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COMMUNICATION

Assessing Changes in the Expression Levels of Cell Surface Proteins with a Turn-on Fluorescent Molecular Probe

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Tri-nitrilotriacetic acid (NTA)-based fluorescent probes were developed and used to image His-tagged-labelled outer membrane protein C (His-OmpC) in live *Escherichia coli*. One of these probes was designed to light up upon binding, which provided the means to assess changes in the His-OmpC expression levels by taking a simple fluorescence spectrum.

There is considerable interest in developing small-molecule-based probes that, by selectively binding short peptide tags, can fluorescently label proteins in their native environment.[1] The main advantages of using these probes over labelling with fluorescent protein (FPs) is their small size, which does not disrupt the normal function of the proteins of interest (POIs), as well as their structural versatility, which enables them to be tailored for different applications.[2] One of the most common tags used in recombinant protein production is the hexa-histidine tag (His-tag), which is widely used to purify His-tagged proteins following their expression.[3] Owing to its prevalence, small size, and ability to coordinate to transition metal ions, fluorescent probes based on nitrilotriacetic acid (NTA), which can coordinate to oligo-histidine tags via metal coordination, have been developed.[2,4] Of particular interest are multivalent probes that can bind the His-tagged POIs with high affinity.[4g-o] The versatility and wide applicability of such probes was demonstrated by Tampé, Piehler, and co-workers,[4m-p] who developed a range of tri-NTA-dye conjugates for studying trafficking and structural dynamics of His-tagged proteins, and for imaging them with super resolution (SR) microscopy.

Our group has been developing fluorescent probes that respond to changes that occur on protein surfaces.[5] Unlike probes that merely label the POI, protein surface sensors[5] change the intensity and/or the wavelength of emission upon binding. Because these changes largely depend on the molecular environment of the fluorescent dyes, such probes could be used to distinguish between protein conformational states and binding interactions,[5e] as well as between structurally similar isoforms that have distinct surface characteristics.[5a-d,5f] The versatility of this approach was demonstrated with protein surface sensors based on tri-NTA-Ni^{2+} complexes that can analyze a wide range of His-tagged labeled proteins.[5e,f] By attaching the tri-NTA unit to fluorescent DNA oligonucleotides, we have recently obtained structurally programmable probes.[5f,6] These DNA-based probes were used to differentiate between His-tagged glycoforms[5f] or label His-tagged cell surface proteins (CSPs) of living bacteria and to provide the bacteria with new properties.[6]

One of the advantages of using turn-on probes (or sensors) over non-responsive probes (or labels) is the ability of the first to track dynamic changes and afford rapid, wash-free detection. Although a few turn-on NTA-based sensors for His-tagged proteins were developed,[7] these systems generally rely on low-affinity mono-NTA binders,[7a-d] or require pre-incubation with a competing quencher that reduces their binding affinity.[7e,f] In our previous work we used responsive tri-NTA-based probes[5e,f] to study structural dynamics[5e] and glycosylation states[5f] of His-tagged proteins. However, these probes only operated *in vitro*. In contrast, our non-responsive probes, which can selectively attach to a His-tagged CSP in living bacteria,[6] can only serve as labels. Herein we present a new tri-NTA-based probe (Fig. 1a, probe **1**) that exhibits a turn-on response upon binding to a His-tagged CSP of living bacteria. To demonstrate the advantage of using this probe over labels, we generated two additional tri-NTA probes that bear non-responsive fluorescent reporters (Fig. 1a, probes **2** and **3**). We show that with **1**, changes in the expression levels of His-OmpC can be straightforwardly assessed by taking a simple fluorescence measurement.

Analytical methods for determining protein expression levels are important for studying their biological function,[8] as well as for determining the optimal condition for recombinant

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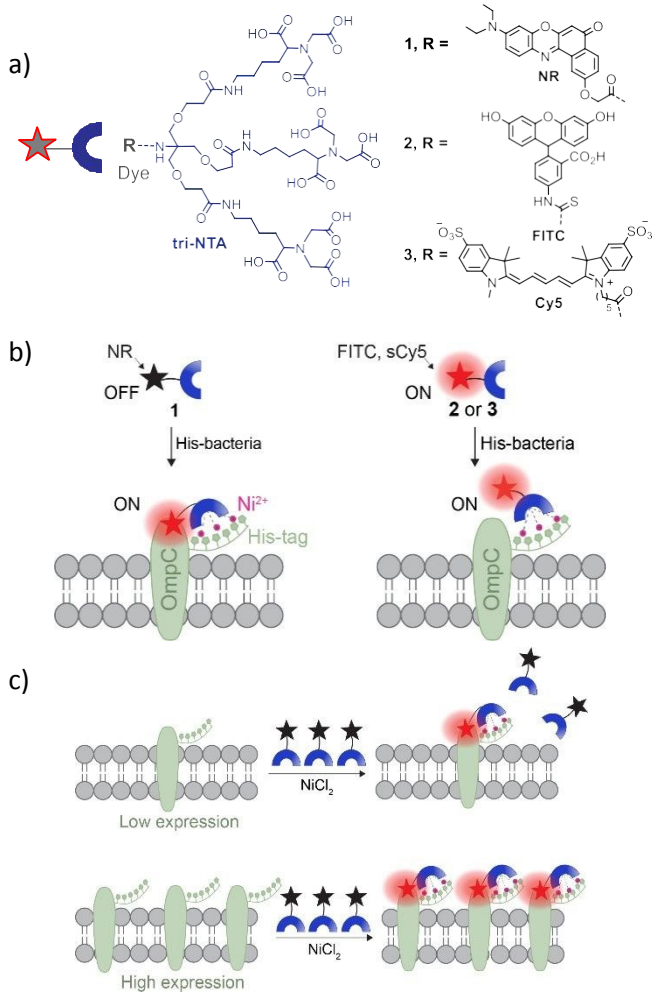

Fig. 1 (a). Chemical structure of turn-on probe **1** and non-responsive probes **2** and **3**. (b) Schematic illustration of the labelling of His-OmpC in living bacteria using probe **1** (left) or probes **2** and **3** (right). (c) Illustration of the way that changes in the OmpC expression levels can be straightforwardly assessed using probe **1**. Bacteria expressing low levels of OmpC (top) will induce a smaller fluorescence response than bacteria with high levels of OmpC expression (bottom).

protein production.[9] Although protein expression levels can be determined using common analytical techniques, such as western blotting (WB) or fluorescence cell imaging, these methods do not provide high-throughput analysis. With WB, the cells must be lysed and the detection process involves gel electrophoresis and cumbersome procedures.[10] Labeling the POI with fluorescent probes, such as NTA-based probes,[2,4,11] or fluorescently labeled antibodies,[12] can be used to assess protein expression in intact cells. These methods, however, require the cells to be washed and plated over coated surfaces, as well as expertise in operating fluorescence microscopes and image processing tools, which are not accessible to many laboratories.

To demonstrate the advantage of using turn-on probes for assessing changes in protein expression levels in intact cells, three probes that share the same tri-NTA unit, but differ in their fluorescent reporters were synthesized (Fig. 1a). According to our design, probe **1**, which is appended with an environmentally sensitive solvatochromic dye (Nile Red, E_x/E_m: 545/655 nm), should act as a turn-on fluorescent sensor that responds to binding to a His-tagged POI (Fig. 1b, left). In contrast, probes **2** and **3**, which are appended with non-responsive fluorescein (E_x/E_m: 465/520 nm) or sCy5 (E_x/E_m: 620/665 nm) dyes, were designed to serve as control probes, namely, fluorescent labels whose emission should not change upon binding to the His-POI (Fig. 1b, right). Our expectation that the binding of **1** to His-tag proteins would induce a turn-on emission signal (Fig. 1b, left) is based on our previous studies showing that molecules linked to specific protein binders tend to 'stick' to the surface of POI.[5,13] In addition, it is based on the ability of solvatochromic dyes to light up upon binding to hydrophobic domains on proteins.[5d-f,14]

The outer membrane protein C (OmpC) of *E. coli* was selected as the protein target for this study because its expression can be controlled by culturing the bacteria under different temperatures.[15] This should enable us to test the main hypothesis in our design, namely, that changes in the His-OmpC expression levels can be straightforwardly analyzed by following changes that occur in the fluorescence of **1** (Fig. 1c). Specifically, we expected that bacteria expressing low levels of His-OmpC (Fig. 1c, top) should activate fewer probes than bacteria with high His-OmpC levels (Fig. 1c, bottom). As a result, bacteria with high expression levels should induce a stronger fluorescence response.

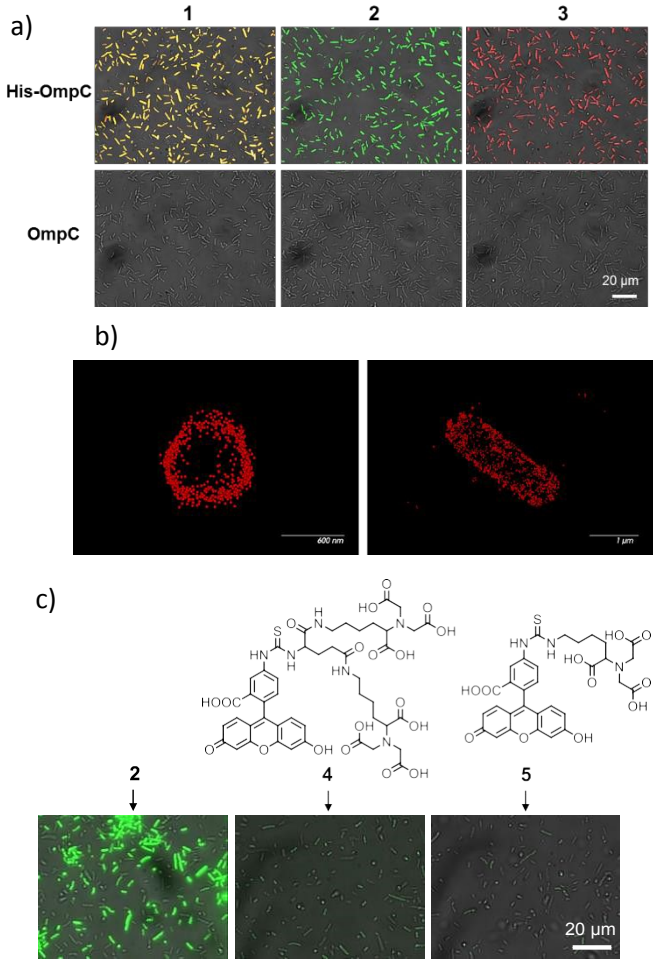

Fig. 2 (a) Merged bright-field and fluorescence images of bacteria expressing His-OmpC (top) and OmpC (that lacks the His-tag) (bottom) following incubation with probes **1**-**3** (500 nM) and washing. (b) STORM images of His-tagged bacteria labelled with probe **3**. Left: Transverse cut. Right: Whole bacteria. (c) Images of bacteria expressing His-OmpC following incubation with 500 nM of probe **2** , **4,** or **5**.

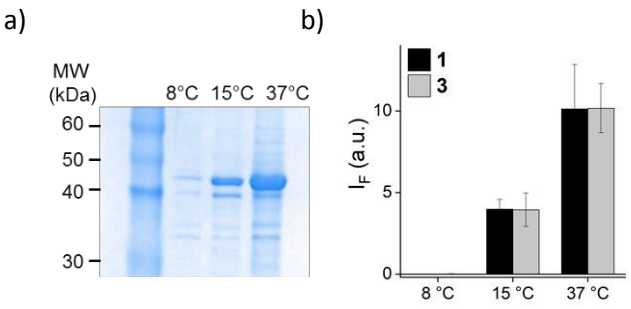

Fig. 3 Assessing changes in His-OmpC expression level in bacteria cultured at 8, 15, or 37 °C. (a) Gel electrophoresis, or (b) Fluorescence imaging. The bars correspond to the fluorescence intensity values measured from living bacteria labelled with probes **1** or **3**.

Initially, we tested the ability of the three probes to fluorescently label His-OmpC by incubating them (500 nM) with the engineered bacteria (His-bacteria) and Ni(II), followed by washing and imaging (Fig. 2a, top). As a control, the probes were also incubated with *E. coli*-expressing OmpC (that lacks His-tag) (Fig. 2a, bottom). The results show that only the His-tagged bacteria were labelled, each with a distinct emission color, indicating specific interactions between the probes and His-OmpC. Super resolution STORM (stochastic optical reconstruction microscopy) imaging with probe **3** showed that, as expected, the labelling occurs only on the outer membrane (Fig. 2b, left) and that His-OmpC spans across the entire bacteria (Fig. 2b, right). The contribution of the multivalent interaction between the three NTA groups and the His-tag to the labelling efficiency of the probes was determined by comparing the fluorescence images taken following incubation of the His-bacteria with 500 nM of **2** (Fig. 2c, left) to the ones taken following incubation with 500 nM of probes **4** (middle) or **5** (right), which possess only two or one NTA group, respectively. The notable increase in fluorescence with an increase in the number of NTA units correlates well with the enhancement in the binding affinities of the three probes ($K_{d\text{-}5}$ = 5578 ± 7 nM, $K_{d\text{-}4}$ =297 ± 1 nM, $K_{d\text{-}2}$ =14.5 ± 0.6 nM) toward the His-tag, which were determined by microscale thermophoresis (MST) (Fig. S1, ESI).

To obtain bacterial cells expressing different levels of His-OmpC on the outer membrane, the bacteria were cultured at 8, 15, or 37 °C. The expected increase in His-OmpC expression[15b] was verified by gel electrophoresis (Fig. 3a and Fig, S2, ESI), showing a notable enhancement in the His-OmpC levels when the temperature increased from 8 to 37°C. Precisely, at 8 °C, there was poor expression, whereas at 37 °C, maximal expression levels were observed. Another way to analyze these changes, which does not require cell lysis and gel electrophoresis, is by labelling His-OmpC with our probes and comparing the fluorescence intensity generated by the different samples. To this end, the bacteria that were cultured under different temperatures were labelled with probes **1** or **3** and the fluorescence generated by the labelled cells was quantified. As shown in Figure 3b, the changes in His-OmpC expression determined by fluorescence imaging are in agreement with the ones observed using the gel electrophoresis analysis.

Next, we determined whether, as expected from our design, the emission of probe **1** would enhance upon binding to the engineered bacteria (Fig. 1b, left) and whether the change in fluorescence could be used to straightforwardly determine changes in the His-OmpC expression (Fig. 1c). For this purpose, we measured the emission spectrum of 100 nM of **1** with Ni (II) before and after adding the engineered bacteria (His-bacteria) (Fig. 4a, left) and compared the optical responses to the responses of probes **2** (Fig. 4a, middle) and **3** (Fig. 4a, right) under the same conditions. As a control, we also measured the responses of the three probes to the bacteria that lack a His-tag. The results show that the emission intensity of probes **2** and **3** slightly changed (1.2- and 1.5-fold, respectively) upon incubation with the His-tagged *E. coli*. However, because similar changes were observed when **2** and **3** were subjected to bacteria that lack His-tag, these experiments indicate that the small changes in the emission resulted from non-specific interactions, which should not be affected by alterations in the His-OmpC expression levels. In contrast, subjecting probe **1** to the His-bacteria led to a hypsochromic shift and a 4.25-fold increase in the emission intensity (at 655 nm). The fact that these profound changes were only induced by the bacteria that express a His-tag indicates that probe **1** acts as a sensor that selectively responds to His-OmpC.

The benefit of using a turn-on sensor (probe **1**) over labels (probes **2** and **3**) for assessing changes in the local concentrations of His-OmpC was demonstrated by recording the emission spectra of the three probes in the presence of the His-tagged bacteria cultured at 8, 15, and 37 °C (Fig. 4b). Unlike with the fluorescence cell imaging experiment (Fig. 3b), in which differences in His-OmpC expression could be determined by both the sensor (**1**) and the label (**3**), there was no change in the emission spectrum of labels **2** (Fig. 4b, middle) or **3** (Fig. 4b, right) upon incubation with the different samples of bacteria. In contrast, probe **1** exhibited an increase in emission (Fig. 4b, left) that is proportional to the enhancement in the His-OmpC expression level (Fig. 3). Although the temperature-dependent increase in His-OmpC expression was also determined by gel

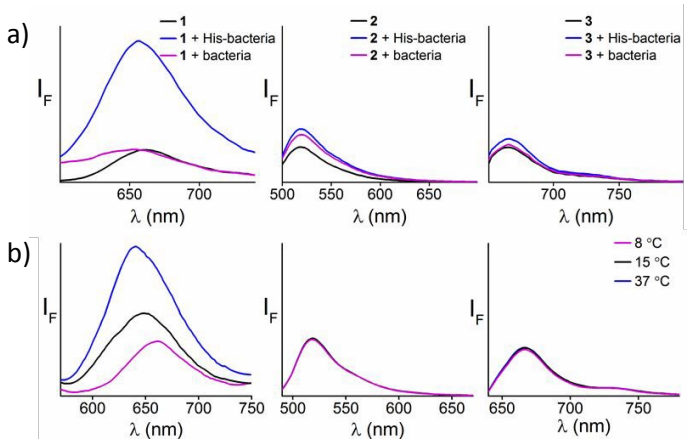

Fig. 4 (a). Fluorescence spectra of probes **1** (left), **2** (middle), and **3** (right) in the absence (black) and presence of bacteria expressing His-OmpC (His-bacteria) or bacteria that express unmodified OmpC (bacteria). (b) Fluorescence spectra of probes **1** (left), **2** (middle), and **3** (right) to His-bacteria cultured at different temperatures.

electrophoresis (Fig. 3a) and cell imaging (Fig. 3b), the current experiment (Fig. 4b, left and Fig. S3, ESI) shows that the same information can be obtained using a simplistic, wash-free protocol. Specifically, with probe **1** the CSP expression levels can be readily analyzed by performing a single incubation step and by taking a single fluorescence measurement.

To summarize, a turn-on fluorescent probe (**1**) integrating a tri-NTA group and a solvatochromic dye was developed and used to analyze changes that occur in the expression levels of His-OmpC in living bacteria. Additional probes that combine NTA groups with non-responsive fluorescence dyes (**2**-**5**) were also developed and used to confirm the principles underlying the sensor design. We showed, for example, that although all probes (**1**-**5**) can label His-OmpC, only sensor **1** displayed fluorescence enhancement upon binding to the engineered bacteria, indicating the important role the environmentally sensitive dye plays in the sensor's response. The contribution of the tri-NTA unit to the sensor's labeling efficiency was also demonstrated by showing that the mono- and bis-NTA probes (**4** and **5**) poorly label the bacteria compared with the tri-NTA probes. Our results also show different applications that can be achieved with the new tri-NTA probe family developed in this study (probes **1**-**3**). We have shown that probes **1**-**3** can be used to label His-tagged CSPs with different colors; probe **3** can be used to image them with super resolution, and probe **1** can straightforwardly analyze changes that occur in their properties. Probe **1** thus adds a new capability to our previous tri-NTA-based probes that could either sense structural changes of His-tagged proteins *in vitro,*[5e,f] or could only label them in living cells.[6] The ability of **1** to determine changes in the expression level of His-tagged proteins in living bacteria indicates the potential to expand the analytical tools currently used to monitor changes in protein expression, such as WB or fluorescence cell imaging. In our future work we aim to test whether such sensors can be used to quantify protein expression levels in living cells and whether they can be applied to investigate the dynamic properties of cell surface receptors.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

1. Y. Takaoka, A. Ojida and I. Hamachi, *Angew. Chem. Int. Ed.* , 2013, **52**, 4088.
2. N. Soh, *Sensors,*, 2008, **8**, 1004.
3. (a) J. A. Bornhorst and J. J. Falke, *Methods Enzymol*, 2000, **326**, 245; (b) J. K. Setlow, *Genetic Engineering: Principles and Methods*, Springer US, 1990; (c) F. Khan, M. He and M. J. Taussig, *Anal. Chem.*, 2006, **78**, 3072.
4. (a) K. Gatterdam, E. F. Joest, V. Gatterdam and R. Tampé, *Angew. Chem. Int. Ed.* , 2018, **57**, 12395; (b) E. G. Guignet, R. Hovius and H. Vogel, *Nat. Biotechnol.*, 2004, **22**, 440; (c) H. Block, B. Maertens, A. Spriestersbach, N. Brinker, J. Kubicek, R. Fabis, J. Labahn and F. Schäfer, in *Guide to Protein Purification, 2nd Edition*, 2009, pp. 439; (d) S. H. Uchinomiya, H. Nonaka, S. H. Fujishima, S. Tsukiji, A. Ojida and I. Hamachi, *Chem. Commun.*, 2009, 5880; (e) S. V. Wegner and J. P. Spatz, *Angew. Chem. Int. Ed.*, 2013, **52**, 7593; (f) Y. T. Lai, Y. Y. Chang, L. Hu, Y. Yang, A. Chao, Z. Y. Du, J. A. Tanner, M. L. Chye, C. Qian, K. M. Ng, H. Li and H. Sun, *Proc. Natl. Acad. Sci. USA*, 2015, **112**, 2948; (g) A. N. Kapanidis, Y. W. Ebright and R. H. Ebright, *J. Am. Chem. Soc.*, 2001, **123**, 12123; (h) Z. Huang, J. Park, D. S. Watson, P. Hwang and F. C. Szoka, *Bioconjug. Chem.*, 2006, **17**, 1592; (i) Z. Huang, P. Hwang, D. S. Watson, L. Cao and F. C. Szoka, *Bioconjug. Chem.*, 2009, **20**, 1667; (j) M. Brellier, B. Barlaam, C. Mioskowski and R. Baati, *Chem. Eur. J.*, 2009, **15**, 12689; (k) N. Zenmyo, H. Tokumaru, S. Uchinomiya, H. Fuchida, S. Tabata, I. Hamachi, R. Shigemoto and A. Ojida, *Bull. Chem. Soc. Jpn.*, 2019, **92**, 995; (l) J. Knezevic, A. Langer, P. A. Hampel, W. Kaiser, R. Strasser and U. Rant, *J. Am. Chem. Soc.*, 2012, **134**, 15225; (m) T. Andre, A. Reichel, K. H. Wiesmuller, R. Tampé, J. Piehler and R. Brock, *ChemBioChem*, 2009, **10**, 1878; (n) S. Lata, A. Reichel, R. Brock, R. Tampé and J. Piehler, *J. Am. Chem. Soc.*, 2005, **127**, 10205; (o) S. Lata, M. Gavutis, R. Tampé and J. Piehler, *J. Am. Chem. Soc.*, 2006, **128**, 2365; (p) A. Kollmannsperger, A. Sharei, A. Raulf, M. Heilemann, R. Langer, K. F. Jensen, R. Wieneke and R. Tampé, *Nat. Commun.*, 2016, **7**, 10372.
5. (a) L. Motiei, Z. Pode, A. Koganitsky and D. Margulies, *Angew. Chem. Int. Ed.* , 2014, **53**, 9289; (b) K. Selvakumar, L. Motiei and D. Margulies, *J. Am. Chem. Soc.*, 2015, **137**, 4892; (c) L. Unger-Angel, B. Rout, T. Ilani, M. Eisenstein, L. Motiei and D. Margulies, *Chem. Sci.*, 2015, **6**, 5419; (d) Z. Pode, R. Peri-Naor, J. M. Georgeson, T. Ilani, V. Kiss, T. Unger, B. Markus, H. M. Barr, L. Motiei and D. Margulies, *Nat. Nanotechnol.*, 2017, **12**, 1161; (e) Y. Nissinkorn, N. Lahav-Mankovski, A. Rabinkov, S. Albeck, L. Motiei and D. Margulies, *Chem. Eur. J.*, 2015, **21**, 15981; (f) R. Peri-Naor, Z. Pode, N. Lahav-Mankovski, A. Rabinkov, L. Motiei and D. Margulies, *J. Am. Chem. Soc.*, 2020, **142**, 15790.
6. N. Lahav-Mankovski, P. K. Prasad, N. Oppenheimer-Low, G. Raviv, T. Dadosh, T. Unger, T. M. Salame, L. Motiei and D. Margulies, *Nat. Commun.*, 2020, **11**, 1299.
7. (a) N. Soh, D. Seto, K. Nakano and T. Imato, *Mol. Biosyst.*, 2006, **2**, 128; (b) M. Kamoto, N. Umezawa, N. Kato and T. Higuchi, *Chem. Eur. J.*, 2008, **14**, 8004; (c) M. Kamoto, N. Umezawa, N. Kato and T. Higuchi, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 2285; (d) Y. Yang, N. Jiang, Y. T. Lai, Y. Y. Chang, X. Yang, H. Sun and H. Li, *ACS Sens.*, 2019, **4**, 1190; (e) A. Murata, S. Arai, S. I. Yoon, M. Takabayashi, M. Ozaki and S. Takeoka, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 6905; (f) S. Arai, S. I. Yoon, A. Murata, M., X. Wu, Y. Lu, S. Takeoka and M. Ozaki, *Biochem. Biophys. Res. Commun.*, 2011, **404**, 211.
8. (a) K. E. Neet and J. C. Lee, *Mol. Cell. Proteomics*, 2002, **1**, 415; (b) P. Braun and J. LaBaer, *Trends Biotechnol.*, 2003, **21**, 383.
9. (a) M. Gilbert and J. S. Albala, *Curr. Opin. Chem. Biol.*, 2002, **6**, 102; (b) C. P. Papaneophytou and G. Kontopidis, *Protein Expr. Purif.*, 2014, **94**, 22; (c) T. W. Overton, *Drug Discov. Today*, 2014, **19**, 590.
10. (a) R. Ghosh, J. E. Gilda and A. V. Gomes, *Expert Rev. Proteomics*, 2014, **11**, 549; (b) M. Mishra, S. Tiwari and A. V. Gomes, *Expert Rev. Proteomics*, 2017, **14**, 1037.
11. R. Wieneke, A. Raulf, A. Kollmannsperger, M. Heilemann and R. Tampé, *Angew. Chem. Int. Ed.*, 2015, **54**, 10216.
12. E. H. Beutner, *Bacteriol Rev*, 1961, **25**, 49.
13. R. Peri-Naor, T. Ilani, L. Motiei and D. Margulies, *J. Am. Chem. Soc.*, 2015, **137**, 9507.
14. (a) G. Loving and B. Imperiali, *J. Am. Chem. Soc.*, 2008, **130**, 13630; (b) G. S. Loving, M. Sainlos and B. Imperiali, *Trends Biotechnol.*, 2010, **28**, 73; (c) D. I. Danylchuk, S. Moon, K. Xu and A. S. Klymchenko, *Angew. Chem. Int. Ed.* , 2019, **58**, 14920; (d) H. Zhu, J. Fan, J. Du and X. Peng, *Acc. Chem. Res.*, 2016, **49**, 2115.
15. (a) B. Lugtenberg, R. Peters, H. Bernheimer and W. Berendsen, *Mol. Gen. Genet.*, 1976, **147**, 251; (b) R. Utsumi, T. Horie, A. Katoh, Y. Kaino, H. Tanabe and M. Noda, *Biosci. Biotechnol. Biochem.*, 1996, **60**, 309.

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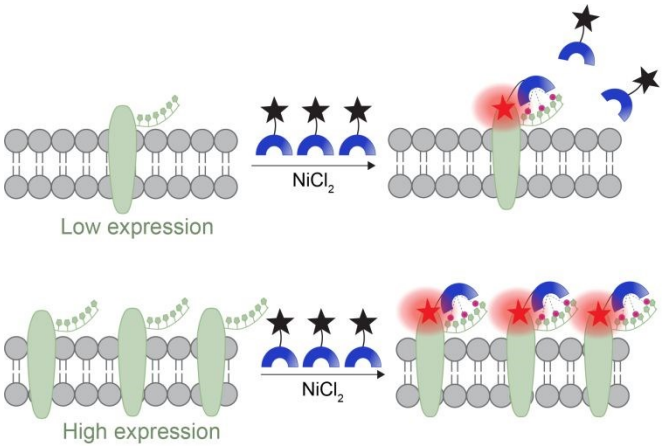

A turn on fluorescent molecular probe was used to assess changes in the expression level of His-tagged cell surface proteins in living bacteria.