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REVIEW ESSAY

Prospects & Overviews

Electrical signalling in prokaryotes and its convergence with quorum sensing in *Bacillus*

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Abstract

The importance of electrical signalling in bacteria is an emerging paradigm. *Bacillus subtilis* biofilms exhibit electrical communication that regulates metabolic activity and biofilm growth. Starving cells initiate oscillatory extracellular potassium signals that help even the distribution of nutrients within the biofilm and thus help regulate biofilm development. Quorum sensing also regulates biofilm growth and crucially there is convergence between electrical and quorum sensing signalling axes. This makes *B. subtilis* an interesting model for cell signalling research. SpoOF is predicted to act as a logic gate for signalling pathway convergence, raising interesting questions about the functional nature of this gate and the relative importance of these disparate signals on biofilm behaviour. How is an oscillating signal integrated with a quorum signal? The model presented offers rich opportunities for future experimental and theoretical modelling research. The importance of direct cell-to-cell electrical signalling in prokaryotes, so characteristic of multicellular eukaryotes, is also discussed.

KEYWORDS

biofilm, communication, electrical, potassium, quorum sensing, YugO

INTRODUCTION

The cell membrane acts as a capacitor in storing energy in the form of electric charge. It is well known that in prokaryotes the proton motive force (pmf) can drive ATP synthesis, flagella rotation and a number of other cellular functions such as organisation of the cell division apparatus in *Bacillus subtilis*.[1] When electric charge, carried by ions, is separated by the impermeable membrane it acts as a store of potential energy and this potential can be made to do useful work when the ions are allowed to flow through ion channels. In eukaryotes it is well-established that this cell-membrane capacitance functions in electrical signalling. This raises the obvious question: does electrical signalling play a role in allowing bacteria to sense their environment and communicate with one-another?

Prokaryotic ion channels have long been a subject of speculation, despite these ion channels providing the foundation to study those present in eukaryotes. For example, structural studies on the potassium channel of *Streptomyces lividans* carried out by Doyle et al. helped elucidate the structure and mechanism of eukaryotic potassium channels.[2] Martinac et al. reviewed the importance of research on prokaryotic ion channels as an aid to understanding ion channels in animals and noted that since prokaryotes are more easily manipulated in genetic studies it is easier to use prokaryote expression systems to produce large quantities of these proteins for structural studies.[3] Although it is recognised that ion channels occur in prokaryotic membranes, there is limited information on their role in cell signalling and communication.

To date a few studies have examined the importance of ligand-gated ion channels in prokaryotes. For example, more than 20 homologues of pentameric ligand-gated ion channels were known in bacteria by 2010 including the proton-gated ion channel of *Gleobacter violaceus*, which belongs to the same family/superfamily as the ionotropic nAchR

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in mammals.[4] Prokaryotes make use of ion channels, including ligand-gated ion channels, in the regulation of osmotic pressure, pH, and ion balance.[5]

There is evidence to suggest that bacteria use ion channels for mechanosensation, for example, *Escherichia coli* has three such channel: MscL (large), McsS/McsK (small/'kalium' since this channel has some specificity for potassium)[6,7] and MscM (mini) named for their single-channel conductancies which descend in the order MscL > MscS > McsM.[8] The primary function of these channels, as currently understood, appears to be osmoregulation and regulation of cell wall synthesis and growth.[8] Interestingly, the MscS channel is gated by both voltage (by membrane depolarisation) and membrane tension.[9]

Despite the examples of mechanosensitive ion channels in bacteria already discussed, uncertainty remains over the importance of these and similar ion-channels in cell-to-cell communication in prokaryotes. This uncertainty may be due to the limited number of ion channel studies carried out using bacteria in their native forms, that is, biofilms. Prindle et al. decided to use *Bacillus subtilis* biofilms to investigate the role of the potassium efflux channel YugO.[10] Their discovery of potential electrical communication related to biofilm growth through the means of potassium signalling, indicated new mechanisms to be identified, as will be discussed in this literature review. Interestingly, it was discovered that an upstream regulator of YugO activity, Spo0A, is additionally part of the quorum-sensing mechanism that appears to regulate biofilm growth in *B. subtilis*.[11] The possible relationship between potassium signalling and quorum sensing will be explored below.

Resistance against antimicrobial agents is currently a major area of concern in clinical research. As biofilms have been shown to have much greater antibiotic resistance than planktonic cells (e.g., see reviews by Saeki et al.[12]; Skilbeck et al.[13]), the understanding of how biofilm growth can be regulated electrically and chemically will prove to be invaluable for identifying new therapeutic targets.[14,15]

YugO IS PART OF AN OPERON WITH MstX REGULATING BIOFILM FORMATION

The role of YugO, a potassium efflux channel, was first investigated following the identification of the *yugO* locus downstream of the gene encoding the membrane associated protein Mistic (MstX), unique to the *Bacillus* genus.[16–18] The two genes are part of the same operon. As the MstX protein is associated with enhancing membrane protein expression,[19] Lundberg et al. investigated its effect as part of its operon, including *yugO*.[16]. Lundberg et al. proposed that MstX may be responsible for facilitating expression of the downstream *yugO* and its insertion into the bacterial membrane.[16] Both of the two strains (*B. subtilis*) used, each with either Δ*yugO* or Δ*mstX* mutations, failed to produce biofilms. Biofilm production was only restored after expression of both genes together.

Whilst researching for this minireview, a FASTA sequence of YugO from the NCBI protein database (accession number SCV39418.1, submitted by Cress and F. Brady, 2016) was submitted to Phyre2 Web Portal for protein modelling, prediction and analysis, to model the structure of a YugO monomer.[20] Phyre2 was able to Model 98% of the structure with greater than 90% confidence using six very similar templates which represent potassium channels in organisms as diverse as *Aplysia*, *Gallus*, *Homo sapiens* and two prokaryotes: *Methanothermobacter thermoautotrophicus* (see Jiang et al.[21]) and *Geobacter sulfurreducens*. This family of potassium channels forms membrane-spanning tetramers, with two transmembrane domains on each subunit. At least some of these channels are gated by calcium, intracellular calcium in the case of the *M. thermoautotrophicus* channel.[21]

MISTIC (MstX) IS HEAVILY INVOLVED IN THE BIOFILM MATRIX PRODUCTION

The membrane-associated protein MstX has been shown to play a role in biofilm formation.[16] Its absence, the result of using an isopropyl *β*-D-1-thiogalactopyranoside (IPTG)-inducible promoter, led to insufficient production of the exopolysaccharide (EPS) layer and therefore no architecturally complex colonies.[16] This EPS layer is a critical component of the biofilm matrix, in which aggregates of bacterial cells are embedded.[22] The *epsA* gene responsible for EPS production showed reduced expression. The effect of inactivating *mstX* was reversed when *epsA*'s expression was induced.[16]

The sequence and NMR-structure of MstX have been deposited in the NCBI protein database by Roosild et al.[17] Phyre2 was used to reconstruct the protein structure from the sequence (a useful exercise for those wishing to study the protein structure in more detail) using the NMR structure as a template with 98% at more than 90% confidence (two residues are modelled ab initio).[17] No other closely matching structures were found. MstX forms a bundle of four helices and, despite its high hydrophilicity, MstX solubilised in lauryl dimethylamine oxide (LDAO) micelles as a monomer and NOE (Nuclear Overhauser Effects) analysis revealed a ring of the detergent molecule interacting around the helical bundle, consistent with MstX being an integral membrane protein.[17] Additionally, paramagnetic probes that selectively partition to hydrophilic or hydrophobic environments also supported the model that MstX is an integral membrane-spanning protein).[17,23]

A NAD+ BINDING SITE LINKS YugO ACTIVITY WITH THE CELL'S METABOLIC STATE

Prindle et al. further studied the regulation of YugO activity by an intracellular TrkA gating domain present in its structure.[10] Whilst researching for this minireview, a BLAST search of conserved domains in YugO was carried out, which also confirmed the presence of the TrkA domain. Prindle et al. deleted this gating domain resulting in impaired signal propagation by YugO.[10] It was hypothesised that the presence of a TrkA domain in the protein indicated that the function of YugO as a potassium efflux channel would be regulated by the metabolic state of the cell. Glutamate depletion was also shown to coincide with YugO activation.[10] A structural analysis of the TrkA domain showed a binding site identical to the NAD+ binding domain of NAD+ dependent dehydrogenases.[24] Intracellular NAD concentrations perhaps

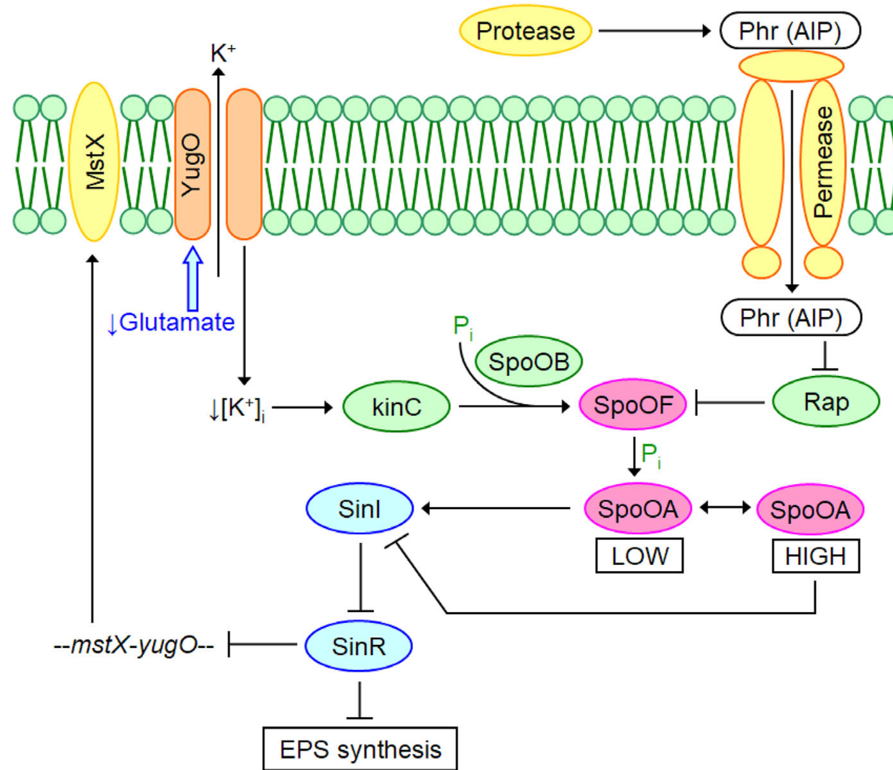

FIGURE 1 The signalling pathways (signal flow diagram) for signalling axes converging on the control of biofilm synthesis (EPS synthesis), illustrating the effects of a drop in intracellular glutamate concentration on YugO activation and potassium efflux and of quorum sensing via the auto-inducing peptide (AIP) Phr on the synthesis of extracellular polysaccharide (EPS) and hence biofilm synthesis. See main text for detailed explanation

increase from reduced activity of glutamate synthase in these bacteria. This enzyme catalyses the production of glutamine from glutamate and NAD+ coenzyme.[25,26] As glutamate is depleted, glutamate synthase becomes less active, increasing the concentration of intracellular NAD+ available.[10] TrkA may also be activated by ATP binding, another indicator of reduced metabolism.[27] The subsequent downstream signalling events are illustrated in Figure 1 and described in more detail below.

POTASSIUM ION SIGNALS DRIVE METABOLIC OSCILLATIONS

Potassium is considered to be the most abundant cation found in the cytoplasm of all cells.[5,28,29] Upon activation during starvation, the YugO channel is opened and a sudden efflux of potassium occurs. This efflux results in the hyperpolarisation of the starving cell.

Potassium that is released into the extracellular matrix is then taken in by neighbouring cells.[10] *Bacillus subtilis* has a low affinity potassium ion importer, KtrCD and can express two high affinity importers, KtrAB and KimA .[29–31] The rapid increase in intracellular potassium within these cells results in their depolarisation.[10] This then impairs glutamate uptake, which, in *B. subtilis*, is normally regulated by a proton-glutamate symporter. This symporter transports a minimum of two protons alongside glutamate from the extracellular to the intracellular environments.[32] In other words, a positive-to-negative electrochemical gradient is necessary for efficient glutamate uptake. Depolarisation abolishes this gradient, reducing the electrical component of the proton motive force.[32] Glutamate uptake is hence reduced, placing these affected cells in a state of nutrient limitation. This enables the original cell, the one that emitted the original signal, to gain better access to nutrients. However, the depolarised cells will eventually reach a state where their own glutamate deprivation activates the same YugO-dependent mechanism activated earlier in the original starved cell. Potassium signals will then be emitted in the reverse direction, giving rise to the periodic oscillations in metabolic potential as observed and modelled by Prindle and colleagues.[10]

When Prindle et al. demonstrated the oscillatory changes in membrane potential of *B. subtilis* biofilms, they used thioflavin-t (ThT) dye, which is used to indicate highly negative membrane potential, that is, hyperpolarised cells. APG-4 fluorescent dye was also used as an indicator of extracellular potassium concentrations.[10] Following exposure to nutrient depletion (glutamate), ThT dye fluoresced in the resulting hyperpolarised cells whilst APG-4 fluorescence was observed in the surrounding extracellular matrix.

KinC IS THE LINK BETWEEN POTASSIUM EFFLUX AND BIOFILM MATRIX GENE EXPRESSION

Nutrient depletion stimulated biofilm matrix production in *B. subtilis*.[33] The sudden decrease in intracellular potassium within

the nutrient-deprived cell has been shown to activate histidine kinase KinC.[34–36] The introduction of endogenous substrates such as surfactin, which is released by *B. subtilis* and is capable of inducing potassium leakage, into biofilm growth media resulted in induced matrix gene expression.[35] The link between potassium efflux and matrix gene expression is KinC. Mutant Δ*kinC* strains eliminated any effect surfactin had on biofilm growth. In particular, it is the PAC-PAS domain that appears to be KinC's critical potassium-sensor domain. This was determined following individually introducing each of the three different alleles encoding the different KinC domains into the Δ*kinC* strains.[35] However, a clear mechanism as to how potassium is sensed by this domain has not yet been determined. Surfactin is also required for sliding motility and colony spreading in *B. subtilis*, by a potassium-dependant mechanism.[37]

KinC REGULATES Spo0A, THE MASTER REGULATOR OF BIOFILM FORMATION

A downstream component of the signalling cascade involving KinC appears to be the master regulator Spo0A (Figure 1) a transcription factor with multiple targets.[34,38,39] Known genes regulated by Spo0A include those associated with biofilm formation, motility and sporulation, that is, genes involved in the development and life-cycle of biofilms.[11]

KinC has been shown to regulate the activity of Spo0A through the means of a major phosphorelay system, consisting of five histidine protein kinases (KinA, B, C, D, and E) and two intermediate proteins (Spo0F and Spo0B).[38–40] The kinases transfer phosphate groups to these intermediate proteins, of which Spo0F is regarded as the response regulator that ultimately transfers the phosphate to Spo0A and activates it.[38,40] This is done via Spo0B, a phosphotransferase protein.[38–40] It is the phosphorylation and activation of Spo0A that is the final result of activating the phosphorelay chain.

SinI IS A TRANSCRIPTION TARGET OF Spo0A

The gene *sinI* is an important target of Spo0A, transcribed when conditions favouring biofilm formation are present, for example, suitable population density and good nutrient availability.[16,41] The promotor region of *sinI* possesses five binding sites, of which one is the activator site. Spo0A binds with strong affinity to this site.[41,42] This site is a 0A-box region, a conserved sequence found amongst all Spo0A targets and is located just upstream of the starting point for *sinI* transcription. Meanwhile, the other four sites are located downstream and are regarded as weak operator sites as *sinI* expression is suppressed when these sites are occupied.[41] Spo0A binds to these sites with weaker affinity than that of the activator site meaning that a greater concentration of active phosphorylated Spo0A is required to inhibit the transcription of *sinI*. This possibly explains how Spo0A coordinates the different stages of the biofilm life-cycle, that is, formation versus sporulation: two "mutually exclusive" cell fates.[43,44]

SinI AND SinR HAVE OPPOSING EFFECTS AS A GENE EXPRESSION-REGULATING DUO

Low concentrations of phosphorylated Spo0A appear to favour a growing phenotype, through binding the only activator site of the *sinI* promotor region and activating *sinI* expression (Figure 1).[45–47] The subsequently synthesised SinI protein is an inhibitor of the negative regulator SinR. [22,48,49] SinR itself represses matrix gene expression.[22,48,49] Matrix genes affected by the activity of SinR include the *eps* gene, responsible for the synthesis of extracellular polymeric substance (EPS), a critical component of the biofilm matrix.[22,49] The double inhibition of SinI—SinR—*eps* (Figure 1) ultimately results in the expression of *eps* and other matrix genes. Going back to YugO, this signalling axis suggests how activation of this potassium efflux channel eventually leads to increased biofilm formation.

It should also be noted that SinR has been shown to negatively regulate the *mstX-yugO* operon.[16] Mutant strains lacking MstX and YugO failed to initiate biofilm formation, most likely due to the unopposed activity of SinR due to a failure to activate SinI. SinI activity is promoted by only low concentrations of phosphorylated Spo0A binding to the activator site of *sinI* and stimulating its transcription.[16] Anti-repression by SinI will activate the *mstX-yugO* operon, suggesting a positive feedback loop that upregulates YugO activity following YugO activation.

In terms of the SinI and SinR duo, it is clear that they each have opposing influences on the extent of multicellularity seen in *B. subtilis* communities.[22] It is the ratio of SinR to SinI that is suggested to determine the fate of the bacterial cells, with emphasis on the fact that SinI is only synthesised and active at low concentrations of phosphorylated Spo0A.

PHOSPHORYLATED Spo0A ALSO REGULATES SPORULATION AT HIGHER CONCENTRATIONS

In contrast, higher concentrations of phosphorylated Spo0A lead to an increased probability of binding to the four operator sites. As these operator sites are inhibitory, *sinI* expression is soon switched off as the expression of sporulation-associated genes becomes favoured.[50] Sporulation of *B. subtilis* generally results in the formation of endospores in which stressed cells transition into spore-like structures instead of producing multiple spore bodies.[51,52] These endospores allow survival of bacteria in conditions of extreme environmental stress, rendering them less susceptible to antimicrobial damage compared to normal vegetative cells.[52,53] Their coats provide barriers for lytic enzymes and radiation damage, whilst their low internal hydration provides protection against heat.[51,53,54] Extreme starvation is a known trigger for sporulation, where endospores enter a metabolically inactive state to minimise their nutrient requirements.[54] Additional factors do affect the properties of these endospores. For example, SR1, a double-stranded RNA in *B. subtilis*, can produce longer spores displaying higher heat resistance when its gene expression is upregulated.[52] SR1's influence has been shown to rely on binding

to *kinA* mRNA and also thought to depend on the cell's metabolic state.[52]

QUORUM SENSING IN *B. SUBTILIS* AND THE POTENTIAL LINK WITH YugO REGULATION

Bacterial populations tend to form biofilms only when there is sufficient cell density where the process of forming these architecturally complex structures would be worthwhile. This allows bacteria to produce "sporing bodies" (towers) elevated above the boundary layer for more efficient dispersal.[13] The mechanism of bacteria detecting their own cell population involves quorum-sensing and this communication is important in biofilm formation.[13,55,56] In Gram-positive bacteria, such as the *Bacillus* genus, this process relies on short peptide chains as autoinducers for cell-cell recognition. Gram-negative bacteria instead generally synthesise acyl-homoserine lactones (AHLs).[13] In *B. subtilis*, quorum sensing is regulated by the *rap*-phr gene pair.[40,57,58] This same system is also found in *B. anthracis*.[56] There is evidence that quorum sensing may in fact be involved in the positive feedback loop regulating YugO activity (Boguslawski et al.[40]; Figure 1).

The *phr* gene encodes a short autoinducer peptide, first synthesised as a precursor (Pro-Phr), that is subsequently exported into the extracellular environment due to the presence of a N-terminal signal sequence.[40] Outside in the extracellular matrix, these precursor peptides encounter proteases, synthesised by cells of the same species, and are cleaved to form a mature Phr peptide. Phr is then able to re-enter the cell through the oligopeptide permease (OPP) system (Figure 1).[40] This permease is an oligomeric ABC transporter which imports short peptides, both for nutrition and the AIP for signalling (see for example Pottahil and Lazazzera,[59] Solomon et al.[60], and references therein).

Another key player is the *rap* gene, which encodes a phosphatase, whose major target is the Spo0F response regulator in the phosphorelay system governing Spo0A's activity.[40] Rap proteins A, B, E, H, I, and J are able to dephosphorylate phosphorylated Spo0F, resulting in the termination of the relay and thereby inhibition of Spo0A activation.[40] SinI thus cannot be synthesised and the repressive actions of SinR on biofilm formation and sporulation will continue.

Phr inhibits Rap activity by directly binding to it and thereby promoting biofilm formation.[40] Rap proteins are also capable of reducing extracellular protease production and so inhibition of Rap results in greater availability of protease and further inhibition by Phr.[57] This is perhaps how bacteria ensure that low cell densities are not incorrectly processed as high: reduced extracellular protease concentrations mean a lack of mature Phr re-entering cells and unopposed inhibition of biofilm development by Rap.

Understanding how quorum sensing regulates the electrical communication mediating biofilm growth could potentially provide an easier approach for externally manipulating biofilm development. Quorum sensing is highly dependent on the interaction with extracellular proteases and Phr peptides. As this occurs outside the cell, it may be a more convenient target for synthetic materials. Manipulation of quorum-sensing and biofilm growth is currently being investigated for its therapeutic potential in treating human, animal, and plant diseases (see for example, Defoirdt et al.[61], Yang et al.[62]) though this requires a detailed understanding of each individual system.

SUMMARY: MODEL DERIVED FROM LITERATURE

Based on the literature that has just been discussed, a signalling circuit operating between a starving cell and a neighbouring cell is illustrated in Figure 2. The main focus of this model is on the interaction between cells in the two states (the 'Starved cell' and 'Neighbouring Cells'). The starved cell is initially depleted of glutamate resulting in an increase in the amount of intracellular NAD+ available. This NAD+ will activate YugO via the TrkA domain and trigger potassium efflux. This efflux will first activate Spo0A (through KinC and the phosphorelay system; shown in Figure 1) and initiate a positive feedback loop. This loop involves double inhibition whereby SinI inhibits SinR, which would otherwise directly inhibit YugO expression, resulting in increased YugO expression as the end result of this loop. The potassium efflux ultimately depolarises the neighbouring cell and impairs glutamate uptake by inhibiting activity of the glutamate-proton symporter.

The neighbouring cell is subsequently "starved" until the same protein and feedback loop activated in the starved cell (Figure 2) is triggered in this cell as well. This process is repeated continuously to produce the oscillations shown experimentally by Prindle and colleagues.[10]

Figure 2 also illustrates the relevance of other signalling circuits, for example, sporulation and quorum sensing, which have been incorporated into the model. The model predicts a high cell density will inhibit Rap, which inhibits Spo0A and EPS synthesis. This double inhibition means that the positive feedback mechanism regulating YugO in the starved cell is likely to be amplified and vice versa for low cell densities. Quorum sensing will also affect the neighbouring cells, but this is not illustrated.

Consideration should be given to a fully sized biofilm. Communicating cell-to-cell in this chain-like or network fashion, potassium waves can pass back and forth along the biofilm as shown by Prindle and colleagues.[10] From a therapeutic perspective, a drug manipulating quorum sensing is most likely to target the peripheral cells as it may be delivered in the surrounding medium, at least until it has time to equilibrate within the biofilm. Conversely, sporulation is more likely to occur in cells with reduced access to nutrients, which may be the internal core of a biofilm or its structures. Simplistically we can think of the "starving cell" in Figure 2 as being situated in the middle of the biofilm and the "neighbouring cells" as being on the periphery.

To summarise, the key *new* findings of the model:

1. There exists convergence between the intracellular potassium ion signalling pathways and extracellular quorum-sensing mechanisms through the primary mediator, Spo0A.
2. Lundberg et al.[16] demonstrated a positive feedback loop involving YugO, KinC, and SinI, which then also interacts with Spo0A. This

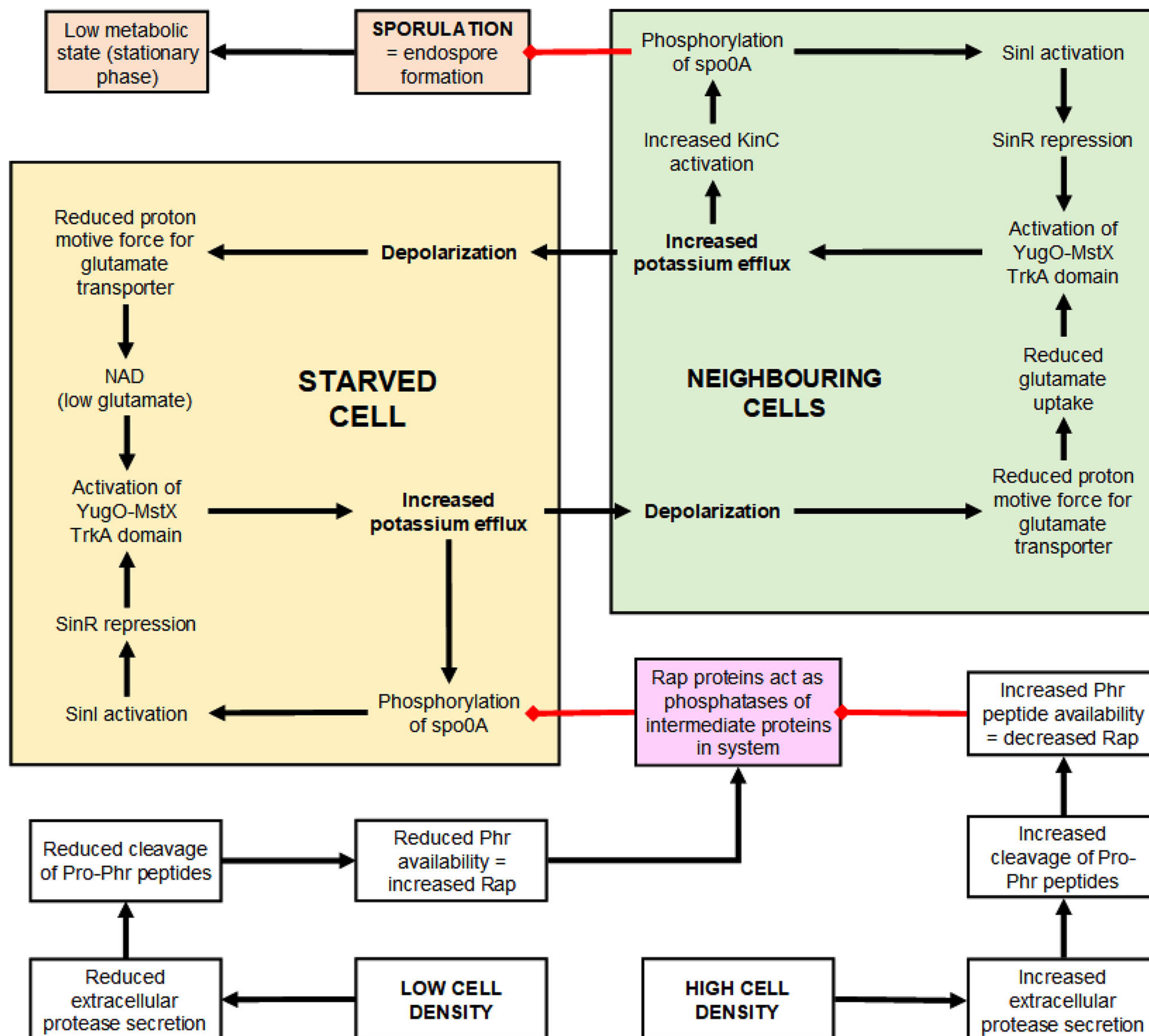

FIGURE 2 The possible pathway of events, derived from the literature review, that may explain the metabolic oscillations found by Prindle et al. alongside the possible role of quorum sensing.[10] Sporulation has also been included. Some aspects mentioned in the review have been simplified, for example, MstX has been removed as it has only been described to activate YugO. KinC, an intermediate component between potassium efflux and Spo0A activation has also been removed, see Figure 1 for more details of the signalling pathway. Arrows indicate the flow of excitatory signals; red diamond-headed arrows indicate the flow of inhibitory signals

loop, as mentioned above, can act in response to different cell densities as part of quorum sensing. This feedback also acts on sporulation, again through the mediator Spo0A, producing a well amplified survival response to cell starvation. This is a key part of the model that integrates quorum sensing, biofilm formation, and sporulation responses altogether and thus opens different avenues to explore.

3. There is a clear need to further investigate this model through altering not just the glutamate as the primary independent variable, but also the concentrations of the extracellular peptide acting as an autoinducer. For example, we can use the model to predict that increasing protease concentrations will result in disinhibition of Spo0A phosphorylation by Phr and potentially amplified oscillations, and vice versa. This will further demonstrate the relationship between YugO and quorum-sensing and allow us to study how this signalling, which constitutes mainly positive feedback, responds to different cell population states.

OTHER ELECTRICAL COMMUNICATION SYSTEMS IN PROKARYOTES?

Electrical communication has been hypothesised in the coordination of cyanobacterial gliding filaments. These filaments can glide in either direction which requires the cells to coordinate their activities. A change in gliding direction requires a signal to travel rapidly along the filament. For example, filaments of Phormidium uncinatum may reach

3000 μm in length and contain up to 1000 cells.[63] Early notions that this might be due to diffusion of protons along the filament seem unfeasible since a theoretical model by Raven predicted that such diffusion would only be effective for a few tens of micrometres; some mechanism would be needed to regenerate the signal at intervals to prevent signal decay.[64] However, this gliding motility is dependent on the proton gradient, and hence metabolic state.[63] This signalling is especially interesting since, superficially at least, it resembles direct electrical signalling between cells in multicellular eukaryotes.

Murvanidze and Glagolev recorded a change in potential along the length of the *Phormidium* filament, but Jaffe and Walsby found no such potential and suggested that the result of Murvanidze and Glagolev could have been an artefact of recording.[65,66] In the review by Hoiczyk a model is proposed in which calcium influx through calcium-voltage gated ion channels is an essential step in gliding, but it is not clear whether the calcium is involved in signalling from cell to cell or is required only to activate the gliding motors.[67]

One has to consider the possibility that bacterial cells in direct physical contact can communicate electrically with one-another. In particular, neighbouring bacterial cells may be bridged by pili or by nanotubes. Certain pili, called nanowires, such as the type IV pilin-based nanowires in *Geobacter sulforreducens* can conduct electric currents and such nanowires are capable of reducing extracellular substrates in respiration.[68,69]

Nanotubes, in contrast to protein-based pili, are protoplasmic appendages lined by unit membrane and containing a cytoplasmic core and have been particularly well studied in *B. subtilis*.[70,71] In the case of *B. subtilis*, the membrane of the appendages is continuous with the cell membrane and the appendages emerge through pores in the cell wall.[71] These appendages can form bridges between neighbouring cells, or they may terminate freely and release membrane vesicles and have been implicated in nutrient, metabolite and protein exchange.[71] Interestingly, the “nanowires” of *Shewanella oneidensis* are actually periplasmic consisting of extensions of the outer membrane and periplasm that can conduct electrons.[72,73]

The hypothesis that these pili / nanowires and/or protoplasmic nanotubes are involved in electrical communication between cells has yet to be tested. This would raise the grade of “multicellularity” in bacteria up one notch, if these junctions do indeed have a role in electrical communication, similar to the gap junctions of animals or the plasmodesmata of plants.

FUTURE DIRECTIONS

Bacillus subtilis is a useful and interesting model Gram-positive prokaryote for cell-signalling research and understanding the regulation of biofilm growth. The full role of electrical communication in this species is still not clear. Of particular interest is the apparent convergence of the quorum-sensing and the glutamate/YugO signalling axes, potentially measurable for instance by EPS synthesis (Figure 1). The pathways converge on SpoOF which would in this case function as a logic gate, for example as an AND or an OR gate. Since SpoOA acts as a two-state toggle switch, either activating or inhibiting SinI, this makes it difficult to predict the behaviour of this signalling circuit. How do quorum-sensing and potassium signals interact to regulate biofilm formation? Under what conditions would the biofilm grow or disperse?

The possible transduction of an oscillatory signal into gene expression, via SpoOF and SinI, warrants further study. The potassium wave travels at about 5 μm/min with a period of about 2 h (see: Martinez–Corral et al.[74]) but each pulse has substantial width lasting about 1 h, long enough to induce gene expression. Even very rapid calcium oscillations can induce gene expression in neurones if the oscillations are sustained (e.g., Sheridan et al.[75]). Alternatively, there may be a molecular toggle switch involved with some degree of cellular memory, activating genes even in response to a transitory signal if the switch persists in the active state. If SpoOF is indeed acting as a logic gate processing inputs from potassium signalling and quorum sensing then it would be informative to study the kinetics of transducing an oscillatory potassium signal and the kinetics of coincident quorum and potassium signals.

Understanding the behaviour of convergent signalling pathways is a crucial step in cell signalling research as understanding moves from single signalling axes to integrated signalling networks. It would likely be beneficial to conduct experimental research alongside computational modelling by network analysis. Theoretical modelling would help establish the minimum circuitry required to simulate actual behaviour, bringing us one step nearer to developing a “virtual cell” and could help elucidate phenomena such as single amplification, integration, allostery, adaptation, and noise reduction. Theoretical models have proven valuable in this respect in understanding chemotaxis in *E. coli*.[76,77] How important is the YugO-KinC-SinI-YugO positive feedback loop identified in Figure 1 in inducing sporulation? *B. subtilis* is potentially a useful model organism in which to investigate oscillatory signal transduction and signal convergence. To-date the most comprehensively analysed and modelled signalling pathway is perhaps the chemotaxis signalling pathway of *E. coli* but the *B. subtilis* biofilm regulation system may offer insights into a very different signalling architecture. *B. subtilis* forms biofilms/pellicles with cells organised into chains that are grouped into parallel bundles with well defined spatial patterns of sporulation. This could facilitate studies on signal convergence, for example, by applying an electrical or starvation signal to one end of a chain, a quorum signal to the other and observing the effects on gene transcription along the chain, perhaps with a reporter for matrix gene expression.[78]

What functions, if any, do nanotubes have in biofilm growth regulation? The possibility of electrical and/or contact signalling in the biofilms of other species needs further investigation. An understanding of the regulation of biofilm growth in pathogens may be useful in a therapeutic setting, particularly given the very high antibiotic resistance of bacterial biofilms. On the one hand, disruption of biofilm signalling may prevent bacteria utilising and sharing resources optimally and thus inhibit biofilm growth. On the other hand, cells deprived of nutrients in biofilms are typically the most resistant cells to antibiotic attack due to activation of their stress response.[79] Quorum sensing has already attracted much attention as a potential target

of exploitation by therapeutics and the interaction between these different communication systems needs further investigation.

It would be informative to see to what extent the YugO system or its homologues occur in other bacteria. Following the identification of two conserved domains (TrkA and an ion transport-2 domain) within the YugO structure, the BLAST tool was also used by the authors to investigate the presence of these domains within other clinically relevant genera. These included both Gram-negative and Gram-positive genera. *Streptococcus*, *Staphylococcus*, *Bacteroides*, *Clostridium*, *Enterococcus*, *Pseudomonas*, and *Escherichia* were included, and all were shown to have either identical or similar proteins to the YugO potassium efflux channel. It is likely that this pathway is not entirely specific to *B. subtilis*, and any model generated may also be used as a prototype applicable to other species, especially pathogens. All in all, it is clear that there is still a lot to be understood about this pathway and many questions to answer. However, this review will hopefully provide a basis for further research.

CONCLUSION

Finally, the signalling model constructed from the literature makes some simple predictions that could be measured experimentally, principally that a strong quorum signal in starving cells inhibits matrix synthesis and promotes sporulation more strongly that either condition alone. It is already established that high population density and good nutrient availability promote biofilm formation suggesting that in a dense population low nutrient status is needed to activate SpoOA sufficiently in order for it to inhibit SinI.[16,41] Experiments in which the strength of both signals is varied would be an informative test of this model. Can the model explain such empirical results sufficiently, or are other signalling circuits also involved?

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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