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COMMENTARY

Setting the Stage: Genes Controlling Mechanosensation and Ca^{2+} Signaling in *Escherichia coli*

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ABSTRACT Although mechanistic understanding of calcium signaling in bacteria remains inchoate, current evidence clearly links Ca^{2+} signaling with membrane potential and mechanosensation. Adopting a radically new approach, Luder et al. scanned the Keio collection of *Escherichia coli* gene knockouts (R. Luder, G. N. Bruni, and J. M. Kralj, J Bacteriol 203:e00509-20, 2021, https://doi.org/10.1128/JB.00509-20) to identify mutations that cause changes in Ca^{2+} transients. They identify genes associating Ca^{2+} signaling with outer membrane biogenesis, proton motive force, and, surprisingly, long-term DNA damage. Their work has major implications for electrophysiological communication between bacteria and their environment.

KEYWORDS Ca^{2+} transients, DNA damage, electrophysiology, mechanosensation, membrane potential, microbial communication

Calcium signaling in bacteria and archaea is an emerging field (1). Although long considered important, progress in the field has been hampered by the small size of microbial cells and the need for high temporal and spatial resolution to be able to detect the signals arising from changes in Ca^{2+} levels, so-called calcium transients. These problems have been resolved with the recent development of fluorescing green fluorescent protein (GFP)-based Ca^{2+} sensors (e.g., GCaMP6f), originally designed for use in neurons (2), as well as rhodopsin-based voltage sensors (e.g., PROPS—proteorhodopsin optical proton sensor) (3), which have recently been adapted for use in *Escherichia coli* (4). The fluorescent derivatives are plasmid encoded and allow live, single-cell imaging to detect both calcium and voltage transients; fluorescent sensors of intracellular pH can also be used. In this issue of the *Journal of Bacteriology*, the paper by Luder et al. (5) describes the technique that they helped develop to scan the complete set of viable *Escherichia coli* mutants in the Keio collection (6) to identify genes whose products are involved in potentially controlling, or simply responding to, changes in calcium and voltage transients. Their elegant study represents a tour de force that reveals both expected "hits" and a new and unexpected link between Ca^{2+} signaling and persistent, i.e., long-term, DNA damage.

Before describing these new findings in a little more detail, we should first recapitulate why Ca^{2+} is an excellent messenger, or signaling molecule. The inorganic chemistry of the Ca^{2+} cation is very revealing about its role in both accompanying and guiding the evolution of living systems throughout the past nearly 4 billion years of earth's 4.56-gigayear (Gya) history (7). Ca^{2+} ions are large and can be hydrated by between 6 and 8 water molecules. As a consequence, the cation also undergoes very rapid reactions with inorganic and organic molecules that are, e.g., 1,000 times faster than Mg^{2+} reactions and more rapid than those detected with any other divalent ion. It especially likes carbonates and phosphates, with which it forms stable and insoluble salts. These facets of Ca^{2+} mean that its solubility in the ocean is limited to around 1 to 5 mM, but this has remained constant since the earth cooled sufficiently approximately 4.4 Gya to allow the oceans to form (8). The Ca^{2+} cation is thus both a blessing and a curse. It is a

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curse because it is always present and it is capable of rapidly precipitating organic molecules, particularly DNA and RNA, once inside cells. Consequently, throughout evolution every cell has had to maintain the concentration of free Ca^{2+} ions within its cytoplasm at an absolute minimum, which is in the range below 10^{-6} to 10^{-7} M; otherwise, every metabolic process, particularly those involving phosphate, would have ceased. This has meant that every living cell on our planet has had to evolve rather rapidly energy-driven Ca^{2+} pumps to eject the cations from the cytoplasm. This also means that Ca^{2+} biochemistry is ancient and thus conserved.

The blessing, or beneficial side, of the Ca^{2+} cation is that with low, millimolar concentrations outside cells and nanomolar concentrations inside, this strong gradient across the cytoplasmic membrane and its fast-exchange reactivity inside and outside cells make Ca^{2+} the ideal means of sensing and communicating environmental changes (7). Being a weak catalyst of acid/base reactions, Ca^{2+} is also frequently found as a metal cofactor in proteases, nucleases, and phosphatases; i.e., it is useful for degrading organic polymers. Later, during the evolution of eukarya, the chemical properties of Ca^{2+} were also adapted for structural roles (8). Ca^{2+} ions also play important structural roles in bacterial spores (9), and there are correlative data indicating roles for calcium in chemotaxis (10), differentiation (11), and pathogenicity (12). However, what has been lacking is a compendium of bacterial genes whose products have been shown to be directly, or even indirectly, involved in Ca^{2+} homeostasis and signaling. This is what is now delivered in the paper of Luder et al. (5), and, as with all well-considered genetic screens, the results reveal some very exciting new avenues for future research.

I should state at the outset that what that study did not reveal is a clear-cut identification of anything like a pump for Ca^{2+} or a Ca^{2+} receptor, but it offers tantalizing hints with respect to how *E. coli* might be responding to the cation. In particular, that study built on a recent publication by the same group (4), which identified a link between voltage-mediated mechanosensation (a bacterium's "sense of touch") and influx of Ca^{2+} ions. After establishing constitutive synthesis of the Ca^{2+} sensor GCaMP6f, the authors first demonstrated that individual *E. coli* cells, when "squeezed" between a glass coverslip and an agarose pad, exhibited voltage-dependent Ca^{2+} transients, pretty much as is known for eukaryal sensory neurons (4). This result indicated that bacterial cells are sensitive to coming into contact with surfaces, which, considering the lifestyles of the many bacteria which prefer surface attachment or an existence within biofilms, is hardly surprising. The authors then demonstrated that dissipation of the membrane potential using the uncoupler CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) caused Ca^{2+} transients to disappear and that treatment with the aminoglycoside antibiotic apramycin caused an increase in the levels of Ca^{2+} transients (see also reference 13). With these controls, they effectively set the boundaries for their test system. The initial correlation between mechanosensation and Ca^{2+} transients was revealed by tethering the cells to a glass surface and giving them a squeeze (4). That study thus provided the impetus to screen the complete Keio collection of *E. coli* mutants for genes that showed either up- or downregulation of Ca^{2+} transients.

In total, approximately 3,500 gene knockouts were initially transformed with a plasmid encoding a fusion of GCaMP6f, which was the Ca^{2+} sensor, and mScarlet, which acted as a sensitivity negative control that discounted potential changes in plasmid copy number or other artifacts. All of these transformants were grown aerobically in 96-well-plate format and were subsequently subjected to a primary screen, whereby they were spotted onto agarose pads and pressed into the bottom of a glass well. A video was then taken of every mutant, and this was analyzed carefully for alterations in Ca^{2+} transient signals. After a huge amount of careful analysis in which time traces of individual cells were monitored, potential "hits" were reimaged in biological triplicate to validate the responses. In total, 143 knockout strains reproducibly exhibited decreased Ca^{2+} transients whereas 32 gene knockouts showed increased transients. Mutants were further phenotypically classified based on their response to a shear force

(measuring changes in voltage transients) and by making use of flow cytometry to monitor changes in membrane potential (tetramethylrhodamine, methyl ester [TMRM] assay) or DNA damage (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL] assay).

Examination of the predicted or known functions of the products encoded by the genes whose respective mutations either positively or negatively influenced Ca^{2+} transients reveals that they fall within four categories: (i) outer membrane (OM) and exopolysaccharide metabolism; (ii) cytoplasmic membrane and membrane potential; (iii) DNA damage/structure; (iv) "others," including iron homeostasis and some potentially Ca^{2+}-dependent enzymes. Perhaps unsurprisingly, and in agreement with the observations made in their earlier study (4), knockouts in genes that impaired lipopolysaccharide, lipid A, and peptidoglycan biosynthesis reduced both voltage and Ca^{2+} transient levels (5). Among the other genes of interest in this category were *pgaD*, defects in which affect biofilm formation, and *wcaB* and *wcaM*, which affect colanic acid production. These findings are all commensurate with the OM and peptidoglycan layers being involved in mechanosensation through shear stress upon contacting a surface. Moreover, the identified genes point to a role for Ca^{2+} signaling in electrical communication within a biofilm.

Numerous knockouts of genes encoding characterized or putative transporters fell within the second category, and many of those, such as complex I and complex II components of the electron-transport chain and the proton-translocating F_1F_o-ATPase, are capable of causing changes in membrane potential. Somewhat unexpected was the discovery of genes encoding an anaerobic dicarboxylate carrier (*dcuA*) and *menD* and *menF* genes, which encode proteins involved in menaquinone biosynthesis. These knockouts reduced Ca^{2+} and voltage transients, while deletions in *dmsB* (anaerobic dimethyl sulfoxide [DMSO] reductase) and *cydB* (high-affinity cytochrome *bd* oxidase) increased the intensity of the transients. The non-proton-translocating cytochrome *bd* quinol oxidase is induced under conditions of O_2 limitation and serves the function of balancing electron flux (14). Removal of this oxidase therefore tends to increase membrane potential due to impaired charge dissipation. Another link with O_2 limitation was the observed increase in voltage and Ca^{2+} transients in an *arcA* mutant. ArcA (aerobic respiration control), the transcriptional regulatory component of an electron-transport-responsive two-component system (15), is responsible for shutting down expression of genes encoding tricarboxylic acid (TCA) cycle and aerobic respiratory enzymes when *E. coli* shifts to anaerobic growth. Consequently, preventing this shutoff occurring leads to accumulation of NADH and, presumably, to increased voltage due to a "backed-up," electron-overloaded respiratory chain (16). Despite the previously published claim that voltage transients and, by implication, Ca^{2+} influx require O_2-dependent respiration and a proton motive force (PMF) (3), these findings suggest that calcium signaling also responds to hypoxia conditions, or to even anaerobic conditions, which also frequently occur in biofilms. Considering the likely evolutionary conservation of calcium signaling, coupled with the fact that all bacteria and archaea maintain a PMF and membrane potential, regardless of their growth mode and given the vast numbers of anaerobes, it will be important in future studies to determine the electrophysiology of microorganisms in O_2-free environments.

The big surprise in the study by Luder et al. was the discovery of a link between persistent, long-term DNA damage and reductions in both voltage and Ca^{2+} transients. It is important that this observation was not based on the characterization of an isolated gene knockout; results obtained with nine different *rec* gene mutations indicated that recombination defects had caused decreased Ca^{2+} signaling. Topoisomerase, ATP-dependent helicase, and DNA polymerase III gene mutations all substantiated the suggestion that anything affecting DNA structure and function is exquisitely sensed. This also correlates with earlier reports of links between DNA damage and decreased membrane potential in mitochondria (17). By making use of mitomycin C, which cross-links DNA strands, Luder et al. were able to verify their observation independently of

introducing mutations. More significantly, however, by employing several elegant approaches, they were able to rule out the possibility that this effect was dependent on the SOS response, whose effects, surprisingly, are diametrically opposed to the reported effects of DNA damage in mitochondria (18). Rather, persistent DNA damage, over time, appears to cause the cells somehow to adapt to the pervading conditions by lowering their membrane potential accordingly; this is a completely new observation. The tools needed to dissect how cells achieve this, and whether this is a more general phenomenon found also in other bacteria, are now available to address these exciting issues.

Finally, as with all genetic screens or selections, several mutations were found to affect Ca^{2+} transients that initially caused scratching of heads but that may have correlative explanations. For example, the genes identified to cause lowering of Ca^{2+} signaling include *wzb*, encoding a protein tyrosine phosphatase; *wzc*, encoding a protein tyrosine kinase; and a gene encoding a NUDIX-type phosphatase. Generally, these enzymes are calcium dependent in other systems (19, 20). However, several genes were identified whose presence suggested metal ion homeostasis; in particular, that encoding Fe^{2+}, or perhaps mediating its delivery for iron-sulfur cluster (Isc) biosynthesis, appears to be linked to Ca^{2+} signaling. A deletion of the *feoB* gene encoding a component of the ferrous iron transporter, as well as knockouts of genes predicted to be involved in Fe^{3+}-siderophore biosynthesis, also affected membrane potential and Ca^{2+} transients. Moreover, deletion of *iscS*, which encodes cysteine desulfurase, a key component of the Isc biosynthetic machinery (21), resulted in reduced levels of transients, while mutations in the negatively regulatory *iscR* gene resulted in increased levels of transients, a finding that lends credence to the authors' previous findings. It is noteworthy that many of the proteins involved in respiration have as cofactors iron-sulfur clusters, which are required for electron transport, and there are reports of roles for these cofactors in recombination and DNA replication and repair (22, 23), so perhaps this is the link with the processes mentioned above.

Despite their being at a very early stage of research, the radically new cross-disciplinary approaches adopted by the Kralj group have already revolutionized our understanding of calcium signaling in bacteria. Their work has now set the stage for the future expansion of this exciting field. It will allow a much more detailed understanding of the role of calcium signaling in bacteria and archaea and the identification of new concepts in electrophysiological communication between microorganisms; it will also possibly reinvigorate research on PMF and membrane potential; and it will reveal the links between the evolutionarily ancient axes of Ca^{2+} signaling, voltage, DNA integrity, and iron-sulfur biochemistry.

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