeruginosa} mutant. Therefore, the minimum non-limiting iron concentration allowing the \textit{ΔpvdAΔpchDΔfpvA} mutant to grow similarly to the wild type, without causing complete repression of iron uptake genes, was initially investigated. To this purpose, the growth of the biosensor strain, the temperature was set at 25 °C during the assay. Interestingly, the lowest cell density (OD\(_{600} \approx 0.25\)) and the smallest volume (50 \(\mu\)L) provided the highest relative light emission, expressed as light counts per cell and hence the biosensor response. This result can be explained by the multiple scattering effect that occurs when the concentration of particles (i.e., cells) is too high. In this case, the measured OD\(_{600}\) does not linearly increase with the cell number, and the Beer–Lambert law is no longer a valid approximation. In addition, the bioluminescent photons are scattered by overlying cells and are deflected away from the photodetector.54 This is because bacteria located in the upper layers shield the light signal emitted by the bacteria underneath. In fact, the higher the volume, the higher the optical path of the well, hence the light shielding by the bacterial suspension. Lowering the reaction volumes and cell densities enhanced the relative performance of the biosensor, expressed as LCPS/OD\(_{600}\). An optimal dose-dependent response of the biosensor was observed using the lowest cell density (OD\(_{600} \approx 0.25\)) and the smallest assay volume (50 \(\mu\)L) within the 5–160 nM PCH concentration range. PCH concentrations of >160 nM caused a moderate increase in light emission (Figure 2I). This phenomenon is likely due to the fact that PCH concentrations higher than 160 nM saturate the FptA/FptX transporters and/or the transcriptional

**Figure 2.** Effect of reporter cell density and assay volume on the biosensor response to PCH. Relative light emission (LCPS/OD\(_{600}\)) by the \textit{P. aeruginosa} \textit{ΔpvdAΔpchDΔfpvA} mutant carrying the \textit{PpchE::lux} fusion, in response to increasing PCH concentrations, ranging from 5 to 5120 nM. LCPS and OD\(_{600}\) were measured every 15 min for 6 h at 25 °C. Bacteria were inoculated at three different cell densities and three final volume combinations: (A) 200 \(\mu\)L, OD\(_{600} = 1\); (B) 200 \(\mu\)L, OD\(_{600} = 0.5\); (C) 200 \(\mu\)L, OD\(_{600} = 0.25\); (D) 100 \(\mu\)L, OD\(_{600} = 1\); (E) 100 \(\mu\)L, OD\(_{600} = 0.5\); (F) 100 \(\mu\)L, OD\(_{600} = 0.25\); (G) 50 \(\mu\)L, OD\(_{600} = 1\); (H) 50 \(\mu\)L, OD\(_{600} = 0.5\); (I) 50 \(\mu\)L, OD\(_{600} = 0.25\). Data are the mean of three independent experiments ± SD.
regulator PchR. Under these conditions, the \( \Delta pvdA \Delta pchD \Delta fpvA \) PchE::lux biosensor rapidly responded to exogenous PCH, achieving the maximum relative light emission in 3 to 3.5 h after the addition of PCH (Figure 2B). No significant evaporation of the biosensor suspension medium (i.e., volume reduction) was noticed during the experimental time course.

**Bioluminescent Cell-Based Biosensor Selectively Responds to PCH, Requires PchR, and Is Repressed by Iron.** Since the maximum light emission of the \( \Delta pvdA \Delta pchD \Delta fpvA \) PchE::lux biosensor was observed 3.5 h after the addition of PCH, the limit of detection (LOD) and the limit of quantification (LOQ) were determined at this time point. A linear dose–response relationship \( (R^2 = 0.98) \) in the range of PCH concentration was observed (Figure 3A). Within this PCH concentration range, the LOD and LOQ were 1.64 ± 0.26 and 5.48 ± 0.86 nM, respectively (Figure 3A). These analytical performances represent a significant advance over previous PCH quantification methods, which required large sample volumes (mL) and exhibited sensitivity in the \( \mu \)M PCH range. To investigate the selectivity of the \( \Delta pvdA \Delta pchD \Delta fpvA \) PchE::lux biosensor, the luminescence emission was measured upon the addition of several iron-chelating compounds, using the standard test conditions (\( OD_{600} \approx 0.25; 50 \mu L \) volume; 3.5 h; 25 °C). Purified PCH from \( P. \ aeruginosa \) PAO1 and chemically synthesized PCH were included as controls. Each iron-chelating compound (listed in Table S3) was added at 160 nM. As expected, the bioluminescence emission induced by the chemically synthesized PCH was similar to that of the native PCH extracted from \( P. \ aeruginosa \) culture supernatants (Figure 3B). Conversely, in the presence of chemically synthesized enantio-PCH, a PCH diastereoisomer was produced by \( P. \ protegens \), and the bioluminescence emission was completely abrogated (Figure 3B). The inability of enantio-PCH to induce the expression of PCH genes in \( P. \ aeruginosa \) and consequently the biosensor response confirms that enantio-PCH is not recognized by \( P. \ aeruginosa \) due to the high specificity of the PCH translocation machinery and of the PchR-mediated gene expression. No response was also observed with other iron-chelating compounds, namely, PVD, deeroxamine (DFO), deferiprone (DFP), and sodium salicylate (SAL, a PCH precursor), further confirming the biosensor selectivity (Figure 3B). The biosensor genetic circuitry is based on the assumption that the production of PCH is controlled by the cytoplasmic transcriptional regulator PchR, which activates the transcription of PCH biosynthesis and transport genes. To define the role of PchR on the biosensor response, the pchR gene was deleted from the \( \Delta pvdA \Delta pchD \Delta fpvA \) PchE::lux biosensor strain, and the expression of the PchE::lux fusion was monitored using the standard assay conditions (\( OD_{600} \approx 0.25; 50 \mu L \) volume; 3.5 h; 25 °C concentration range). As expected, the deletion of pchR completely abrogated PCH-inducible PchE::lux expression in the 5–5.120 nM PCH range, confirming that PchR is strictly required in PCH gene expression and thus for the PCH-mediated response of the biosensor (Figure 3C).

To verify the ability of iron to repress the expression of the PchE::lux fusion, the biosensor was exposed to 160 nM of PCH, in the presence of two different FeCl\(_3\) concentrations (i.e., 5 and 10 \( \mu \)M). The bioluminescence signal emission was completely shut off with 5 \( \mu \)M FeCl\(_3\) (Figure 3D).

Taken together, these results indicate that the biosensor response depends on the concentration of PCH and iron, in line with the regulatory mechanisms, which control PCH production and uptake.
PVD Interference with PCH Measurement. Several studies in animal models and humans showed that PCH biosynthesis and uptake genes are expressed during P. aeruginosa infection,\textsuperscript{25–28} though the presence of PCH could be detected fluorometrically in a few CF sputum samples.\textsuperscript{15,16} Fluorescence spectroscopy can detect PCH concentrations of >1 μM, and therefore, with this method, it is not possible to detect PCH in P. aeruginosa isolates, which produce lower PCH concentrations (i.e., <1 μM).\textsuperscript{11} Moreover, PCH fluorescence is quenched upon Fe(III) binding,\textsuperscript{57} implying that the Fe(III)-PCH complex escapes fluorometric detection. Another commonly used procedure for PCH quantification entails solvent extraction and purification by HPLC.\textsuperscript{39} Both these methods are affected by significant analyte loss and unavoidably underestimate the actual concentration of PCH produced by P. aeruginosa.

The ability of the biosensor to specifically and rapidly respond to low PCH concentrations encouraged its use for direct PCH quantification in P. aeruginosa culture supernatants. Calibration of the biosensor can easily be obtained with commercially available PCH (Santa Cruz Biotechnology Inc., CA). To this purpose, the supernatants of wild-type PAO1 and ΔpvdA and ΔpchD single and double mutants were collected after 24 h of growth in the iron-poor medium DCAA, and the biosensor bioluminescence emission was measured. As expected, no bioluminescence emission was recorded in culture supernatants of PCH-defective mutants, indicating that no compound other than PCH can trigger the biosensor (Figure 4A). Surprisingly, the bioluminescence emission of the PAO1 culture supernatant was much lower than that of the pvdA mutant (Figure 4A). This result is in contrast with previous data showing that wild-type PAO1 and PVD defective mutants produce a similar amount of PCH under iron-limited conditions\textsuperscript{24} and suggests that high PVD levels can interfere with the biosensor response. To verify this hypothesis, PCH was extracted from the supernatant of PAO1, ΔpvdA, ΔpchD, and ΔpvdAΔpchD and analyzed by TLC. The PCH spot of PAO1 was quantitatively similar to that of the pvdA mutant (Figure S4A), suggesting that PCH production in these two strains is comparable under the test conditions. Since PAO1 produces a high amount of PVD when grown in DCAA (Figure S4B), we hypothesized that PVD may hamper PCH measurements by chelating iron traces in the growth medium. Indeed, previous work has demonstrated that PchR binds PCH in its iron-loaded form, while it does not in its apo-form.\textsuperscript{21,58} Therefore, iron withholding by PVD could negatively affect PCH gene expression, and consequently bioluminescence emission. To verify this hypothesis, the biosensor response was measured in the presence of increasing concentrations of purified PCH and PVD (62.5–4000 and 20–20000 nM, respectively). The choice of using 20000 nM as the maximum PVD concentration derives from the experimental observation that P. aeruginosa PAO1 produces ≈200 μM PVD in DCAA culture supernatants (Figure S4B) and that a 1:10 dilution (i.e., 5 μL of culture supernatants added to 45 μL of biosensor suspension) is used for the assay. PVD concentrations of ≥2500 nM significantly reduced the biosensor luminescence emission (Figure 4B), even in the presence of elevated PCH concentrations. Conversely, PVD concentrations of ≤1250 nM had a negligible effect on the biosensor luminescence emission at all PCH concentrations tested. The above findings suggest that an appropriate dilution of P. aeruginosa culture supernatants can overcome PVD interference in the PCH biosensor assay. To corroborate these results, the culture supernatants of wild-type PAO1 and ΔpvdA and ΔpchD single and double mutants were serially diluted, and the bioluminescence emission of the ΔpvdAΔpchDΔpvdA PchE::lux biosensor was measured after 3.5 h at 25 °C. Interestingly, the luminescence emission progressively increased with serial dilution of the PAO1 supernatant, reaching the same values of the PVD-deficient ΔpvdA mutant at 1:32 dilution (Figure S5A). Moreover, the biosensor luminescence emission in the presence of the ΔpvdA mutant supernatant was nearly constant up to the 1:32 dilution, suggesting that PCH levels in supernatants of the PVD-defective mutant exceeded the upper detection limit of the biosensor, resulting in its saturation.

The above results indicate that an appropriate dilution of culture supernatants is mandatory for PCH quantification. To calculate the concentration of PCH produced by P. aeruginosa, 1:256, 1:512, and 1:1024 dilutions were used, and the PCH concentration was calculated by using a standard calibration curve (Figure S5B). Interestingly, the PCH concentrations estimated by using the three different dilutions gave comparable results (Figure S5C), as expected for PCH concentrations below the biosensor saturation point (i.e., 160 nM, Figure 2I and Figure 3C). Therefore, the lowest sample dilution (1:1024) was selected for further experiments (see also the Supporting Information, text S1). By diluting the supernatant, the PCH concentrations determined for PAO1 and the ΔpvdA mutant culture supernatants (≈70 μM/OD<sub>600</sub>) were in line with previous experiments (Figure 4C) and literature data.\textsuperscript{59}
Indeed, the supernatant of two test strains (Figure S4B). Indeed, the can be ascribed to the di

LesB58, culture supernatants were diluted 1:1024, and 5 concentration of PCH produced by PAO1, ATCC 27853, and to PCH measurement. On this basis, to calculate the exact eliminating PVD interference by diluting the supernatant prior (Figure S4B). These

PVD concentration in the LesB58 supernatant is 10-fold lower iron-poor conditions is almost the same (

64-fold dilution (Figure S5D). The di

luminescence emission, which remained constant up to the determined an initial increase (2-fold dilution) of bio-

supernatant dilution. Contrarily, the supernatant of LesB58 showing biosensor saturation and PVD interference for strains were cultured for 24 h in DCAA, and the supernatants

different PVD levels in the medium (Figure S4B).

Figure 5. Bioassays for PCH detection in liquid and solid media. (A) PCH concentrations (μM/OD₆₀₀) in the culture supernatants of P. aeruginosa strains PAO1, ATCC 27853, LesB58, and TR1 grown in DCAA for 24 h. Supernatants were 1:1024 diluted, and PCH concentration was calculated using a PCH calibration curve, as outlined in the Supporting Information, text S1. Data are representative of three independent experiments ± SD. (B) Top: qualitative detection of PCH production on CAA agarose plates, as outlined in the Supporting Information, text S2. P. aeruginosa strains were spotted on CAA agarose plates, grown for 24 h at 37 °C, and killed by chloroform vapor. Then, CAA agarose plates were overlaid with the P. aeruginosa ΔpvdΔpchDΔfpvA PpchE::lux biosensor strain and incubated for 1 h at 37 °C. The PCH grey halo was detected using a ChemiDoc XRS+ Imaging System (Bio-Rad). The gray scale denotes pixel intensity. Bottom: PVD production by the P. aeruginosa strain, prior to the biosensor overlay, detected as fluorescence emission upon UV light exposure. Images are representative of one of several independent tests providing similar results.

Figure 6. Biosensor response to PCH-spiked biological fluids. Relative light emission (LCPS/OD₆₀₀) of the P. aeruginosa ΔpvdΔpchDΔfpvA PpchE::lux biosensor in response to increasing concentrations of PCH (5 to 5120 nM) in undiluted and diluted (10⁻¹ and 10⁻²) biological fluids, after 3.5 h of incubation at 25 °C. Each value represents the mean ± SD of three independent experiments.

PCH Quantification in P. aeruginosa Clinical Strains and Biological Fluids. To check the ability of the biosensor to measure PCH levels produced by clinical P. aeruginosa strains, P. aeruginosa ATCC 27853 60 and the Liverpool-

epidemic strain LesB58 61 were preliminarily tested. P. aeruginosa ATCC 27853 and LesB58 produce different PVD types (i.e, type II and III PVD, respectively) and release quite different PVD levels in the medium (Figure S4B). P. aeruginosa strains were cultured for 24 h in DCAA, and the supernatants were diluted. The biosensor response to the supernatant of ATCC 27853 was similar to that observed with PAO1, showing biosensor saturation and PVD interference for ≤1:32 supernatant dilution. Contrarily, the supernatant of LesB58 determined an initial increase (2-fold dilution) of bio-

luminescence emission, which remained constant up to the 64-fold dilution (Figure SSD). The different response profile can be ascribed to the different PVD concentrations in the supernatants of two test strains (Figure S4B). Indeed, the amount of PVD produced by PAO1 and ATCC 27853 under iron-poor conditions is almost the same (~200 μM), while the PVD concentration in the LesB58 supernatant is 10-fold lower (Figure S4B). These findings further corroborate the need of eliminating PVD interference by diluting the supernatant prior to PCH measurement. On this basis, to calculate the exact concentration of PCH produced by PAO1, ATCC 27853, and LesB58, culture supernatants were diluted 1:1024, and 5 μL of each supernatant were used for the assay, as recommended in the Supporting Information, text S1. Preliminary dilution lowers the PVD concentration to <40 nM, thereby preventing any interference of PVD with biosensor response to PCH (Figure 4B). The clinical P. aeruginosa isolate TR1 was included as a negative control, being a natural PCH-defective mutant. 62 Interestingly, PCH production was slightly lower in P. aeruginosa LesB58 than in ATCC 27853 and PAO1, while no PCH was detected in the supernatant of the PCH-negative strain TR1 (Figure 5A). Altogether, these results indicate that, with an appropriate setting (Supporting Information, text S1), the biosensor is a valuable tool to quantify PCH production by P. aeruginosa clinical isolates.

Lastly, the newly generated whole-cell biosensor was adapted to generate a rapid screening system of PCH-producing P. aeruginosa strains on solid media. To this aim, several P. aeruginosa strains (listed in Table S1) were spot-

inoculated on CAA agarose plates and grown at 37 °C for 24 h until ~3 mm colonies became visible. Colonies were inspected under UV light exposure to detect PVD production (blue halo), and then bacteria were killed with chloroform vapors. Thereafter, a thin (~1 mm) agarose layer containing the ΔpvdΔpchDΔfpvA PpchE::lux biosensor was overlaid onto the test colonies and bioluminescence emission was detected after 1 h incubation at 37 °C. To validate the plate-based screening system, wild-type P. aeruginosa PAO1 and PCH and/ or PVD defective mutants were tested. Noteworthy, the same signal was registered for wild-type P. aeruginosa PAO1 and
is extremely variable depending on the practice, considering that the concentration of secreted PCH growth conditions (from incompatible with PCH production by laboratory media and CF sputa, respectively), appropriate conditions. The biosensor specificity of the extremely high sensitivity of performances. Both factors, however, do not undermine the biosensor validity by virtue of the extremely high sensitivity of the assay (lower detection limit = 1.64 nM). Here, pilot testing of sputa from eight anonymous CF patients revealed that PCH was detectable in six of them (range 86.9 ± 4.6–24,390.0 ± 91.2 nM), showing an excellent correlation between the presence of PCH and P. aeruginosa culture-positivity of sputa (Table S5).

Altogether, these results indicate that the ΔpvdAΔpchDΔfpvA PchE::lux biosensor is suitable for PCH quantification in both P. aeruginosa culture supernatants and biological fluids and can be used for the rapid screening of PCH-producing P. aeruginosa isolates on a solid medium (Supporting Information, text S2).

■ CONCLUSIONS

In summary, we generated a whole cell-based biosensor for PCH quantification and tested its proficiency under laboratory conditions. The biosensor specificity is guaranteed by the unique selectivity of the PCH transport machinery and the PchR activator of the PchE::lux reporter fusion. In fact, both the FptA receptor and the PchR regulator are strictly PCH-selective. Accordingly, evidence was provided that the biosensor does not respond to PCH stereoisomers or precursors, as well as to a variety of iron-chelating compounds, excluding the possibility of false positive readouts.

Two main factors, namely, iron and PVD carryover in the test sample, were shown to interfere with the biosensor performances. Both factors, however, do not undermine the biosensor validity by virtue of the extremely high sensitivity of the assay (lower detection limit = 1.64 ± 0.26 nM PCH), which requires the sample to be diluted several fold (≥10⁻³) prior to testing, so that interference by iron and PVD would be negligible. It should also be taken into account that PCH is produced by P. aeruginosa only under conditions of severe iron limitation [<5 μM Fe(III)], implying that the presence of sufficient (≥ 5 μM) iron levels in biological samples would be incompatible with PCH production by P. aeruginosa. In practice, considering that the concentration of secreted PCH is extremely variable depending on the P. aeruginosa strain and growth conditions (from ~800,000 to ~1000 nM in optimized laboratory media and CF sputa, respectively), appropriate serial dilutions of the sample should be made to adjust the PCH concentration in the linear response range of the biosensor (5–40 nM; Figure 3A). Of note, a calibration curve can be easily prepared using pure PCH, which is commercially available.

The luminescent biosensor offers several advantages over previous PCH detection and/or quantification methods, which rely on chromatographic or spectrophotometric analysis of partially purified organic extracts. Briefly, (i) the assay protocol is straightforward and allows PCH to be quantified in 3.5 h with negligible sample handling; (ii) up to 28 samples can contemporarily be tested in triplicate using a single 96-well microplate, also including standard samples for system calibration; (iii) the microtiter plate format would allow the assay to be scaled down to a smaller format for higher throughput (e.g., using 384-well microplates) and is amenable to automation (e.g., using automatic dispensers); (iv) once dispensed with the reporter strain, the microtiter plates can be stored frozen at −80 °C for months with negligible loss of the assay performances (Figure S6); (v) the assay combines high sensitivity (LOQ in the nM PCH range) with simplicity, since the luciferase-based reporter system is characterized by a high signal/noise ratio and does not require an exogenous substrate for signal emission. Bioluminescence background levels in living cells are extremely low, making bioluminescence up to 50 times more sensitive than fluorescence, which would be impracticable in testing the intrinsically fluorescent Pseudomonas species. Moreover, P. luminescens luciferase is endowed with remarkable chemical and physical stability. The inclusion of P. aeruginosa into the list of risk group 2 bacterial pathogens could represent a limitation to the use of the luminescent biosensor in biosafety level 1 laboratories. However, the P. aeruginosa PAO1 ΔpvdAΔpchDΔfpvA mutant carries stable genetic knock-outs of both PCH and PVD biosynthesis genes, together with a deletion of the PVD receptor gene. Altogether, these mutations impair iron uptake and result in an avirulent phenotype in animal models of infections, downgrading the risk associated with biosensor manipulation.

In conclusion, the ΔpvdAΔpchDΔfpvA PchE::lux whole cell-based biosensor represents an innovative tool to detect and quantify the PCH siderophore. The preliminary setup of the test conditions allows the fast, easy, accurate, and cost-effective determination of nanomolar PCH concentrations in P. aeruginosa liquid cultures and biological fluids, as well as the qualitative screening of PCH-producing P. aeruginosa colonies on an agar plate, hopefully facilitating future investigations on the role of PCH in the pathogenesis of P. aeruginosa infection.

■ ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.1c01023.

Supplementary methods, figures, tables, and description of the analytical protocol (PDF)

■ AUTHOR INFORMATION

Corresponding Authors
Daniela Visaggio – Department of Science, Roma Tre University, Rome 00146, Italy; Santa Lucia Foundation IRCCS, Rome 00179, Italy; Email: daniela.visaggio@uniroma3.it
Paolo Visca – Department of Science, Roma Tre University, Rome 00146, Italy; Santa Lucia Foundation IRCCS, Rome
Authors
Mattia Pirolo — Department of Science, Roma Tre University, Rome 00146, Italy; Present Address: Department of Veterinary and Animal Sciences, University of Copenhagen, DK-1165 Frederiksberg, Denmark
Emanuela Frangipani — Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino 61029, Italy; orcid.org/0000-0001-5619-7912
Massimiliano Lucidi — Department of Science, Roma Tre University, Rome 00146, Italy
Raffaella Sorrentino — Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Naples 80138, Italy
Emma Mitidieri — Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Naples 80138, Italy
Francesca Ungaro — Department of Pharmacy, University of Naples Federico II, Naples 80131, Italy; orcid.org/0000-0003-0850-9533
Andrea Lagrati — Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan 20126, Italy
Francesco Peri — Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan 20126, Italy; orcid.org/0000-0002-3417-8224
Complete contact information is available at: https://pubs.acs.org/10.1021/acssensors.1c01023

Author Contributions
E.F. and P.V. conceived and designed the study. D.V., M.P., M.A., and E.M. performed the experiments. E.F., R.S., F.U., F.P., and P.V. analyzed the results. D.V., E.F., F.P., and P.V. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Author Contributions
D.V. and M.P. contributed equally to this work.

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Notes
The authors declare no competing financial interest.

ABBREVIATIONS
CAA, casamino acids; DCAA, deferrated casamino acids; DFP, deferrirone; DFO, deferoxamine; DMSO, dimethyl sulfoxide; Fur, ferric uptake regulator protein; HPLC, high-performance liquid chromatography; LB, Luria-Bertani broth; LCPS, light counts per second; LOD, limit of detection; LOQ, limit of quantification; PVD, pyoverdine; PCH, pyochelin; S, slope of calibration curve; SAL, salicylate; S/N, signal/noise ratio; SD, standard deviation; TLC, thin layer chromatography

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