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A Spectrofluorophotometrical Method Based on Fura-2-AM Probe to Determine Cytosolic Ca^{2+} Level in *Pseudomonas syringae* Complex Bacterial Cells

Simone Trabalza[1, 2], Roberto Buonaurio[1], Alberto M. Del Pino[1], Carlo A. Palmerini[1], Harrold A. van den Burg[2] and Chiaraluce Moretti[1, *]

[1]Department of Agricultural, Food and Environmental Science, University of Perugia, Perugia, Italy; [2]Molecular Plant Pathology, Swammerdam Institute for Life Sciences (SILS), University of Amsterdam, Amsterdam, the Netherlands

*For correspondence: chiaraluce.moretti@unipg.it

[Abstract] Calcium signaling is an emerging mechanism by which bacteria respond to environmental cues. To measure the intracellular free-calcium concentration in bacterial cells, $[Ca^{2+}]_i$, a simple spectrofluorometric method based on the chemical probe Fura 2-acetoxy methyl ester (Fura 2-AM) is here presented using Pseudomonad bacterial cells. This is an alternative and quantitative method that can be completed in a short period of time with low costs, and it does not require the induction of heterologously expressed protein-based probes like Aequorin. Furthermore, it is possible to verify the properties of membrane channels involved in Ca^{2+} entry from the extracellular matrix. This method is in particular valuable for measuring $[Ca^{2+}]_i$ in the range of 0.1-39.8 µM in small cells like those of prokaryotes.

Keywords: Fura 2-AM, Cytosolic calcium concentration, Spectrophotometer, Pseudomonad, Live cell signaling

[Background] Ca^{2+} is an emerging intracellular messenger of bacteria that impacts a wide array of cellular processes such as the maintenance of cell integrity, cell division (Dominguez *et al*., 2015), motility (Tisa and Alder, 1995; Gode-Potratz *et al*., 2010; Cruz *et al*., 2012; Guragain *et al*., 2013; Parker *et al*., 2015; Fishman *et al*., 2018), type III secretion (DeBord *et al*., 2003; Dasgupta *et al*., 2006; Gode-Potratz *et al*., 2010; Fishman *et al*., 2018), gene expression (Dominguez *et al*., 2015), quorum sensing (Werthén and Lundgren, 2001), biofilm formation (Patrauchan *et al*., 2005; Sarkisova *et al*., 2005; Rinaudi *et al*., 2006; Cruz *et al*., 2012; Das *et al*., 2014; Zhou *et al*., 2014; Parker *et al*., 2016) or biofilm suppression (Bilecen and Yildiz, 2009; Shukla and Rao, 2013). Recently, it was demonstrated that the intracellular Ca^{2+} concentration controls virulence of *Pseudomonas savastanoi* pv. *savastanoi* (*Psav*) (Moretti *et al*., 2019). Furthermore, several known virulence genes were upregulated in the presence of increasing Ca^{2+} concentrations in *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 (Fishman *et al*., 2018), and *Xylella fastidiosa* (Parker *et al*., 2016). Measurement of the intracellular free-calcium concentration, $[Ca^{2+}]_i$, in prokaryotes has, therefore, become of great interest to study its role as intracellular messenger of bacteria in response to environmental cues. Monitoring the $[Ca^{2+}]_i$ within bacterial cells, which is indispensable for understanding the correlations between the transport of Ca^{2+} across the plasma membrane and cellular processes was thus far difficult, as it was hampered by the

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small size of the bacterial cells, the semi-selective nature of the bacterial cell wall, the low membrane permeability, and the toxicity of many Ca^{2+} chelators used (Gangola and Rosen, 1987; Knight *et al.*, 1991; Futsaether and Johnsson, 1994; Norris *et al*., 1996; Herbaud *et al*., 1998; Jones *et al.*,1999; Torrecilla *et al.*, 2001). A well-established method to determine changes in the $[Ca^{2+}]_i$ in prokaryotes is based on the heterologous expression of Aequorin (a calcium-activated photoprotein) bacterial cells (Watkins *et al*., 1995). This method employs the expression of recombinant Aequorin (from a plasmid or integrated in the bacterial genome), which emits light upon Ca^{2+} binding. This method is rather time-consuming, requires the availability of molecular biology tools, and is technically challenging. In fact, the method is better suited for eukaryotic cells even if it was successfully used to monitor $[Ca^{2+}]_i$ in several bacterial species (Naseem *et al*., 2007; Guragain *et al*., 2016). To overcome these limitations of Aequorin, we developed an alternative and complementary spectrofluorometric method based on the chemical probe Fura 2-AM {1-[2-(5-carboxyoxazol-2-yl)-6-amino-benzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy) ethan-*N*,*N*,*N'*,*N'*-tetraacetic acid}. Importantly, both the assay solution used and the Fura 2-AM do not compromise cell viability at the concentrations here used (Gangola and Rosen, 1987; Futsaether and Johnsson, 1994; Tisa and Alder, 1995; Norris *et al.*, 1996; Jones *et al.*, 1999), meaning that this method allows quantification of $[Ca^{2+}]_i$ in response to external cues and different conditions without the need of advanced equipment (Moretti *et al.*, 2019). It must be pointed out that Fura 2-AM is a probe that diffuses across the cell membrane of viable bacterial cells and its subsequent rapid de-esterification by cellular esterases yields Fura 2, which retains the ability to bind the cytosolic Ca^{2+} while it losses the ability to diffuse across the cell membrane (Figure 1). When Fura 2 forms a complex with Ca^{2+}, the intensity of the fluorescence at λ=510 nm increases with increasing Ca^{2+} concentration (Grynkiewicz *et al.*, 1985) (Figure 2). In addition, Fura 2 is unable to permeate bacterial cells itself due to the selective permeability of the cell walls and membranes (Grynkiewicz *et al.*, 1985). Of notice, since the measurements make use of a fluorescence signal that only becomes apparent inside cells (when Fura 2-AM is converted to Fura 2), it is not necessary to use unloaded viable cells as negative control. In fact, if the cells are not viable, the cell membrane loses its integrity and the probe would not be trapped in the cells but rather remain dispersed in the incubation medium.

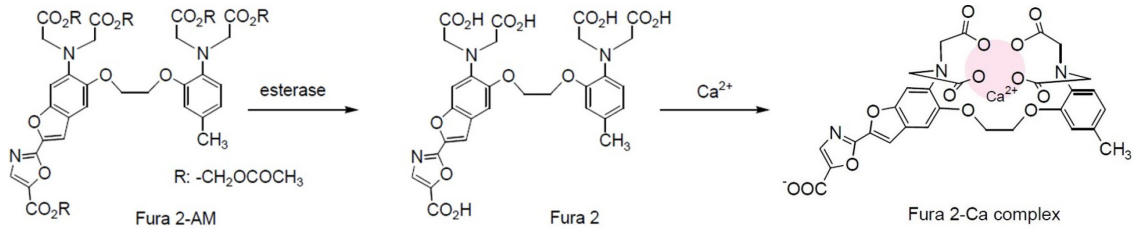

Figure 1. The fate of Fura 2-AM in cells. Fura 2-AM is de-esterified by cellular esterases and transformed in Fura 2, which is able to form a complex with cytosolic calcium (Ca^{2+}) and cannot passively cross the cell membrane.

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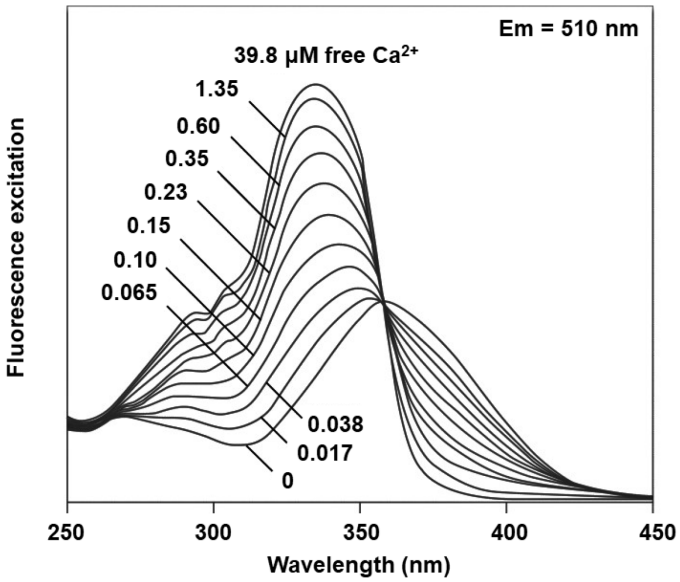

Figure 2. Excitation spectra of Fura 2. Excitation spectra of Fura 2 in solution containing 0 to 39.8 µM of free calcium (Ca^{2+}). Modified from Grynkiewicz *et al*. (1985).

We find that the fluorescent probe Fura 2-AM is highly sensitive allowing us to determine changes in cytosolic Ca^{2+} levels in *Pseudomonas savastanoi* pv. *savastanoi* DAPP-PG 722 (Moretti *et al*., 2019) and *Pseudomonas syringae* pv. *tomato* DC3000 (Trabalza *et al*., in preparation) cells by using a spectrofluorometer equipped with a stirred semi-micro cuvette.

Materials and Reagents

1. Inoculation loops 10 µl (Laboindustria S.P.A., catalog number: 21131)
2. Petri dish Ø 90 (Laboindustria S.P.A., catalog number: 21050)
3. Pipette tips (Mettler Toledo, catalog numbers: 17007956, 17007952)
4. High clarity polypropylene (PP) conical centrifuge tube 50 ml (Falcon, catalog number: 352070)
5. NIR Quartz SUPRASIL 300 Rectangular Macro Cell with Lid, volume 3.5 ml (PerkinElmer, catalog number: B0631015)
6. PIREX Media bottles, graduated, Corning, 500 ml (VWR, PIREX, catalog number: 1395-500)
7. *Pseudomonas savastanoi* pv. *savastanoi* (*Psav*) DAPP-PG 722 strain (Moretti *et al*., 2014), stored at -80 °C in 15% glycerol
8. *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 strain (Gizjen, 2008), stored at -80 °C in 15% glycerol
9. MilliQ double distilled water
10. Tris base (Sigma-Aldrich, catalog number: T1503); prepare 0.12 M aqueous solution, adjust pH to 8.0 with 1 M HCl, autoclave and store at room temperature
11. Fura 2-AM (Sigma-Aldrich, catalog number: F0888); prepare 2 mM solution in DMSO in aliquots of 50 µl and freeze at -20 °C. Store at -20 °C, wrap it in aluminum foil to avoid photodegradation.

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Avoid repeated freezing and thawing of the aliquots

12. EGTA (Sigma-Aldrich, catalog number: E3889); prepare a 0.5 M aqueous stock solution, adjust pH to 8.0 with 0.5 M NaOH, autoclave and store at room temperature. Prepare a 2 mM aqueous solution from the stock solution
13. Sodium chloride (Sigma-Aldrich, catalog number: S7653)
14. Tryptone (Sigma-Aldrich, Millipore, catalog number: T7293)
15. Yeast extract (Sigma-Aldrich, catalog number: Y1625)
16. Calcium chloride (Sigma-Aldrich, catalog number: 449709); prepare a 50 mM aqueous solution and autoclave at 121 °C for 20 min
17. Hank's Buffered Salt Solution (HBSS) buffer; prepare 1 L aqueous solution with 8.18 g/L NaCl, 0.4 g/L KCl (Sigma-Aldrich, catalog number: P9541), 5.96 g/L HEPES (Sigma-Aldrich, catalog number: H3375), adjust pH to 7.4 and autoclave at 121 °C for 20 min
18. EDTA (Sigma-Aldrich, catalog number: E9884); prepare a 0.5 M aqueous stock solution, adjust pH to 8.0 with NaOH, autoclave at 121 °C for 20 min and store at room temperature. Prepare a 0.1 mM aqueous solution from the stock solution
19. Triton X-100 (Sigma-Aldrich, catalog number: X100); prepare a 1% aqueous solution and autoclave at 121 °C for 20 min
20. Luria Bertani (LB) medium (see Recipes)

Equipment

1. 1 L measuring cylinder (DWK Life Sciences, catalog number: 21 390 54 08)
2. BRAND magnetic stirring bar (Sigma-Aldrich, Aldrich, catalog number: BR137630)
3. Magnetic stirrer (Heidolph MR 2000, catalog number: 200-505-20000-00)
4. Eppendorf Research Plus G pippetes (Sigma-Aldrich, Eppendorf, catalog number: EP3123000918)
5. Autoclave (EXAPro, Lequeux, catalog number: P80602001)
6. Shaking incubator SI500 (Stuart, catalog number: FV-79520-00)
7. Eppendorf® Centrifuge 5804R (Sigma-Aldrich, Sigma, catalog number: EP022628146)
8. Heratherm™ Incubator (Thermo Scientific, catalog number: 51028112)
9. LS-50B Luminescence Spectrometer (PerkinElmer, catalog number: 17931)
10. Laminar flow cabinet Gelaire BSB 6A (Gelaire)

Software

1. FL WinLab Software, version 3 (PerkinElmer)
2. Prism 8 (GraphPad, https://www.graphpad.com/scientific-software/prism/)

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Procedure

A. Bacterial growth (Day 1: ~15 min followed by 16 h incubation)

Note: Do not use any antibiotics in the growth medium, because it can interfere with the experiments. Do not use more than 10% of maximum tube volume to ensure growth of the bacterium. Conduct all steps under sterile conditions.

1. Prepare a fresh culture of *Psav* DAPP-PG 722 or *Pto* DC3000 strain by inoculating bacterial cells, scraped with a inoculation loop from a 15% glycerol stock at -80 °C, on Petri dish containing LB agar medium and incubate for 16 h at 27 °C.

B. Bacterial growth (Day 2: ~15 min followed by 16 h incubation)

Note: Do not use any antibiotics in the growth medium, because it can interfere with the experiments. Do not use more than 10% of maximum tube volume to ensure growth of the bacterium. Conduct all steps under sterile conditions.

1. Inoculate a loop of bacterial cells into a 50 ml tube containing 5 ml LB broth. Incubate the tubes in the shaking incubator for 16 h at 27 °C and 200 rpm (until the OD_{660} = 0.8).

C. Cell preparation with Fura 2-AM (Day 3: ~3.5 h)

Note: Do not centrifuge the bacterial suspension at >16,000 × g to avoid any cell damage. Wrap the tube containing the Fura 2-AM with aluminum foil or work in the dark to avoid photodegradation of the probe.

1. Collect the bacterial cells from the 5 ml cell culture by centrifugation (15,585 × *g,* 3 min) at room temperature (RT).
2. Discard the supernatant and resuspend the cells in 5 ml of sterile 0.12 mM Tris HCl (pH 8.0).
3. Adjust the bacterial suspension to 1 × 10^8 CFU ml^{-1} by bringing the OD_{660} at 0.06.
4. Collect again 10 ml of this bacterial suspension by centrifugation (15,585 × *g,* 3 min) at RT.
5. Discard the supernatant and resuspend the cell pellet in 5 ml 0.12 mM Tris HCl (pH 8.0) (= 5 × 10^9 CFU), add 0.5 ml of a 2 mM EGTA solution (pH 8.0), and incubate at 25 °C for 5 min in an air incubator to render the bacteria receptive to the Ca^{2+} probe.
6. Add 20 µl of 2 mM $CaCl_2$ solution to quench the EGTA.
7. Pellet the cells by centrifuging them at 15,585 × *g* for 3 min at RT.
8. Discard the supernatant and resuspend the cells in 5 ml HBSS supplemented with 2 µl of 2 mM Fura 2-AM stock solution. Note, add the Fura 2-AM freshly to the HBSS prior to usage from a fresh stock solution.
9. Incubate this cell suspension in HBSS + Fura2-AM in the incubator without shaking at 25 °C for 2 h.
10. Pellet the cells by centrifuging them at 15,585 × *g* for 3 min at RT.
11. Add 5 ml HBSS (without the dye), resuspend the cells and incubate for 1 min at RT.

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D. Measurement of the Ca^{2+} levels (Day 3: ~1.5 h)

Note: Set the LS-50B luminescence spectrometer with FL WinLab Software using the User's Guide.

1. Pour 1 ml of the cell suspension in the NIR Quartz SUPRASIL 300 Rectangular Macro Cell containing the BRAND magnetic stirring bar which reduces the swirling and guarantees the correct reading of the samples.
2. Place the Rectangular Macro Cell in the LS-50B luminescence spectrometer, wait until the Traffic Light bottom becomes green to initialize the spectrometer (Figure 3A) and click on it to start the measurement. Similar results could be obtained with analogous spectrometer.
3. Wait about 50 s (a time useful for the signal stabilization) and add 30-240 µl of a 50 mM $CaCl_2$ solution to reach a final concentration ranging from 0.5 to 4 mM (Figure 4).
4. Add 1 ml of 1% Triton X-100 to disrupt the cells, record the time and wait until the signal stabilizes. Triton X-100 is added at the end of the measurement because it allows one to determine the maximum Ca^{2+} level in the system.
5. Add 1 ml of 0.1 mM EDTA (pH 8.0) to chelate the Ca^{2+} ions in solution and record the exact time appearing in the display as it is important for the final Rmin determination, wait until the signal stabilizes and press the Traffic Light buttom (Figure 3A), make sure it becomes red to acquire the signals and save the value. By adding EDTA the Ca^{2+} ions are chelated allowing one to determine the minimum Ca^{2+} level in the system.
   Note: Triton X-100 and EGTA are added at the end of the determination so the measurement is performed with intact cells. If the cells were not intact, the minimum and maximum Ca^{2+} value could not be determined.
6. To automatically determine the Ca^{2+} concentration according to the formula of Grynkiewicz *et al*. (1985) [see also Data analysis], set the calibration values Rmin and Rmax (Figure 3B) in the Calibration Tab layout of the FL WinLab Software, as reported in the User's Guide.
   Note: Without this software it is necessary to determine the Rmin and Rmax for each sample as indicated in point 1 of Data analysis.
7. Press the Convert to [ion] button (Figure 3B) to convert the raw dataset of the signals into Ca^{2+} ion concentration dataset using the calibration values set before and save the generated file. Without FL WinLab Software Ca^{2+} ion concentration has to be calculated using the formula of Grynkiewicz *et al*. (1985).

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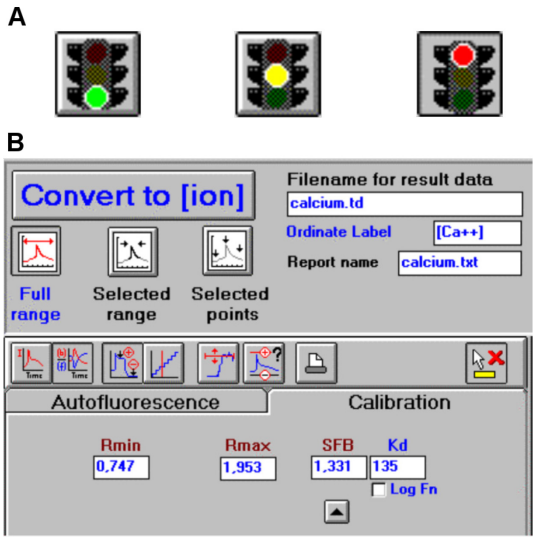

Figure 3. FL WinLab Software. A. The Traffic light button allows to start and stop the analysis and describes the status of the instrument. B. The Calibration Tab layout permits to manage the raw data and the Convert to [ion] button to visualize the data (from the FL WinLab User's Guide, PerkinElmer, Inc., UK).

8. Calculate the difference between the Ca^{2+} ions present before and after $CaCl_2$ addition (Figure 4).

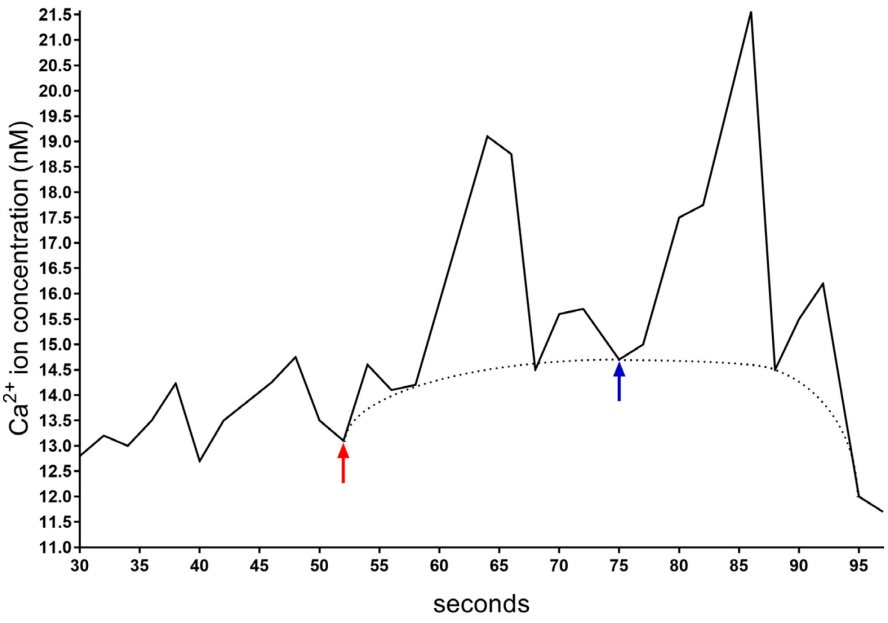

Figure 4. Example of a plot generated by FL WinLab Software used to calculate $\Delta[Ca^{2+}]_c$ before and after $CaCl_2$ addition. The presence of the peaks in the trace is due to the change of the wavelength that LS-50B Luminescence Spectrometer shifts every two seconds. Therefore, the calculation of the Ca^{2+} concentration has to be performed considering the baseline (dotted line). After about 50 s, when the signal is stabilized, the $CaCl_2$ is added (red arrow) and the

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$\Delta[Ca^{2+}]_c$ is calculated between the highest Ca^{2+} concentration value reached in the baseline (blue arrow) and the value when the $CaCl_2$ is added (red arrow).

9. Incubate the cells in HBSS buffer supplemented with Fura 2-AM (basal condition) or in HBSS buffer supplemented with 2 mM Fura 2-AM and different carbon sources (*e.g.*, 5 mM glucose, fructose or sucrose), 50 µM ATP, or other conditions of interest. Of note, one should optimally add these compounds only after 50s from the start of the measurement (Figure 5).

Data analysis

1. The free intracellular Ca^{2+} concentration was calculated using the formula of Grynkiewicz *et al*. (1985):

$$[Ca^{2+}] = K_d \beta \frac{R - Rmin}{Rmax - R}$$

K_d: Dissociation constant for the Ca-Fura-2 complex
β: Fluorescence intensity ratio with an excitation wavelength of 380 nm, with and without Ca^{2+}
R: Ratio of fluorescence intensities obtained
R_{min} and R_{max}: Fluorescence intensity ratio excited at 340 and 380 nm, in the absence (min) and in the presence (max) of Ca^{2+}, respectively

2. To validate the results, multiple biological replicates are necessary. Here, ten independent experiments were carried out and the data points and error bars (Figure 5) represent the mean and standard error, respectively. In *Psav* DAPP-PG 722 cells it has been observed that under basal conditions (*i.e.*, HBSS buffer) an increase in external Ca^{2+} results in an increase in the cytosolic Ca^{2+} concentrations. The cytosolic Ca^{2+} concentrations rapidly increase in response to external Ca^{2+} concentration in the medium (Figure 5). This trend was suppressed when different carbon sources (glucose, fructose or sucrose) or ATP were added in a combination with Ca^{2+} (Figure 5).

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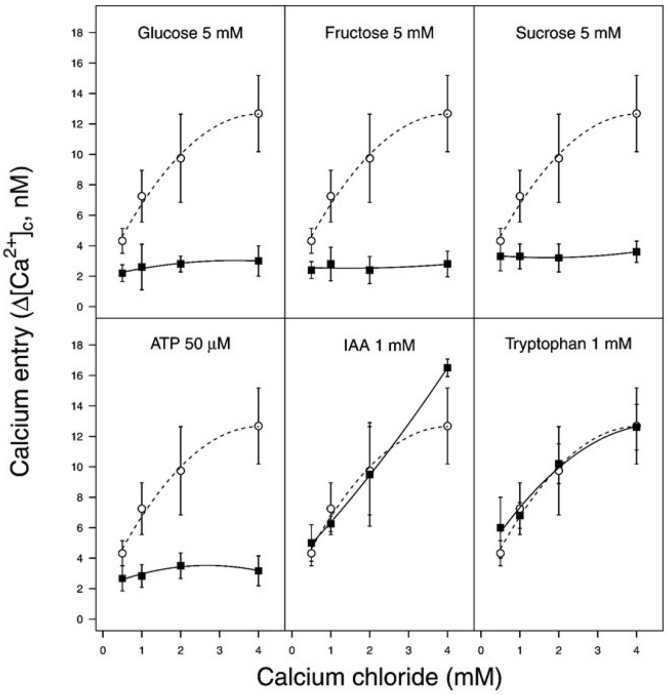

Figure 5. An increase of cytosolic Ca^{2+} levels in *Pseudomonas savastanoi* pv. *savastanoi* (*Psav*) DAPP-PG 722. *Psav* bacterial cells incubated in HBSS medium alone (basal condition, closed squares) or in the presence of glucose, fructose, sucrose, indole 3 acetic acid (IAA) or tryptophan (open circles) over a concentration range of extracellular calcium chloride. Each point represents the mean of 10 independent experiments ± SE. From Moretti *et al*. (2019).

Recipe

1. Luria Bertani (LB) medium (1 L)
   a. Add 800 ml of MilliQ water into a 1 L measuring cylinder
   b. Put the measuring cylinder on magnetic stirrer with a magnetic rod
   c. Add 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl
   d. Add MilliQ water to 1 L
   e. Pour 250 ml medium into four 500 ml PYREX® glass bottles
   f. Autoclave at 121 °C for 20 min

Acknowledgments

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Competing interests

The authors declare no conflict-of-interest and have no competing financial interests.

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Informed consent was obtained from all individual participants included in the study.

References

1. Bilecen, K. and Yildiz, F. H. (2009). Identification of a calcium - controlled negative regulatory system affecting *Vibrio cholerae* biofilm formation. *Environ Microbiol* 11(8):2015-2029.
2. Cruz, L. F., Cobine, P. A. and De La Fuente, L. (2012). Calcium increases *Xylella fastidiosa* surface attachment, biofilm formation, and twitching motility. *Appl Environ Microbiol* 78(5): 1321-1331.
3. Das, T., Sehar, S., Koop, L., Wong, Y. K., Ahmed, S., Siddiqui, K. S. and Manefield, M. (2014). Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation. *PLoS One* 9(3): e91935.
4. Dasgupta, N., Ashare, A., Hunninghake, G. W. and Yahr, T. L. (2006). Transcriptional induction of the *Pseudomonas aeruginosa* type III secretion system by low Ca^{2+} and host cell contact proceeds through two distinct signaling pathways. *Infect Immun* 74(6): 3334-3341.
5. DeBord, K. L., Galanopoulos, N. S. and Schneewind, O. (2003). The *ttsA* gene is required for low-calcium-induced type III secretion of Yop proteins and virulence of *Yersinia enterocolitica* W22703. *J Bacteriol* 185(12):3499-3507.
6. Dominguez, D. C., Guragain, M. and Patrauchan, M. (2015). Calcium binding proteins and calcium signaling in prokaryotes. *Cell Calcium* 57(3): 151-165.
7. Fishman, M. R., Zhang, J., Bronstein, P. A., Stodghill, P. and Filiatrault, M. J. (2018). Ca^{2+}-induced two-component system CvsSR regulates the Type III Secretion System and the extracytoplasmic function sigma factor AlgU in *Pseudomonas syringae* pv. *tomato* DC3000. *J Bacteriol* 200(5): e00538-17.
8. Futsaether, C. and Johnsson, A. (1994). Using Fura-2 to measure intracellular free calcium in *Propionibacterium acnes*. *Can J Microbiol* 40(6):439-445.
9. Gangola, P. and Rosen, B. P. (1987). Maintenance of intracellular calcium in *Escherichia coli*. *J Biol Chem* 262(26): 12570-12574.
10. Gizjen, M. (2008). Diane Cuppels and the history of *Pseudomonas syringae* pv. *tomato* DC3000. *IS-MPMI Report* 1: 4-5.
11. Gode-Potratz, C. J., Chodur, D. M. and McCarter, L. L. (2010). Calcium and iron regulate swarming and type III secretion in *Vibrio parahaemolyticus*. *J Bacteriol* 192(22): 6025-6038.
12. Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260(6):3440-3450.
13. Guragain, M., Lenaburg, D. L., Moore, F. S., Reutlinger, I. and Patrauchan, M. A. (2013). Calcium homeostasis in *Pseudomonas aeruginosa* requires multiple transporters and modulates swarming motility. *Cell Calcium* 54(5): 350-361.
14. Guragain, M., Campbell, A. K. and Patrauchan, M. A. (2016). Measurements of intracellular calcium concentration in *Pseudomonas aeruginosa*. *Bio-protocol* 6(23): e2041.

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15. Herbaud, M. L., Guiseppi, A., Denizot, F., Haiech, J. and Kilhoffer, M. C. (1998). Calcium signalling in *Bacillus subtilis.* *Biochim Biophys Acta* 1448(2): 212-226.
16. Jones, H. E., Holland, I. B., Baker, H. L. and Campbell, A. K. (1999). Slow changes in cytosolic free Ca^{2+} in *Escherichia coli* highlight two putative influx mechanisms in response to changes in extracellular calcium. *Cell Calcium* 25(3): 265-274.
17. Knight, M. R., Campbell, A. K., Smith, S. M. and Trewavas, A. J. (1991). Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* 352(6335): 524-526.
18. Moretti, C., Trabalza, S., Granieri, L., Caballo‐Ponce, E., Devescovi, G., Del Pino, A. M., Ramos, C., Venturi, V., van den Burg, H. A., Buonaurio, R. and Palmerini, C. A. (2019). A Na^{+}/Ca^{2+} exchanger of the olive pathogen *Pseudomonas savastanoi* pv. *savastanoi* is critical for its virulence. *Mol Plant Pathol* 20(5):716-730.
19. Moretti, C., Cortese, C., Passos da Silva, D., Venturi, V., Ramos, C., Firrao, G. and Buonaurio, R. (2014). Draft Genome Sequence of *Pseudomonas savastanoi* pv. *savastanoi* Strain DAPP-PG 722, Isolated in Italy from an Olive Plant Affected by Knot Disease. *Genome Announc* 2(5): e00864-14.
20. Naseem, R., Davies, S. R., Jones, H., Wann, K. T., Holland, I.B. and Campbell, A. K. (2007). Cytosolic Ca^{2+} regulates protein expression in *E. coli* through release from inclusion bodies. *Biochem Biophys Res Commun* 360(1): 33-39.
21. Norris, V., Grant, S., Freestone, P., Canvin, J., Sheikh, F. N., Toth, I., Trinei, M., Modha, K. and Norman, R. I. (1996). Calcium signalling in bacteria. *J Bacteriol* 178(13): 3677-3682.
22. Parker, J. K., Chen, H., McCarty, S. E., Liu, L. Y. and De La Fuente, L. (2016). Calcium transcriptionally regulates the biofilm machinery of *Xylella fastidiosa* to promote continued biofilm development in batch cultures. *Environ Microbiol* 18(5): 1620-1634.
23. Parker, J. K., Cruz, L. F., Evans, M. R. and De La Fuente, L. (2015). Presence of calcium-binding motifs in PilY1 homologs correlates with Ca-mediated twitching motility and evolutionary history across diverse bacteria. *FEMS Microbiol Lett* 362(4).
24. Patrauchan, M. A., Sarkisova, S., Sauer, K. and Franklin, M. J. (2005). Calcium influences cellular and extracellular product formation during biofilm-associated growth of a marine *Pseudoalteromonas* sp. *Microbiology (Reading)* 151(Pt 9): 2885-2897.
25. Rinaudi, L., Fujishige, N. A., Hirsch, A. M., Banchio, E., Zorreguieta, A. and Giordano, W. (2006). Effects of nutritional and environmental conditions on *Sinorhizobium meliloti* biofilm formation. *Res Microbiol* 157(9): 867-875.
26. Sarkisova, S., Patrauchan, M. A., Berglund, D., Nivens, D. E. and Franklin, M. J. (2005). Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonasaeruginosa* biofilms. *J Bacteriol* 187(13): 4327-4337.
27. Shukla, S. K. and Rao, T. S. (2013). Effect of calcium on *Staphylococcus aureus* biofilm architecture: a confocal laser scanning microscopic study. *Colloids Surf B Biointerfaces* 103: 448-454.

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28. Tisa, L. S. and Adler, J. (1995). Cytoplasmic free-Ca^{2+} level rises with repellents and falls with attractants in *Escherichia coli* chemotaxis. *Proc Natl Acad Sci U S A* 92(23): 10777-10781.
29. Torrecilla, I., Leganés, F., Bonilla, I. and Fernández‐Piñas F. (2001). Calcium transients in response to salinity and osmotic stress in the nitrogen‐fixing cyanobacterium *Anabaena* sp. PCC7120, expressing cytosolic apoaequorin. *Plant Cell Environ* 24(6):641-648.
30. Watkins, N. J., Knight, M. R., Trewavas, A. J. and Campbell, A. K. (1995). Free calcium transients in chemotactic and non-chemotactic strains of *Escherichia coli* determined by using recombinant aequorin. *Biochem J* 306(3):865-869.
31. Werthén, M. and Lundgren, T. (2001). Intracellular Ca^{2+} mobilization and kinase activity during acylated homoserine lactone-dependent quorum sensing in *Serratia liquefaciens*. *J Biol Chem* 276(9):6468-6472.
32. Zhou, G., Li, L. J., Shi, Q. S., Ouyang, Y. S., Chen, Y. B. and Hu, W. F. (2014). Efficacy of metal ions and isothiazolones in inhibiting *Enterobacter cloacae* BF-17 biofilm formation. *Can J Microbiol* 60(1): 5-14.

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