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Non-invasive, ratiometric determination of intracellular pH in Pseudomonas species using a novel genetically encoded indicator

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Summary
The ability of Pseudomonas species to thrive in all major natural environments (i.e. terrestrial, freshwater and marine) is based on its exceptional capability to adapt to physicochemical changes. Thus, environmental bacteria have to tightly control the maintenance of numerous physiological traits across different conditions. The intracellular pH (pH_i) homoeostasis is a particularly important feature, since the pH_i influences a large portion of the biochemical processes in the cell. Despite its importance, relatively few reliable, easy-to-implement tools have been designed for quantifying in vivo pH_i changes in Gram-negative bacteria with minimal manipulations. Here we describe a convenient, non-invasive protocol for the quantification of the pH_i in bacteria, which is based on the ratiometric fluorescent indicator protein PHP (pH indicator for Pseudomonas). The DNA sequence encoding PHP was thoroughly adapted to guarantee optimal transcription and translation of the indicator in Pseudomonas species. Our PHP-based quantification method demonstrated that pH_i is tightly regulated over a narrow range of pH values not only in Pseudomonas, but also in other Gram-negative bacterial species such as Escherichia coli. The maintenance of the cytoplasmic pH homoeostasis in vivo could also be observed upon internal (e.g. redirection of glucose consumption pathways in P. putida) and external (e.g. antibiotic exposure in P. aeruginosa) perturbations, and the PHP indicator was also used to follow dynamic changes in the pH_i upon external pH shifts. In summary, our work describes a reliable method for measuring pH_i in Pseudomonas, allowing for the detailed investigation of bacterial pH_i homoeostasis and its regulation.

Introduction
Microbial cells have a remarkable ability to maintain physiological homoeostasis under a wide variety of environmental conditions. Thereby, the tight control of the intracellular pH (pH_i, i.e. cytoplasmic pH) is of particular importance (Slonczewski et al., 2009), because pH_i influences enzyme activity, protein stability, nucleic acid structure and many other biological processes (Booth, 1985). It is well known that few species of adapted microbes can grow under extreme pH conditions. Bacterial life is possible even at pH values close to 0 (Nordstrom et al., 2000) or above pH = 13 (Roadcap et al., 2006). Nonetheless, the cytoplasmic pH value is remarkably similar for most bacteria, even when exposed to extreme pH environments (Krugliw et al., 2011; Martinez et al., 2012). Generally, bacteria have evolved a series of mechanisms to maintain this tight regulation of pH_i homoeostasis. A major strategy to this effect is to regulate the activity of proton (H^+) transporters, such as H^+-pumping respiratory chain complexes and H^+-coupled ATPases or cation/H^+ antiporers (Krugliw et al., 2011). As H^+ ions are transported either way across the cell membrane by these mechanisms, major changes in the transmembrane pH gradient (ΔpH) are also generated. Since both ΔpH and the transmembrane electrical potential (ΔΨ) are directly connected to the H^+-motive force (PMF; given that PMF = ΔΨ – 60 × ΔpH at 25°C), the demand of pH homoeostasis directly influences the...
overall energetic status of the cell (Deamer and Nichols, 1989).

Several methods have been developed to monitor the pH in bacteria (Slonczewski et al., 2009; Chen and Lo, 2016). Many of them rely on the measurement of in vivo $^{31}$P nuclear magnetic resonance (NMR) spectra (Roberts et al., 1981; Sijbesma et al., 1996) or on the ratiometric fluorescence of specific chemical dyes (Molenaar et al., 1991; Breeuwer et al., 1996; Riondet et al., 1997; Awaji et al., 2001; Hunter and Beveridge, 2005; Rupprecht et al., 2017). While NMR is a laborious and not widely accessible technique, some of the fluorescent chemical probes commercially available are usually expensive, short-lived and their use for pH determination requires a number of time-consuming manipulations. In addition to these drawbacks, some fluorescein-based fluorophores can rapidly leak out of cells once they are liberated in the cytosol via intracellular hydrolysis (Han and Burgess, 2010). Other chemical dyes also pose problems due to their (i) low permeability (which in turn result in uneven loading of dyes within a cell population), (ii) high chemical instability and (iii) cytotoxicity. Derivatives of fluorescent proteins that respond to changes in pH, including the model green fluorescent protein (GFP), have been described in the past decades (Olsen et al., 2002; Mahon, 2011; Martinez et al., 2012; Rupprecht et al., 2017; Reifenrath and Boles, 2018). The expression of genes encoding fluorescent proteins and the detection of the resulting fluorescence signals are an inexpensive and fast technique, enabling online measurements in vivo, requiring considerably less technical effort than other techniques deployed for pH determination. The dependence of the fluorescence intensity on the pH in GFP derivatives is based on the direct protonation of the phenolate moiety of the fluorophore derived from tryptophan, an extremely fast exchange reaction (McAnaney et al., 2005). Thus, fluorescence determinations allow for the assessment of the rate of pH change in response to rapid shifts in extracellular pH. Additionally, the continuous expression of the protein allows for live-imaging over a longer timescale than is possible using chemical fluorophores (Grillo-Hill et al., 2014).

Given the importance of assessing cytoplasmic pH values to understand the principles governing metabolic homoeostasis and energy conservation mechanisms in bacteria, the improvement of non-invasive methods for calculating the pH is needed. In the present work, we propose a standard, non-invasive method for the determination of pH in Pseudomonas species, as well as other Gram-negative bacteria such as Escherichia coli. This procedure is based on the enhanced expression of a pH-sensitive variant of GFP, and the ratiometric assessment of pH against cells equilibrated to a range of physiologically relevant pH values. The usability of this novel tool for the quantification of pH in Gram-negative bacteria is illustrated by investigating the changes in pH homoeostasis brought about by internal metabolic perturbations in the cell factory platform Pseudomonas putida KT2440 and by antibiotic treatment in the opportunistic pathogen P. aeruginosa. The PHP indicator was also used to explore the response of pH in E. coli upon several perturbations. Moreover, the intrinsic compatibility of our tool with plasmids following the Standard European Vector Architecture (SEVA) (Silva-Rocha et al., 2013) allows to modulate the replication and expression of PHP in other bacteria. As indicated by the examples below, the PHP indicator constitutes a flexible, non-invasive and easy-to-implement tool to study both endogenous and exogenous pH perturbations in bacteria.

Results and discussion

Construction, benchmarking and calibration of the PHP indicator as a reliable tool to calculate the cytoplasmic pH in Pseudomonas

Our earlier attempts to use previously characterized ratiometric fluorescent proteins as pH indicators in Pseudomonas species (and, in particular, P. putida and P. aeruginosa) did not yield satisfactory signal-to-noise ratios. We attributed this lack of reliable fluorescent signal to two main reasons: (i) deficient (and potentially stochastic) expression of the gene(s) encoding the pH indicator(s) and (ii) some of the currently available ratiometric fluorescent protein indicators have been tailored for eukaryotic systems (Tournu et al., 2017; Reifenrath and Boles, 2018). In order to overcome these issues, we constructed a broad-host-range vector for the calibrated expression of a variant of the pH-sensitive pHluorin2 indicator protein (Mahon, 2011). pHluorin2 is a derivative of GFP that carries 11 point substitutions in its amino acid sequence, including the F64L mutation, known to enhance protein folding (Heim et al., 1995; Mahon, 2011). The sequence encoding pHluorin2 was codon-optimized for Pseudomonas species, and a synthetic ribosome binding site was added to ensure sufficient expression, as indicated in Experimental Procedures (see also Sequence S1 in the Supporting Information). The synthetic module, which we termed PHP (pH indicator for Pseudomonas), was placed under the transcriptional control of the constitutive EM7 promoter ($P_{EM7}$) in the pSEVA2513 vector [kanamycin (Km)-resistant, oriV (RSF1010)]. The resulting plasmid, termed pS2513-PHP, ensures the constitutive production of the PHP fluorescent protein in the bacterial cytosol regardless of the growth stage of the cells.

We first set to compare the performance of the original pHluorin2 protein and the PHP indicator in P. putida...
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KT2440 by comparing the fluorescence of the two proteins in individual bacterial cells by flow cytometry (Fig. 1). To this end, two identical, pSEVA2513-based plasmid systems were used, in which the expression of either pHluorin2 or PHP is driven by the same constitutive promoter under an equivalent vector copy number (Fig. 1A). These plasmids were individually transformed into P. putida KT2440, and the fluorescence stemming from either pHluorin2 or PHP was measured in exponentially growing cultures in LB medium by means of flow cytometry (Fig. 1B). Both the PHP and pHluorin2 fluorescence could be easily detected with the instrument settings used for GFP and related fluorescent proteins. As indicated by the histogram plots of cell count versus level of fluorescence of the indicators, cells expressing pHluorin2 exhibited a bimodal distribution of fluorescence, with approximately 21% of the cell population displaying fluorescence levels similar to that of a negative control (i.e. P. putida KT2440 transformed with an empty pSEVA2513 vector). Cultures of strain KT2440 transformed with plasmid pS2513-PHP, in contrast, had a clearly unimodal fluorescence distribution, with > 99% of the bacterial population testing positive for the PHP signal. Moreover, the geometric mean value of fluorescence was considerably higher for PHP (fluorescence x mean = 42.5) than for the original pHluorin2 indicator (fluorescence x mean = 22.3). Next, we investigated the signal-to-noise ratio, a parameter reflecting the efficiency of fluorescence detection (Giesecke et al., 2017), for the two indicator proteins. The signal-to-noise ratio was 40% higher for the PHP indicator than for pHluorin2 (Fig. 1C) – an advantageous property that addresses one of the main problems previously experienced with fluorescent protein-based pH indicators in Pseudomonas species. The coefficients of variation, a measure of the height versus width of fluorescence histograms and therefore an indication of the dispersion of the signal (Sharrow, 2001), were calculated for the two pH indicators according to the distribution of positive (i.e. pHluorin2+ or PHP+) cells of Fig. 1A. Again, the PHP protein outperformed pHluorin2, with a coefficient of variation which was approximately half of the original pHluorin2 indicator (Fig. 1C). Taken together, these results accredit the value of PHP as a uniformly accumulated fluorescent protein in Pseudomonas, with fluorescence characteristics similar to both GFP and pHluorin2.

Next, we experimentally verified the PHP expression by means of fluorescence microscopy of exponentially growing E. coli DH5α, P. putida KT2440 and P. aeruginosa PA14 carrying plasmid pS2513-PHP in LB medium. As observed in Fig. 2A, a homogeneous distribution of fluorescence was spotted across the individual cells of the three bacterial species examined, both during exponential growth and during the stationary phase – consistent with the results of flow cytometry experiments (Fig. 1). Subsequent tests in LB medium and M9 minimal medium indicated that the presence of plasmid pS2513-PHP did not affect the growth of E. coli or the two Pseudomonas species tested, neither in terms of specific growth rates nor the final cell density achieved in these cultures (data not shown). Moreover, flow cytometry analysis of the corresponding bacterial populations showed that the fluorescence signal followed a
Fig. 2. Assessment of intracellular pH (pHi) in different Gram-negative bacteria.

A. Fluorescent microscopy from exponentially growing cultures of *E. coli* DH5α, *P. putida* KT2440 and *P. aeruginosa* PA14 transformed with plasmid pS2513-PHP and grown in LB medium. Scale bars are indicated in each case.

B. Fluorescence excitation spectrum of the PHP indicator in *P. aeruginosa* PA14/pS2513-PHP cultured in LB medium. The intracellular pH (pH) of the cells was collapsed to the extracellular pH as outlined in *Experimental Procedures*. The mean values obtained from three independent experiments are shown. A.U., arbitrary units.

C. Calibration curve of the PHP indicator in *P. aeruginosa* PA14/pS2513-PHP cultured in LB medium supplemented with 20 μM carbonyl cyanide m-chlorophenyl hydrazine (CCCP). The data for the calibration curve were obtained as the ratio between the excitation peaks at 405 nm and 485 nm (RF(405/485)) plotted against the pH values of equilibrated cells (blue circles). These values were fitted using the Boltzmann sigmoid best-fitting curve to obtain the calibration curve (orange triangles), which was used to calculate the pH values in *P. aeruginosa* PA14 during exponential growth (red crosses). The lowest and highest pH values tested (pH = 5.5 and 8.5) are highlighted. Data points are representative of independent triplicates, and the red crosses (corresponding to six individual determinations) are shown in the curve to indicate the narrow dispersion of experimentally calculated pH values.

unimodal distribution in all three species both in complex and minimal culture media supplemented with glucose as the sole carbon source – indicating that the PHP protein can be used for studying pH across different culture conditions. The next question is how does the fluorescence stemming from PHP correlates with changes in pH, as indicated below.

All ratiometric derivatives of GFP (e.g. pHluorin and pHluorin2) rely on the bimodality shown in their excitation spectra (Llopis *et al*., 1998; Esposito *et al*., 2008), which exhibit two distinct maxima at an excitation wavelength ($\lambda_{\text{excitation}} \sim 395$ nm and $\lambda_{\text{excitation}} \sim 475$ nm (Miesenböck *et al*., 1998; Mahon, 2011). We first aimed at scanning the excitation spectrum of the engineered PHP protein in *P. aeruginosa* PA14 cells harbouring plasmid pS2513-PHP. To this end, we recorded the culture’s fluorescence emission at an emission wavelength ($\lambda_{\text{emission}} = 515$ nm in a standard microtiter plate fluorimeter. In order to check the effect of pH on the two excitation peaks of the PHP indicator, cells (previously grown in LB medium) were pelleted and resuspended in M9 minimal medium buffered at different physiologically relevant pH values (ranging from pH = 5.5 to pH = 8.5; see *Experimental Procedures* for details) in which the pHi was collapsed to equalize the extracellular pH. This effect was achieved by the addition of benzoate (a permeant acid) and methylamine (a permeant base), and this pH collapse was experimentally verified by using the pH-responsive, ratiometric chemical fluorophore 5(6)-carboxyfluorescein diacetate succinimidyl ester (data not shown). We observed that upon acidification, the excitation peak of PHP at $\lambda_{\text{excitation}} \sim 395$ nm decreased with the corresponding increase in the peak at $\lambda_{\text{excitation}} \sim 475$ nm (Fig. 2B). Conversely, the excitation peak at $\lambda_{\text{excitation}} \sim 395$ nm increased when pHi alkalized, while the intensity of the peak at $\lambda_{\text{excitation}} \sim 475$ nm concomitantly decreased. Almost identical spectra were obtained in PHP-expressing *E. coli* DH5α and *P. putida* KT2440 (data not shown), confirming the expected PHP’s sensitivity to changes in pH in the range of physiological pH values in different bacterial species. Although the bimodal pattern in both excitation peaks of PHP was essentially the same across the different Gram-negative bacteria tested herein, we observed that the $\lambda_{\text{excitation}}$ values of 405 nm and 485 nm reported by Martinez *et al.* (2012) gave rise to the same results regarding calculated pHi values. We thus adopted these experimentally determined $\lambda_{\text{excitation}}$ values (405 and 485 nm) for all subsequent experiments. In summary, our results thus far indicate that the spectral properties of the PHP fluorescent protein are very similar to those of pHluorin2, the parental ratiometric protein from which it derives (Mahon, 2011), across different bacterial species.

The next step was to use this dual excitation attribute of PHP to obtain a calibration curve by plotting the ratio between the fluorescence peaks at $\lambda_{\text{excitation}} = 405$ nm and $\lambda_{\text{excitation}} = 485$ nm (RF(405/485)) against the pH values of equilibrated cells in the set of pH-modified buffers described above. At first, we harvested exponentially growing *P. aeruginosa* PA14/pS2513-PHP cells incubated in LB medium. Once again, the pHi of the cells was rapidly collapsed to equalize the pH of the different buffered solutions as detailed above. This allowed us to calculate highly reproducible sigmoid-shape calibration curves using the Boltzmann best-fitting equation as indicated in *Experimental Procedures* (Fig. 2C). The same
procedure was applied to obtain calibration curves in cells sampled from exponentially growing cultures of E. coli DH5α/pS2513-PHP and P. putida KT2440/pS2513-PHP (Fig. S1A and S1B in the Supporting Information respectively), and of P. aeruginosa CH2682/pS2513-PHP (data not shown) in LB medium.

We observed some inconsistencies in the \textit{R}_{405/485} values when the pH$_i$ of \textit{Pseudomonas} cells was collapsed in buffers with pH $>$ 7.5. The differences in pH sensitivity of GFP-borne indicators under alkaline conditions have been reported to be caused by the accessibility of chromophores to H$^+$ ions in the solvent (Bencina, 2013). In order to overcome this problem, we set to supplement all \textit{Pseudomonas} cultures with a sublethal amount of the hydrazone protonophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP). At low concentrations, this compound is known to translocate H$^+$ ions across the cell wall, thereby dissipating the H$^+$ gradient (i.e. ApH) across the cytoplasmic membrane without significantly compromising viability (Ghoul \textit{et al.}, 1989; Clark \textit{et al.}, 2015). Note that CCCP addition was necessary for \textit{Pseudomonas} cultures as these species are known to display a very limited membrane permeability (see, for instance, the section below on antibiotic treatment). Protonophores (e.g. CCCP) increase membrane permeability by altering the electrochemical gradient, thus establishing an equilibrium across the cell membrane (Yu \textit{et al.}, 2015).

The addition of CCCP at 20 μM to \textit{Pseudomonas} cultures tackled the problem observed during incubation of the cells in alkaline-buffered solutions, and the presence of this protonophore did not impact cell growth across all the conditions tested herein, both in complex and minimal culture media. By applying this strategy, we could obtain highly reproducible sigmoid-shape calibration curves for all \textit{Pseudomonas} species harbouring plasmid pS2513-PHP, as shown for \textit{P. aeruginosa} PA14 (Fig. 2C) and \textit{P. putida} KT2440 (Fig. S1B in the Supporting Information). Moreover, addition of CCCP to \textit{E. coli} cultures did not affect the calibration curves obtained for this species (data not shown), indicating that this strategy can be used in other Gram-negative bacteria to collapse the pH$_i$. With these tools and procedures at hand, we set to calculate pH$_i$ in \textit{E. coli} and in all \textit{Pseudomonas} species under different culture conditions as indicated below.

**physiological cytoplasmic pH values are conserved across Gram-negative bacterial species**

The calibration curves constructed for wild-type \textit{P. aeruginosa} strains (both PA14 and CH2682), \textit{P. putida} KT2440 and \textit{E. coli} DH5α were used to calculate the pH$_i$ in actively growing cells cultured in LB medium (Table 1). Cell pellets of the four species, promptly harvested from the corresponding cultures, were resuspended in M9 medium salts lacking any perment acid or base. Under these conditions, cells were able to maintain their pH$_i$ homeostasis. We observed that the pH$_i$ values calculated for \textit{E. coli} DH5α, \textit{P. aeruginosa} PA14 and \textit{P. putida} KT2440 were highly similar across all strains tested – with an average value of pH$_i$ = 7.65 ± 0.14, close to neutrality. Furthermore, the pH$_i$ values obtained correspond to those previously reported for neutrophilic bacteria using other detection methods, including fluorescent pH indicator proteins (Olsen \textit{et al.}, 2002; Sezonov \textit{et al.}, 2007; Kruilwich \textit{et al.}, 2011; Martinez \textit{et al.}, 2012; Gao \textit{et al.}, 2016). A slightly higher pH$_i$ value was observed in the CH2682 clinical isolate of \textit{P. aeruginosa} (the cytoplasmic pH of this strain was consistently $>0.2$ units above the values observed in the prototypical laboratory strain PA14). Conversely, the lowest pH$_i$ value was observed for \textit{E. coli} DH5α cells. The external pH values determined in the culture supernatants indicated that cells were able to maintain stable pH$_i$ values while the external pH suffered changes (Table 1), e.g. due to the secretion of metabolic by-products by the cells. A case in point is that of \textit{E. coli}, for which the pH$_i$ was approximately 1.5 units above the external pH. \textit{E. coli} is known to secrete acetate and other low-molecular-weight organic acids to the culture medium (Nikel \textit{et al.}, 2008; Bernal \textit{et al.}, 2016), which is probably the reason explaining the acidification of the extracellular medium in these cultures. All the cultures of \textit{Pseudomonas} species, in contrast, had pH values close to neutrality – displaying differences between pH$_i$ and extracellular pH smaller than in \textit{E. coli}.

In order to validate these results against a different protocol for determination of pH$_i$, we calculated the pH$_i$ values of both \textit{P. aeruginosa} PA14 and \textit{P. putida} KT2440 by applying the well-established method of staining the cells with the pH-responsive fluorophore 5(6)-carboxyfluorescein diacetate succinimidyl ester (Riondet \textit{et al.}, 1997). Exponentially growing cells of each species cultured in LB medium were harvested and treated with the chemical fluorophore as indicated in \textit{Experimental Procedures}. The pH$_i$ values calculated with this method (pH$_i$ \textit{P. aeruginosa} PA14 = 7.78 ± 0.19 and pH$_i$ \textit{P. putida} KT2440 = 7.59 ± 0.12) had a variation of $<3\%$ with respect to those obtained via the PHP indicator – yet, the dispersion of experimental pH determinations across replicates of the same experiment was, on average, higher for the cells treated with 5(6)-carboxyfluorescein diacetate succinimidyl ester than for the cells carrying pS2513-PHP. Taken together, these results demonstrate that PHP is a reliable indicator of pH$_i$ in \textit{Pseudomonas} species, indicating that cells tightly maintain a pH$_i$ when cultured in favourable conditions (i.e. 

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fast growth in a rich culture medium), and the next question was to evaluate how they respond to internal and external metabolic perturbations in terms of pH homoeostasis.

**The cytoplasmic pH of P. putida fluctuates depending on the glucose consumption routes**

In the model platform bacterium *P. putida* KT2440 (Beneditti *et al.*, 2016; Nikel and de Lorenzo, 2018; Calero and Nikel, 2019), the initial steps for the catabolism of glucose occur simultaneously through the phosphorylation of the sugar to glucose-6-P by the enzyme glucokinase (Gk), or by its oxidation to gluconate by means of glucose 2-dehydrogenase (Gcd) (del Castillo *et al.*, 2007; Nikel and de Lorenzo, 2014; Nikel *et al.*, 2015; Nikel and Chavarría, 2016). The oxidation of glucose in the periplasm yields low-molecular-weight organic acids, e.g. gluconate and 2-ketogluconate (Hirsh *et al.*, 2003; Sánchez-Pascuála *et al.*, 2019). Hence, we set to study how the use of this peripheral pathway influences the pH, in strain KT2440 and its glycolytic mutant derivatives. We transformed the pS2513-PHP plasmid into two *P. putida* mutants, each devoid of one of the two aforementioned enzymes, and calculated the pH, in exponential and stationary phase cultures grown in M9 minimal medium containing glucose as the only carbon source (i.e. glycolytic conditions). In a Δgk mutant of strain KT2440, the bulk of hexoses (80–90%) is oxidized into gluconate, whereas in a Δgcd mutant, sugars are exclusively phosphorylated to glucose-6-P (Dvorák *et al.*, 2017; Sánchez-Pascuála *et al.*, 2017, 2019). Once again, cultures of *P. putida* KT2440 grown in the aforementioned medium supplemented with CCCP were used to calculate the calibration curve for pH, quantification in all strains (Fig. S2 in the Supporting Information).

As indicated in Fig. 3, cytoplasmic pH values calculated for exponentially growing *P. putida* KT2440 in M9 minimal medium with glucose (pH = 7.66 ± 0.09) were similar to the values calculated when the same bacteria were grown in LB medium (Table 1). Additionally, the cytosolic pH of wild-type cells grown in the presence of CCCP showed a drop in pH, decreasing to levels approaching the extracellular culture medium (i.e. around neutrality). This pH, range was observed to be maintained around the same values along the growth curve, even after the cultures reached the stationary phase (after 24 h of cultivation; see Fig. 3). The pH indicator thus captured the increase in pH, as cells transitioned from exponential growth into the stationary phase, a well-described phenomenon in Gram-negative bacteria (Lambert *et al.*, 1997; Kurbatov *et al.*, 2006; Reva *et al.*, 2006).

Interestingly, we observed significant differences in the cytoplasmic pH between the Δgk and Δgcd derivatives of *P. putida* (Fig. 3). The Δgk strain had a slightly higher (albeit statistically significant) pH, than the Δgcd mutant during exponential growth; this difference became more evident during stationary phase (e.g. pH, = 8.11 for the Δgk strain and pH, = 7.61 for the Δgcd strain after 24 h of cultivation). Forcing the carbon flow through oxidation (in the Gk-deficient strain) leads to the generation of gluconate (and 2-ketogluconate, the further oxidized product of this metabolic branch of hexose processing). Gram-negative bacteria, including *Pseudomonas* species, are known to respond to slight acidic stress by increasing their pH, (Kruithof *et al.*, 2011), and this general response could partially account for the results. In addition, the ΔpH (and PMF), which is another determining factor of the differences in pH, across the strains tested (Casey *et al.*, 2010), is likewise expected to be affected in these mutants. In turn, the differences in ΔpH result in an alteration of the energetic status of the cells (Führer *et al.*, 2005; Voskuil *et al.*, 2018). Interestingly, the Gcd-deficient *P. putida* strain was able to stably maintain pH, values around neutrality during the whole cultivation period. Experimental determination of gluconate and 2-ketogluconate confirmed that there were no detectable acidic by-products secreted into culture supernatants, while cultures of both the wild-type strain and its Δgk derivative reached gluconate and 2-ketogluc- 

| Bacterial strain | Relevant characteristics | Specific growth rate (h⁻¹) | Cytoplasmic pH | Extracellular pH |
|------------------|--------------------------|----------------------------|----------------|-----------------|
| *E. coli* DH5α   | Laboratory strain typically used for cloning | 1.46 ± 0.05                 | 7.54 ± 0.11    | 6.05 ± 0.02     |
| *P. putida* KT2440 | Wild-type strain, platform bacterial host | 1.89 ± 0.06                | 7.77 ± 0.11    | 7.51 ± 0.01     |
| *P. aeruginosa* PA14 | Wild-type strain, wide host virulence spectrum | 0.92 ± 0.02               | 7.63 ± 0.08    | 7.12 ± 0.02    |
| *P. aeruginosa* CH2682 | Wild-type strain, clinical isolate | 0.57 ± 0.03               | 7.87 ± 0.07    | 7.54 ± 0.02    |

All strains, carrying plasmid pS2513-PHP, were grown in LB medium, and the cytoplasmic pH was determined during exponential growth. The pH of the LB medium was determined to be 7.05 ± 0.02 just prior to inoculation of the cultures. These results represent the mean value of each parameter ± standard deviation of triplicate measurements from at least three independent experiments.

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with antimicrobial agents, as indicated in the sections below.

The PHP indicator can be used to track time-dependent changes in cytoplasmic pH upon a shift in extracellular pH

We investigated if the PHP indicator could reflect adaptive changes in pH, upon a sudden shift in extracellular pH values, both acidic and alkaline. To this end, both wild-type *E. coli* MG1655 and *P. putida* KT2440 harbouring plasmid pS2513.PHP were grown in M9 minimal medium containing glucose, and the cell suspensions were challenged by exposure to a buffer at pH = 5.5 or pH = 8.5 (see Experimental Procedures for details). The pH values were calculated from fluorescence readings taken every 10 sec over 5 min to capture the short-term dynamics of cytoplasmic pH homeostasis upon the acidic or alkaline challenge (Fig. 4). Control experiments were run for both bacterial species by adding benzoic acid to the cell suspension in order to collapse pH, an operation which, as expected, resulted in an almost immediate drop in pH, to match the pH value of the external medium (i.e. pH = 6.95 ± 0.05). The pH values recorded for *E. coli* and *P. putida* did not recover after exposure to sodium benzoate (nor did they recover during 1 h post-treatment, data not shown). In experiments without any perturbation in extracellular pH, pH values were stably maintained during the assay within the values previously observed for these bacteria (i.e. in the range of 7.5–7.8 units; see also Table 1). When *E. coli* was challenged with a buffer at pH = 5.5, the cells did not react with a sudden drop in pH, in contrast with the response to a pH = 8.5 shift, which entailed a sudden increase in pH, followed by a slow, steady decrease of the pH, to the values pre-shift (Fig. 4A). From 9 min onwards, the pH of base-challenged cells matched that of untreated control cultures (data not shown). Overall, these observations are similar to the results previously reported by Padan *et al.* (2005) and Šeputienė *et al.* (2006), indicating that the cytoplasmic pH of *E. coli* K-12 is more sensitive to alkaline challenges than to acidification of the surrounding culture medium. The pattern of pH values in *P. putida* KT2440 upon pH shifts was much more rigid in comparison (Fig. 4B). Exposure of the bacterial cells to pH = 5.5 or pH = 8.5 did not result in any major change in pH, over the whole experimental period, and the fluctuations observed in PHP fluorescence (hence, in pH, values) were within the range of the experimental error for these determinations. Taken together, the results of these experiments reflect the increased membrane permeability of *E. coli* as compared to that in *P. putida*. In fact, the outer membrane of several *Pseudomonas* species is known to exhibit from 100- to 400-

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**Fig. 3.** The cytoplasmic pH of *P. putida* is altered by the pathway used for glucose consumption. Wild-type *P. putida* KT2440 and its isogenic mutants in the enzymes glucokinase (Δglk) and glucose 2-dehydrogenase (Δgdh) were transformed with plasmid pS2513.PHP and grown in M9 minimal medium supplemented with glucose at 20 mM as the sole carbon source. The intracellular pH (pHi) was investigated during the exponential and stationary (24 h post-inoculation) phase of growth. The pHi of wild-type cells cultured in the presence of 20 μM carbonyl cyanide m-chlorophenylhydrazine (CCCP) is also shown in the figure. Independent triplicates of the experiments were carried out, and the level of statistical significance in the pairwise comparisons indicated in the comparisons across experimental conditions is indicated as **P < 0.01** and ***P < 0.001** (ANOVA).
fold lower permeability to hydrophilic compounds than that of *E. coli* (Yoshimura and Nikaido, 1982; Llamas et al., 2000; Sohlenkamp, 2017). Our results are also in line with the observations reported by Reva et al. (2006), suggesting that *P. putida* KT2440 displays increased resistance to pH shifts as compared to Gram-negative *P. aeruginosa*. Since the PHP indicator enabled to follow changes in pH upon alkalization or acidification of the external milieu, we also explored if this procedure could be used to capture the dynamic changes in pH brought about by antibiotic treatment as disclosed below.

**Response of the cytoplasmic pH of *P. aeruginosa* to treatment with different antibiotics**

The next phenotype that we addressed was the changes of pH due to addition of clinically relevant antibiotics often used for the treatment of *P. aeruginosa* infections. To this end, we first grew *P. aeruginosa* PA14 harbouring plasmid pS2513-PHP in LB medium to reach the mid-exponential phase of growth [i.e. optical density at 600 nm (OD_{600}) = 0.4–0.5]. At this point, we treated the cultures with three different antibiotics at one-fifth (0.2 ×) or at full (1 ×) minimal inhibitory concentrations (MIC) of the antibiotics, and we followed the changes in pH continuously over the course of 4 h (Fig. 5). The antibiotics chosen for this purpose belong to three different functional classes based on their mode of action: ceftazidime (CAZ, a cephalosporin), amikacin (AMK, an aminoglycoside) and ciprofloxacin (CIP, a fluoroquinolone) (Lambert, 2002; Morita et al., 2014; Bassetti et al., 2018).

No significant changes in the cytosolic pH were observed after exposure of *P. aeruginosa* PA14 to 0.2 × (1.6 µg ml⁻¹) and 1 × (8 µg ml⁻¹) MIC concentrations of CAZ when compared to untreated cells (Fig. 5A). However, a clear decrease in the pH values was observed after the treatment of the same cells with AMK, albeit only when the antibiotic concentration matched the full MIC (4 µg ml⁻¹, Fig. 5B). The drop in pH could be observed very early on (becoming noticeable just 1 h after treatment) and was retained until the end of the experiment. Remarkably, the pH in cells exposed to 1 × MIC concentration of AMK dropped to levels close to pH = 6.7. CIP also caused a reduction in the pH, which was evident from 2 h post-antibiotic treatment with 1 × MIC (0.125 µg ml⁻¹), and after 3 h when cells were challenged with 0.2 × MIC (0.025 µg ml⁻¹, Fig. 5C). This decrease was, however, not as strong as the pH drop observed in AMK-treated cultures. Interestingly, it was not possible to continue the pH assessment in cultures amended with 1 × MIC of CIP beyond 2 h after treatment. In this particular case, the integrity of the cells was likely compromised due to the harsh antimicrobial treatment, making it very difficult to obtain consistent cell pellets after centrifugation of the bacterial suspension. This phenomenon has been reported for *P. aeruginosa* when exposed to different antibiotics, including treatment with CIP (Day et al., 1993; Lee et al., 2017).

The results above shed light on the mechanism of action of antibiotics on *P. aeruginosa* cells, an area of active research. Bartek et al. (2016) suggested that antibiotic exposure with different types of bactericidal compounds increases the cytosolic pH in *Mycobacterium smegmatis*. Furthermore, they proposed that there is a general mechanism by which antibiotics kill bacteria through disruption of cellular biosynthetic processes (caused by the primary inhibition of either nucleic acids, proteins or cell wall synthesis) which in turn drives an imbalance in H⁺ homoeostasis leading to a potentially lethal increase in the pH. In our experiments, we have used three different types of antibiotics, both at MIC and sub-MIC concentrations. However, we could not observe an increase in pH, along the 4 h of antibiotic treatment. Conversely, exposure to high concentrations of AMK or CIP caused a drop in the cytosolic pH (Fig. 5). This effect could be caused by the permeabilization of the cell membrane due to a physiological response to the
damage caused by the aforementioned antibiotics, a phenomenon previously characterized in Gram-negative bacteria (Dougherty and Saukkonen, 1985; Davis, 1987; Novo et al., 2000). The differential permeability of the cell membrane of *Pseudomonas* species to chemicals is a decisive factor that also dictates tolerance or sensitivity to antimicrobials (Novo et al., 2000; Shen et al., 2019). On the other hand, the phenomenon of pH increase upon antibiotic treatment could be affected by the nature of the bacteria tested [e.g. Gram-positive versus Gram-negative species (Eichenberger and Thaden, 2019)], and also the experimental conditions used [e.g. addition of antibiotics at the onset of the cultivation or during active growth of the bacterial cells]. Taken together, our results suggest that *P. aeruginosa* may react differently to treatment with diverse types of antibiotics, differing in their mechanism of action, at the level of pH homoeostasis.

Conclusions

In this work, we described the construction and calibration of a non-invasive pHi indicator and we have demonstrated its efficacy in *E. coli* and *Pseudomonas* species by using a standard protocol that can be easily reproduced in different bacteria (Fig. 6). We have shown the efficacy of this new tool in *P. aeruginosa* and *P. putida* by quantifying the alterations in pHi homoeostasis elicited after internal (redirection of glucose consumption pathways) and external (antibiotic treatment) metabolic perturbations, as well as pH shifts for both *E. coli* and *P. putida*. Although the vector platform containing the pH indicator was primarily designed for *Pseudomonas* species, it can be easily adapted to other Gram-negative bacteria (unlike other pH ratiometric protein-bearing plasmids), since our system was constructed following the rules set in the SEVA format (Silva-Rocha et al., 2013). Plasmids constructed according to this format allow for the easy construction of different parts at the user’s will. Unique oriV modules can be swapped to further broaden the (already sizeable) host range of the plasmids. Typically, different intracellular amounts of pHi will be expected by increasing or decreasing the plasmid copy number. Other manipulations are also possible: different antibiotic markers can be exchanged into the vector depending on the resistance profile of the bacterium under scrutiny. Simple approaches for the online assessment of intracellular properties such as pHi will thus enable both fundamental and applied studies, e.g. biosensor-based evolution of phenotypic traits (Fernández-Cabezón et al., 2019).

Experimental procedures

Bacterial strains and culture conditions

All strains used in this work are derivatives of *E. coli* strains MG1655 (Blattner et al., 1997) and DH5α (Manoil and Beckwith, 1985), *P. putida* KT2440 (Bagdasarian et al., 1981; Belda et al., 2016), and *P. aeruginosa* strains PA14 (Friedman and Kolter, 2004) and CH2682 (Hornischer et al., 2018). *E. coli* DH5α was used both for the propagation and construction of plasmids and for pH calculations, along with the well-characterized K-12 strain MG1655 (Blattner et al., 1997). The *P. putida* Δglk and Δgcd mutants are derivatives of the parental strain KT2440 with specific mutations in the genes encoding the enzymes that execute the first steps of glucose utilization (Sánchez-Pascuala et al., 2017, 2019). *P. aeruginosa* CH2682 is a meropenem-resistant clinical strain isolated from a patient with a rectal infection (Hornischer et al., 2018).

Unless indicated otherwise, bacteria were cultured in 50-ml Erlenmeyer flasks containing 10 ml of LB medium

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Fig. 6. General procedure for in vivo assessment of intracellular pH (pHi) in *Pseudomonas* species by means of the PHP indicator. Plasmid pS2513-PHP is introduced into the bacteria of interest by chemical transformation, mating or electroporation. The expression of PHP is driven by the constitutive P\_CAT promoter, and vector pSEVA2513 carries the orf(RSP1010) origin of replication, indicated as a purple circle. The kanamycin-resistance (Km\^r) determinant and the T1 and T0 transcriptional terminators are likewise highlighted in the plasmid. Cells are grown in the appropriate culture medium, and a small culture sample is harvested by centrifugation. The cell pellet is resuspended in M9 medium salts adjusted to different pH values (ranging from pH = 5.5–8.5, see Experimental Procedures) to a final OD\_600 = 0.4–0.5. A 200-\*\(\mu\)l aliquot of each cell suspension is transferred into a 96-well microtiter plate, and OD\_600 and fluorescence (excitation = 405 nm and 485 nm; \(\lambda_{\text{emission}} = 515\) nm) are measured in a plate reader. Finally, the \(R_{\text{pHi-cal}}\) values are plotted versus the pH values of pH-equilibrated cells (calibration curve), and the pH is calculated using a Boltzmann sigmoid best-fitting equation.

(Green and Sambrook, 2012) with agitation at 180 rpm at either 30\(^\circ\)C (*P. putida* strains) or 37\(^\circ\)C (*E. coli* and *P. aeruginosa* strains). In some experiments, *P. putida* strains were also grown in 10 ml of M9 minimal medium, containing 8.5 g l\(^{-1}\) Na\(_2\)HPO\(_4\)\(\cdot\)2H\(_2\)O, 3 g l\(^{-1}\) KH\(_2\)PO\(_4\), 1 g l\(^{-1}\) NH\(_4\)Cl, 0.5 g l\(^{-1}\) NaCl, 0.5 g l\(^{-1}\) MgSO\(_4\)\(\cdot\)7H\(_2\)O and 2.5 ml l\(^{-1}\) of trace element solution (Abril \textit{et al.}, 1989; Nikel and de Lorenzo, 2013) with glucose as the sole carbon source at 3.6 g l\(^{-1}\) (i.e. 20 mM). *P. putida* strains were also grown under gluconeogenic conditions by using succinate at 20 mM as the sole carbon source. Km was added when needed to ensure plasmid maintenance either at 50 \(\mu\)g ml\(^{-1}\) (for *E. coli* strains and *P. putida* KT2440 and its derivatives) or 500 \(\mu\)g ml\(^{-1}\) (for all *P. aeruginosa* strains). Additionally, the MIC of the antibiotics CAZ, AMK and CIP was determined in *P. aeruginosa* strains using macrodilutions in Mueller–Hinton broth as described previously (Wiegand \textit{et al.}, 2008).

For growth rate measurements, overnight pre-cultures of all the strains were grown for 16 h in 3 ml of LB medium supplemented with Km. One millilitre of each pre-culture was harvested by centrifugation (5000 g, 5 min) and washed twice with 1 ml of fresh LB. The cells were resuspended in fresh LB medium containing Km, diluted to a starting OD\_600 = 0.02 and distributed (200 \(\mu\)l each well) into a Honeycomb 2\(\text{TM}\) plate (Thermo Fisher Scientific, Waltham, MA, USA). The plate was incubated into an automated Bioscreen C MBR\(\text{TM}\) plate reader (Oy Growth Curves Ab, Helsinki, Finland), and the growth of each sample was constantly monitored by measuring its OD\_600. The growth rates of each strain were calculated from the exponential fraction of the growth curves (Nikel and Chavarría, 2016). The extracellular pH of culture media was measured in 1-ml aliquots of culture broth harvested at the times indicated, after separation of the bacterial biomass by centrifugation (5000 g, 5 min) and filtering the supernatant with a 0.2-\(\mu\)m filter-membrane (Filtropur S0.2; Sarstedt, Nümbrecht, Germany), with a SevenCompact\(\text{TM}\) pH meter S210 system (Metler-Toledo A/S, Glostrup, Denmark). In experiments involving *P. putida* and its glycolytic mutant derivatives, the concentration of gluconate and 2-ketogluconate in culture supernatants was determined by high-pressure liquid chromatography as explained by Nikel \textit{et al.} (2015).

Construction of a vector for the constitutive expression of the gene encoding the PHP indicator

The DNA sequence of the pfluorin2 gene was codon-optimized for *P. putida*, and the resulting coding sequence was ordered as a synthetic DNA fragment (Integrated DNA Technologies, Leuven, Belgium). For the sake of clarity, we refer to the optimized sequence as PHP throughout the text. The entire PHP sequence was PCR-amplified from the synthetic DNA with primers 5’-AAA AAG CTT AGG AGG AAA AAC ATA TGA GCA-3’ and 5’-TTT ACT AGT TTA CTT GTA GAG TTT AC-3’ containing the restriction sites HindIII and Spel (underlined) and a ribosome binding site (italics) adjacent to the start ATG codon, indicated in boldface in the forward primer (see also Sequence S1 in the Supporting Information). The PCR product and the expression vector pSEVA2513 were double-digested with the restriction enzymes HindIII and Spel and column purified (QIAquick\(\text{TM}\) PCR purification kit; Qiagen, Hilden, Germany). Both DNA fragments were ligated to give rise to plasmid pS2513-PHP, used throughout this study for pH\(_i\) determinations. The correct plasmid sequence was confirmed by sequencing of plasmid DNA retrieved from selected *E. coli* DH5\(\alpha\) clones. Plasmid pS2513-PHP was...
later introduced into P. putida and P. aeruginosa strains by electroporation (Choi et al., 2006; Volke et al., 2019; Wirth et al., 2019). Plasmid pS2513-PHP has been deposited in AddGene (https://www.addgene.org/) with catalogue # 122590.

Fluorescence microscopy of bacterial cells expressing PHP

Overnight cultures of bacteria transformed with plasmid pS2513-PHP were grown in LB medium supplemented with Km at the appropriate concentrations (see above). Next, 1 ml of each pre-culture was pelleted by centrifugation (5000 g, 5 min) and washed twice with 1 ml of fresh LB. Erlenmeyer flasks (50 ml) filled with 10 ml of fresh LB medium (without Km) were inoculated with the washed cells to an OD600 of 0.05 and grown until mid-exponential phase (OD600 = 0.4–0.5). Cells were fixed by mixing an aliquot of the culture with 5% (v/v) paraformaldehyde in a 1:1 (v/v) ratio, followed by a 30 min incubation at room temperature. Finally, 10 μl of the cell suspensions were placed onto 1.5% (w/v) agarose pads as described by Young et al. (2011) and visualized with a 100 × immersion oil objective in a Nikon Eclipse™ Ti microscope (Nikon Instruments, Amsterdam, Netherlands) equipped with a eGFP ET filter set and a Hamamatsu Orca Flash 4.0 camera (Hamamatsu Photonics K.K., Shizuoka, Japan). Fluorescence images were analysed with the Fiji software package (Schindelin et al., 2012).

In vivo assessment of cytoplasmic pH by flow cytometry and fluorescence spectroscopy using the pHluorin2 or PHP indicators and fluorescent chemical probes

Overnight grown pre-cultures of each bacterium carrying plasmid pS2513-PHP or plasmid pS2513-pHluorin2 were prepared in the indicated culture media (LB or M9 minimal medium, as specified in each case) added with the necessary antibiotics as described above for the fluorescence microscopy experiments. Unless stated otherwise, 10 ml cultures of each strain were prepared in the same media devoid of antibiotics and grown until mid-exponential phase (OD600 = 0.4–0.5). Next, 1 ml aliquots were taken from these cultures, and the OD600 of each sample was adjusted (if necessary) to 0.4 before centrifugation (5000 g, 5 min) and cell resuspension in 1 ml of M9 salts devoid of any carbon source, which yielded a cell density of approximately 10^9 cells ml^-1. For flow cytometry experiments, the resulting cell suspension was directly subjected to analysis in a MACSQuant VYB flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). Both pHluorin2 and PHP were assessed by using the same instrument settings. The fluorescence emission at 525 nm was detected using a 525/40-nm band pass filter array upon excitation with a diode-pumped solid-state laser at 488 nm (Nikel et al., 2016).

Size-related forward scatter signals gathered by the flow cytometer were used by the FlowJo software v. 9.6.2 (FlowJo LLC, Ashland, OR, USA) to calculate the geometric mean of fluorescence and coefficients of variation of the positive bacterial populations as indicated elsewhere (Benedetti et al., 2016; Akkaya et al., 2018).

For the fluorimetry determinations, 200 μl of each diluted sample were distributed into a 96-well microtiter plate (Corning & Costar, NY, USA) and incubated for 10 min at 30°C. After incubation, both the OD600 of the cell suspension and the fluorescence emission of PHP (λ_emission = 515 nm) were recorded at λ_excitation of either 405 nm or 485 nm with an EnSpire™ Plate Reader (Perkin-Elmer, Waltham, MA, USA). In order to generate a calibration curve to calculate pH_i, another set of cultures containing 10 ml of either LB medium or M9 minimal medium were inoculated with each bacterial strain. In the case of P. putida and P. aeruginosa strains, CCCP was added to the cultures at 20 μM (in the case of the standard curve for E. coli, the addition of CCCP was not necessary). The cultures were also grown to mid-exponential phase as detailed before. One millilitre of each cell culture was spun down and resuspended into 1 ml of M9 medium salts containing 50 mM sodium benzoate and 50 mM methylamine HCl, and adjusted to the desired pH with 50 mM of the appropriate buffering agent [2-(N-morpholino)ethanesulfonic acid (MES) for pH = 5.5 to 6.5, 3-(N-morpholino)propanesulfonic acid (MOPS) for pH = 7.0 and 7.5, and N-Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) for pH = 8.0 and 8.5]. The effect of adding sodium benzoate or methylamine on collapsing pH_i was evaluated with the fluorescent probe 5(6)-carboxyfluorescein diacetate succinimidy (see section below), and it was found that pH_i equaled the pH of the surrounding medium within ± 0.5 units range. For both the PHP- and pHluorin2-dependent determinations of pH_i, culture samples (200 μl) were distributed into a 96-well microtiter plate and both OD600 and fluorescence were also recorded as described in the preceding section. A Boltzmann sigmoid best-fitting curve was applied to generate the calibration curves from the λ_excitation = 405 nm and λ_excitation = 485 nm excitation ratios (R405/485) obtained by incubating the cells into the buffered solutions, essentially as described by Martínez et al. (2012). The equation used to adjust the best-fitting curve is as follows:

\[ R(pH) = \frac{R_1 - R_2}{1 + e^{(pH-pK_i)/\phi}} + R_2, \]

where \( R(pH) \) is the function representing the Boltzmann sigmoid best-fitting curve; \( R_1 \) and \( R_2 \) are the lower and upper asymptotes of the curve (representing
minimum and maximum values of $R_{205/485}$ respectively; pH is the actual pH$_i$ value; pH$_0$ is the half-value for pH$_i$ within the range assessed; and $\phi$ is the slope of the fitting curve. The pH$_i$ values are expressed as the mean value of two measurements from at least three independent replicates $\pm$ SD.

In order to benchmark the PHP indicator against a well-established protocol, the pH$_i$ of the cells was calculated in some experiments by using the ratiometric fluorescent probe 5(6)-carboxyfluorescein diacetate succinimidyl ester according to the standard protocols of Riondet et al. (1997) and Olivares Pacheco et al. (2017). Briefly, cells, grown as indicated in the text, were collected at an OD$_{600}$ of 0.4–0.5 and centrifuged at 5000 g for 5 min at room temperature. The bacterial pellets were resuspended at a cell density of approximately $10^8$ cells ml$^{-1}$ in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.0) containing 1 mM EDTA and incubated for 10 min at 30°C with 2.5 mM 5(6)-carboxyfluorescein diacetate succinimidyl ester (Thermo Fisher Scientific, Waltham, MA, USA). Following this incubation, the samples were washed twice and resuspended in phosphate-buffered saline buffer (pH 7.0) containing 1 mM MgCl$_2$. In order to eliminate any possible excess from the non-conjugated probe, samples were treated with 10 mM glucose in phosphate-buffered saline buffer. Cells were then centrifuged at 5000 g for 5 min, and bacterial pellets were washed twice and resuspended in a buffer composed of 50 mM Tris, 50 mM MES, 140 mM choline chloride, 1 mM MgCl$_2$, 10 mM KCl, 10 mM NaHCO$_3$ and 0.5 mM CaCl$_2$ (pH 7.0). One hundred fifty microlitres of each sample was placed on black 96-well plates (NuncIon Δ Surface; Sigma-Aldrich Co., St. Louis, MO, USA) and incubated at 30°C in a microtiter plate spectrophotometer (Infinite 200 Pro™; Tecan Trading AG, Mannedorf, Switzerland). Fluorescence intensities were measured at excitation wavelengths $\lambda_{\text{excitation}} = 490$ nm (pH-sensitive wavelength) and $\lambda_{\text{excitation}} = 440$ nm (pH-insensitive wavelength). The emission wavelength used in all of these experiments was $\lambda_{\text{emission}} = 525$ nm. The excitation and emission intensities were used to calculate the corresponding ratios, which were associated to pH$_i$ through a linear regression in a standard curve prepared with cells exposed to a set of calibrated buffers following the procedure described by Riondet et al. (1997). The pH$_i$ values calculated with the chemical probe are expressed as the mean value of three measurements from at least three independent replicates $\pm$ SD.

In vivo tracking of the dynamic changes of cytoplasmic pH upon pH shifts in the extracellular milieu

These experiments were carried out with 10 ml cultures of E. coli MG1655 or P. putida KT2440, each carrying plasmid pS2513-PHP, grown in M9 minimal medium added with 20 mM glucose from overnight pre-cultures prepared as stated above. Once the cells reached mid-exponential phase (OD$_{600}$ = 0.4–0.5), 150-μl aliquots of each culture were distributed into 96-well microtiter plates and incubated for 10 min at 30 or 37°C (as appropriate for each strain) with orbital agitation at 180 rpm. The plate was transferred to the EnSpire™ Plate Reader, and the fluorescence emission was recorded continuously every 10 s as described previously. After 1 min incubation, 50 μl of acidic (pH = 5.5; buffered with 200 mM MES) or basic (pH = 8.5; buffered with 200 mM TAPS) M9 minimal medium containing 20 mM glucose was added to each well. Additionally, 50 μl of 250 mM sodium benzoate was added to a set of samples in order to fully collapse the pH$_i$ of the cells as indicated above. The recording of the fluorescence emission was resumed immediately after these additions. The pH$_i$ for each sample was calculated from the $R_{205/485}$ values obtained for the fluorescence emission plotted against the standard curves prepared as described above.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Calibration curves for E. coli and P. putida carrying the PHP indicator in a complex culture medium.

Fig. S2. Calibration curve for P. putida carrying the PHP indicator in a minimal culture medium.

Table S1. Intracellular pH assessment of P. putida KT2440 and mutants in glycolytic genes in glucose cultures.

Sequence S1. Synthetic DNA encoding the PHP indicator.