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Studying Intracellular pH of Bacteria with Fluorescent Tools

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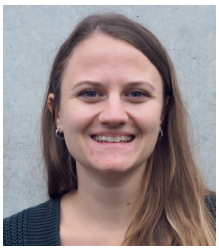
Abstract: This perspective discusses the relevance of studying the intracellular pH of pathogenic bacteria. Acidic environments trigger phenotype switches, increasing stress tolerance and antibiotic persistence – key challenges in treating bacterial infections. Understanding these phenotypic adaptations under clinically relevant stress conditions is important for elucidating bacterial survival mechanisms. Here, we discuss fluorescent tools to monitor pH homeostasis in bacterial cells and how advances in this field could shed light on pathogen resilience to antibiotics and human immune responses.

Keywords: Bacteria · Fluorescent tools · Persistence · Phenotype switch · pH sensing



Dorothea Kossmann received her BSc in Chemistry from the University of Freiburg and performed her Master's thesis in the group of Prof. Jörg Pietruszka at HHU Düsseldorf. After an internship in the Medicinal Chemistry Department of Hoffmann – La Roche, she joined the group of Prof. Pablo Rivera Fuentes at the University of Zurich to pursue her PhD studies. Within the NCCR AntiResist initiative (SNSF), her research focuses on developing chemical tools to study the physiology of bacteria.

1. Introduction

Antimicrobial resistance results in antibiotic treatment failures and is a major global health threat. A complete sterilization of a bacterial population by antibiotics is almost never achieved, with antibiotic resistance being the most studied cause of treatment failure.[1] However, even when bacteria are sensitive to the antibiotic, a small subpopulation of cells is always tolerant to lethal drug concentrations.[2] Unlike resistance, tolerance is not genetically encoded but is induced by fluctuating environmental conditions under which bacteria adapt their physiological state, like metabolism, growth phases, and stress resistance.[3,4] These multi-drug tolerant phenotypic subpopulations, called persisters, survive treatments and can regrow, causing relapsing infections (Fig. 1A).[1,2] Persistence is a transient, nonheritable phenotype, and subsequent generations of cells remain susceptible.[4,5]

1.1 *Phenotypic Heterogeneity*

Besides the stochasticity of biological processes, phenotypic heterogeneity is induced by environmental conditions such as heat, acid, antibiotics, and hyperosmotic stress.[1] Especially during infections, complex environments induce a broad phenotypic heterogeneity within a genetically identical bacteria population (Fig. 1B).[6] Unlike resistance, persistence is challenging to measure due to missing quantitative indicators and the complexity of physiological states with limited lifetimes.[5,7] Even though research on persisters is increasing, the molecular mechanism of their formation is not fully understood.[7,8] Some unresolved questions include: What is the persister status in patients? And how does the host immune system contribute to their formation?[7] To address clinically relevant questions, persisters must be studied under conditions that mimic in-patient conditions, as those phenotypes might not be observed under standard laboratory conditions (Fig. 1B).[6] Additionally, methods to analyze single cells with minimal cellular perturbation are required.[9] Cell physiology can be studied on several levels, including transcription, translation, metabolism, and pH homeostasis.[9,10]

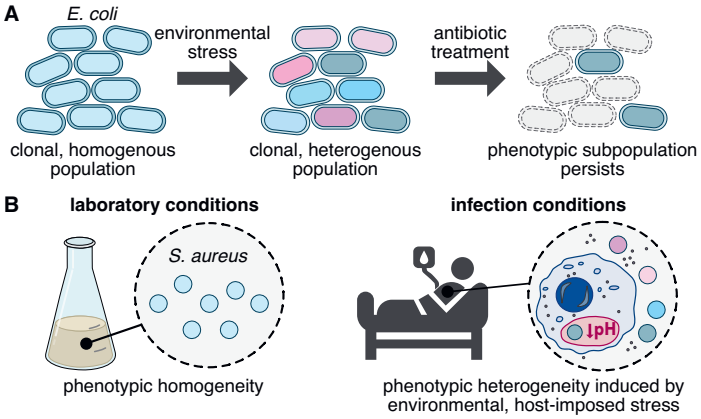


Fig. 1. Phenotypic heterogeneity of bacterial populations. **A** Schematic phenotype switches of a clonal *Escherichia coli* population induced by environmental stress. The colors represent different phenotypes. Upon antibiotic treatment, only persistent cells survive. **B** Comparison of *Staphylococcus aureus* phenotypes obtained under standard laboratory conditions compared to infections. During infections, bacteria are exposed to host-imposed stress, resulting in phenotypic heterogeneity.

1.2 *pH-Induced Phenotype Switch*

The pH homeostasis of living systems is crucial for maintaining protein functions and regulating multi-stress resistance responses.[10,11] Neutralophilic bacteria such as *E. coli* maintain their cytoplasmatic pH neutral, even if exposed to harsh external pH conditions.[12,13] However, changes in environmental pH influence transcriptional and translational processes, activating bacterial virulence or phenotype switches.[14,15] The host immune

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system can induce such a phenotypic switch. Macrophages, one of the first human defense systems, take up bacteria in phagosomes and degrade them by acidification.[16] Unintentionally, this low pH exposure can trigger persistence and has the opposite effect of clearing the infection. For instance, *S. aureus* has been reported to persist in low-pH cellular compartments of macrophages, allowing it to escape the immune system and regrow despite harsh conditions (Fig. 1B).[15] Persistence is not only induced by exposure to low pH, it has also been observed that cells becoming persisters exhibit a lower intracellular pH than clonal cells, which will die.[8] Consequently, intracellular pH may serve as a potential marker for the identification of persister cells.

2. Fluorescent Tools to Study pH in Bacteria

Fluorescent tools are powerful for studying processes and dynamics in biological systems and can be analyzed with flow cytometry or microscopy. Such techniques allow for the visualization of single-cell traits and enable access to the heterogenicity of biological systems, unlike methods that rely on bulk analysis of a whole population.[17] Fluorescent probes with dual-signal detection offer advantages over single-signal readouts, as ratiometric analysis provides a normalized, concentration-independent signal that reduces artifacts from instrument fluctuations, enhancing accuracy and sensitivity.[18] This self-calibration is crucial, as the bioavailability of small-molecule probes or protein expression can vary depending on the bacteria's environment and physiological state.[19]

2.1 *Small-Molecule-Based Sensors*

Most pH sensors are designed for mammalian cells, which favor uncharged, lipophilic molecules, whereas bacterial cells prefer positively charged, zwitterionic, and highly polar compounds.[19,20] These differences limit the use of many sensors in bacteria due to decreased uptake and increased efflux. This article highlights widely used probes successfully applied in bacterial imaging. Many pH sensors are based on the non-toxic xanthene dye fluorescein, which has high brightness and displays pH-sensitive absorption and emission due to ionic changes (Fig. 2A).[21] The highest emission of fluorescein is observed at neutral pH since the dianion with a strong donor-acceptor system is the main fluorescent species.

The fluorescein derivative bis-(carboxyethyl)-carboxyfluorescein (**BCECF**, Fig. 3) with three additional negative charges has shown increased cellular retention compared to fluorescein, which leaks out of cells.[22,23] To increase uptake by passive diffusion, the negative charges can be masked with diacetates or acetoxymethyl (**AM**), which upon cleavage by nonspecific esterases release the pH-sensitive form.[23,24] **BCECF-AM** is widely used to study intracellular pH in bacteria, as shown in bacteria-host cell studies with *S. aureus*.[25] Efflux of small molecule probes can be minimized with reactive conjugation groups such as thiol-reactive chloromethyl, amine-reactive succinimidyl ester, or isothiocyanate groups (Fig. 2B).[24,26,27] For instance, fluorescein isothiocyanate (**FITC**) is a widely used dye with pH-dependent emission near neutral pH.[27]

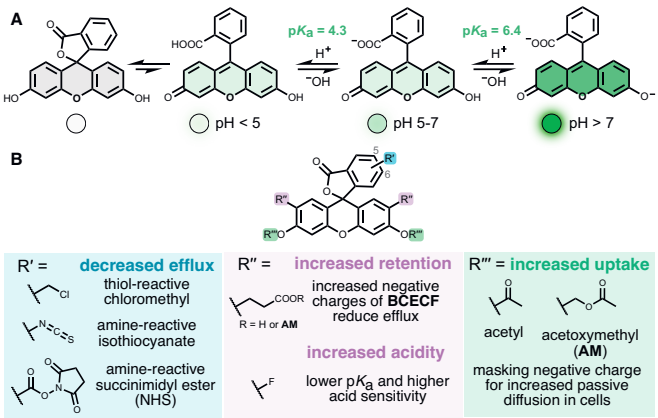

Fig. 2. Properties of fluorescein probes. **A** The pH equilibrium of fluorescein. At low pH, the spirocyclic form is mainly present and with increasing pH the anion (pK_a 4.3) and the dianion (pK_a 6.4) are formed. The dots below represent the relative emission brightness at the indicated pH. **B** Fluorescein scaffold modifications to reduce efflux, increase uptake and cellular retention, or tune the acid sensitivity.

Fluorescein derivatives, such as **BCECF** (pK_a 7.0) or **FITC** (pK_a 6.8), are unsuitable for sensing low pH since the fluorescent dianion species is only present in low concentrations (Fig. 2A). The acid strength can be tuned by introducing electron-withdrawing groups on the molecular scaffold. The fluorinated fluorescein derivative **Oregon Green** exhibits increased acid sensitivity (pK_a 4.7) and enhanced photostability and quantum yield (Fig. 3). The additional carboxylic acid group limits bacterial uptake, but replacing it with an *n*-propyl amide demonstrates good penetration into *E. coli*.[28]

In contrast to fluorescein derivatives, seminaphthorhodafluors (**SNARF**) emit red-shifted light and reduce overlaps with cellular autofluorescence signals. **SNARF-1** (pK_a 7.5) can sense pH changes around neutrality and has been applied in host-pathogen interaction studies (Fig. 3).[25] Modifying its structure by introducing a fluoride in *ortho*-position to the hydroxy group provides a more acid-sensitive derivative **SNARF-4F** (pK_a 6.4), which has mainly been applied for extracellular pH sensing of biofilms.[29,30] However, while using **SNARF** dyes, it is important to note that intracellular interactions can significantly change the pK_a and the spectral properties, and the probe should always be calibrated in the studied system.[31]

Another popular acidic pH sensor with a large dynamic range is **pHrodo** (pK_a 6.8). It shows increased fluorescence with decreasing pH and has been used to study bacterial phagocytosis.[32] Whereas the protonation of xanthene-based probes results in a weaker donor-acceptor system with decreased fluorescence, **pHrodo** exhibits a more complex sensing mechanism. In the deprotonated form, the first excited state (S_1) is a charge transfer state that decays in a non-radiative process. After protonation, S_1 becomes a localized excited state, enabling fluorescence emission.[33] The main disadvantage of **pHrodo** is its single signal readout, which prevents ratiometric analysis. The spectral changes are not only induced by pH but also by its intracellular concentration and interactions.[34] Thus, **pHrodo** serves only as a qualitative pH indicator, unsuitable for quantitative or comparative measurements between cells.

2.2 *Protein-Based Sensors*

The pH-sensitive green fluorescent protein (GFP), called **pHluorin**, is a widely used tool for intracellular pH studies and is constantly optimized.[35,36] It exhibits a reversible excitation ratio change between the protonated and deprotonated chromophore, with a pK_a of 7.1 (Fig. 4).[35] Since most mutants are tailored for eukaryotic systems, the expression of the system has to be optimized for other organisms, as shown for *E. coli*.[37] Red-shifted biosensors, like the pH-sensitive mutant **mCherryEA** (pK_a 7.3), have been developed and applied in high-throughput pH screenings of *E. coli* mutants.[38,39] This sensor has a beneficial 3-fold signal increase compared to **pHluorin** and is more sensitive.[39]

Although ratiometric pH sensing can be obtained with a single protein like **pHluorin**, the fluorescence intensity might vary between cells due to differences in protein expression. Fusion proteins, such as **mCherry-pHluorin** address this variability by combining non-pH-sensitive and pH-sensitive fluorophores.[40] This fusion has been used to compare cytoplasmic pH re-

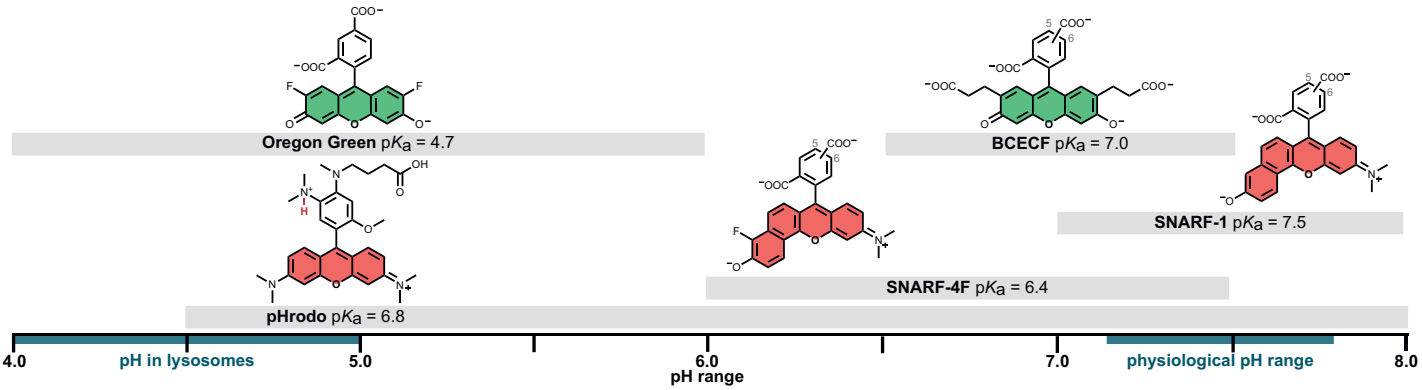

Fig. 3. Chemical structure of commonly used small-molecule pH sensors and their sensing range. **Oregon Green** (pK_a = 4.7, pH range 4.0-6.0), **BCECF** (pK_a = 7.0, pH range: 6.5-7.5), **pHrodo** (pK_a = 6.8, pH range: 4.5-8.0), **SNARF-4F** (pK_a = 6.4, pH range: 6.0-7.5) and **SNARF-1** (pK_a 7.5, pH range 7.0-8.0).

sponses in susceptible versus persistent *E. coli*, revealing that persistent cells exhibit a more acidic pH even before antibiotic treatment.[8]

In addition, protein sensors offer the advantage of targeting specific locations and allow extracellular pH sensing if located in the outer membrane or periplasm.[41] For instance, a pH-sensitive protein tagged to cell wall-binding domains of *S. aureus* showed that bacteria are exposed to low pH in macrophages.[15]

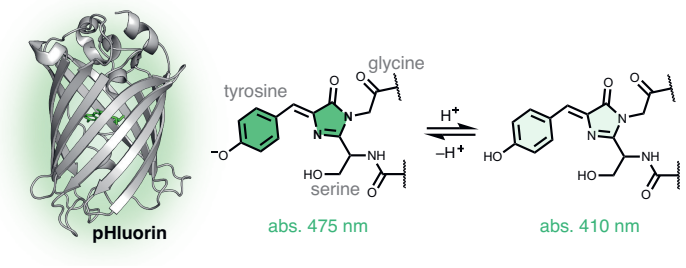

Fig. 4. Protein structure of the **pHluorin**-based sensor (Lime mutant, PDB: 7YV3). The amino acid residues tyrosine, serine, and glycine form the pH-sensitive chromophore. Protonation and deprotonation result in a ratiometric emission change between pH 5.5 to 7.5.

2.3 *Advantages and Disadvantages of Small-Molecule- and Protein-Based Sensors*

Protein sensors have several advantages, including selectivity and the ability to target specific subcellular locations. However, their application can be laborious and unsuitable for clinical isolates as they require genetic encoding that modifies native strains.[42] Moreover, their fluorescence intensity is influenced by buffer composition, bacterial viability, and expression levels, which could introduce sensing artifacts.[43] Additionally, chromophore maturation requires molecular oxygen, which might be limited in complex environments, leading to dim signals.[44] Although protein sensors have been optimized and successfully applied in several studies, these systems may need further optimizations to achieve sufficient expression and fluorescence if applied to different bacterial strains.

In contrast, small-molecule fluorophores can be applied to various bacterial strains less laboriously, allowing the imaging of non-genetically modified microorganisms, even in clinical samples.[45] However, the efficiency of bacterial staining depends on the uptake and retention of the chemical probes. Choosing a probe for bacterial studies often requires compromises between high cell accumulation, beneficial spectral properties, and the desired pH sensing range.

3. Conclusion and Future Prospects

Monitoring bacterial intracellular pH under host-mimicked conditions can provide valuable insights into how pathogens develop resilience to antibiotics.[6] To observe relevant phenotypes, it is crucial to study native clinical isolates using tools that minimize cellular perturbation. Unlike protein sensors, small-molecule probes allow non-invasive staining without genetic modification. Since most sensor probes have been tailored for mammalian cell imaging, more research on bacteria-specific probes could be beneficial. A universal small-molecule probe that effectively stains both Gram-positive and Gram-negative bacteria would be a valuable tool for studying the physiology of clinical strains. The *eNTRy* guidelines could be considered when designing such probes to achieve a high bacterial uptake. According to these guidelines, molecules with an ionizable amine (*N*), low three-dimensionality (*T*), and a high rigidity (*R*) with less than five rotatable bonds, small molecular weight (>600 Da), and high polarity are likely to accumulate in bacteria.[19,20,46] Efflux prevention could be achieved by installing a cross-linking functional group, such as chloromethyl or isothiocyanate.[26,27] Optimal spectral properties should include high brightness and selective pH dependence with a broad dynamic range from neutral to acidic pH. Dual excitation or emission signals are essential for ratiometric analysis, ideally in the red region. Further desired properties include selective bacterial staining over mammalian cells and low toxicity to prevent stress or phenotype changes.

Bacteria remain an ambitious target for small molecules, but developing a robust probe could be beneficial for studying bacterial heterogeneity. Such tools may provide insights into clinical phenotypes and better understand how pathogens escape the human immune system and antibiotic treatments.

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