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Quorum sensing and bacterial cross-talk in biotechnology

John C March and William E Bentley*

Only a decade ago, the secretion and perception of small signalling molecules that in turn are transduced to coordinate behaviour of a 'minimal unit' of microorganisms was termed quorum sensing by EP Greenberg and colleagues. Since then, an explosion (or exponential growth) in understanding and prevalence of quorum-sensing systems has ensued, with sightings ranging from virulence in human and plant pathogens to degradative capacity of activated sludge. Not surprisingly, regulatory mechanisms span traditional inducer/repressor motifs homologous to the *lac* operon to the recently discovered interfering RNAs. Further characterisation of signalling circuits, coupled with creative niche applications, suggest a wealth of opportunity for advancing commercial biotechnology.

Addresses

Center for Biosystems Research, University of Maryland Biotechnology Institute, and Department of Chemical Engineering, University of Maryland, 5115 Plant Sciences Building, College Park, MD, 20742, USA
*e-mail: bentley@eng.umd.edu

Current Opinion in Biotechnology 2004, **15**:495–502

This review comes from a themed issue on
Biochemical engineering
Edited by Manuel Carrondo and John G Aunins

Available online 11th September 2004

0958-1669/\$ – see front matter

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DOI 10.1016/j.copbio.2004.08.013

Abbreviations

AHL acyl-homoserine lactone
AI-2 autoinducer-2
GFP green fluorescent protein
HSL homoserine lactone
RIP RNA III inhibiting peptide

Introduction

Researchers in biotechnology constantly seek novel platforms from which to address problems: platforms that, in a broad sense, enhance efficacy, while maintaining or intensifying specificity. Most recently, microbial quorum sensing has emerged as such a technology. Because microbial communities occupy a confined space, over time concentrations of extracellular signalling molecules accumulate, providing stimulus for unique and varied cellular responses as well as protection from competing microbial communities. Referred to as 'quorum sensing' for its often reported and coincident dependence on high population density [1], extracellular signalling provides a new

basis for control over molecular and cellular processes as well as population behaviour, perhaps in a manner more consistent with that of native physiology. Quorum sensing might be the foundation upon which the more sophisticated intracellular communication found in higher order organisms has evolved. If this is the case, methods that incorporate native signalling architecture might realise greater control with less collateral damage to non-target processes. Although such is the goal of work in this field, much is to be done for its realisation.

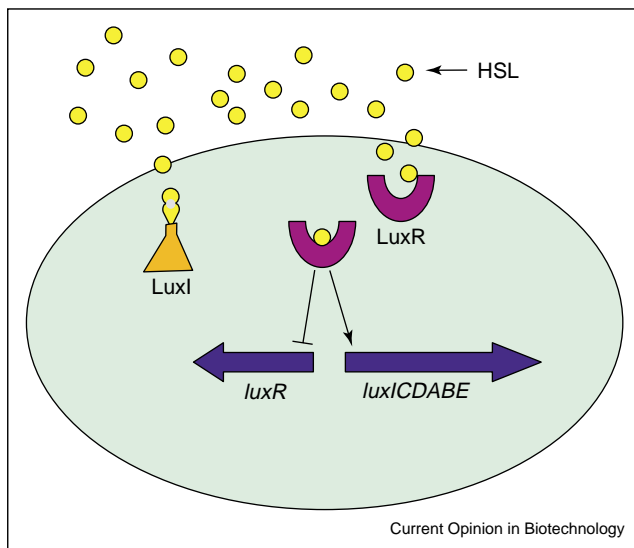
This review discusses some of the more significant breakthroughs in the areas of defining quorum sensing and harnessing quorum signalling for biotechnology that have appeared in the literature over the past two years. A cursory overview of quorum sensing mechanics is provided, with greater discussion of some of the specific functions carried out by extracellular signalling that have emerged recently.

Defining quorum sensing

To be considered a signalling molecule, a compound has to effect a reaction in a population of cells that is distinct from the manner in which the cells would behave individually. There are two kinds of quorum sensing: species-specific and interspecies. Species-specific quorum sensing in Gram-negative bacteria is mediated by acyl-homoserine lactones (AHLs) with various moieties distinguishing signals among species [2]. In Gram-positive bacteria, species-specific quorum sensing is mostly facilitated through small peptides [3]. More recently discovered interspecies communication has been linked to autoinducer-2 (AI-2), a furanosyl borate diester [4]. A recent review of cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica* has a concise description of AI-2 signalling [5].

Mechanisms of quorum sensing vary between organisms, hence here we describe the system of *Vibrio fischeri* and provide relevant differences for each organism as necessary. *V. fischeri* is a luminescent marine bacterium that infects higher order organisms (most notably luminescent fish and squid species) within compartments (light organs) completely occupied by the bacterium. When so confined, the population density of *V. fischeri* reaches upwards of 10^{11} cells per ml and luminescent genes are activated through a quorum mechanism, with the luminescence of the bacterial consortium being exhibited by the host fish or squid in a fascinating symbiotic manner. Figure 1 illustrates the quorum sensing system of *V. fischeri*. *LuxI* encodes for an autoinducer synthase that synthesizes the autoinducer *N*-(3-oxohexanoyl)-homoser-

Figure 1



Quorum sensing in *V. fischeri*. The *luxI* gene encodes for an autoinducer synthase (Lux I), which produces the autoinducer *N*-(3-oxohexanoyl)-homoserine lactone (HSL). HSL exits the cell and re-enters freely against a gradient when the external concentration reaches a threshold value. Upon re-entry into the cell HSL binds to the gene product of *luxR* (LuxR), a transcription factor. The HSL-LuxR complex binds upstream of the *luxICDABE* operon, facilitating the transcription of all the necessary components of the luciferase system in addition to an exponential increase in *luxI* transcription. LuxR also binds to the *luxR* promoter in a positive feedback loop (the presence of LuxR inhibits its synthesis).

ine lactone (HSL). *LuxR* encodes a transcription factor that binds with the autoinducer and then the bound complex binds upstream of the *luxICDABE* operon, enabling transcription of all the necessary components of the luciferase system as well as the exponential increase in LuxI synthesis [6]. LuxR also binds to the *luxR* promoter in a positive feedback inhibition loop. In other *Vibrio* species, the system is more complex, with additional sensing and phosphorylation components involved upstream of *luxR* [7^{••}]. It should be noted that a recent paper by Lenz *et al.* [7^{••}] sheds new light on quorum-sensing mechanisms, including the role of small RNA molecules (sRNA) in regulating the quorum circuit in *Vibrio harveyi* and *Vibrio cholerae*.

Functions of quorum sensing

A classic approach to solving problems from an engineering viewpoint is to break a system under consideration down into functional elements, and to assess the specific contribution of each element to a coordinated phenomenon: a technique termed functional decomposition. For many organisms of interest within biotechnology, the role of quorum sensing is complicated and unobvious. Indeed, research aimed at elucidating quorum functionality has perhaps left more questions than answers as to how these

systems can best be put to use. Three reviews [8,9[•],10] published in 2003 as part of series on quorum sensing edited by E Peter Greenberg discuss advances made in understanding quorum sensing as it relates to infectious disease. Quorum sensing is believed to regulate competence development, sporulation, antibiotic synthesis, virulence factor induction, cell differentiation, and nutrient flux along with other physiological events in pathogenic bacterial infections [8,9[•],10]. More recently, quorum-sensing was linked through proteomic analysis to increased pathogenic competence in tubercular strains of *Pseudomonas aeruginosa* [11].

Webb and co-workers [12] reviewed work on programmed cell death and microcolony differentiation in biofilms. As biofilms age, cellular differentiation and death enhance nutrient sequestration and allow for biofilm sustenance when nutrients become scarce. Although the functions of cell differentiation and programmed cell death are apparently at odds, they can be explained as an evolutionary advancement that allows biofilms of prokaryotes to behave and adapt as multicellular organisms, a behavior that appears to be coordinated through quorum sensing [12].

Many human or plant pathogens exist as biofilms in their hosts, but occupy a different modality both pre- and post-infection. Quorum sensing is thought to regulate this transition. For example, in the human pathogen *V. cholerae* quorum signalling enables the cells to negotiate the acidic human gut environment without compromising their ability to infect [13]. Biofilms formed by *V. cholerae* protect the cells from stomach bile and low pH. Cells deficient in the quorum-sensing regulator HapR exhibit thicker biofilms than wild-type strains. HapR facilitates disruption of the biofilm by inhibiting expression of the *Vibrio* polysaccharide synthesis (*vps*) operon through CqsA (an autoinducer synthase) when the function of protecting the cells from stomach bile is no longer as necessary as the function of infecting the host [13]. The authors conclude that quorum sensing promotes cellular exit from biofilms as a route to infection. Hence, *hapR* mutants are less likely to colonise their host than wild-type *V. cholerae*. Similarly, infection of plant hosts by *Xanthomonas campestris* requires the quorum-sensing gene cluster *rpf*, components of which synthesize and sense DSF (diffusible signal molecule) [14]. The DSF/*rpf* system regulates the activity of a β -mannanase that is possibly required for planktonic (non-aggregated) growth in culture, although the exact substrate for the enzyme was not determined [14].

In addition to regulating intraspecies survival and differentiation in bacterial communities, quorum sensing also relates interspecies information between symbionts and competitors [15[•],16]. Acyl-homoserine lactone (AHL) signals from *P. aeruginosa* can enter mammalian cells and activate artificial transcription factors, although it is

yet unclear which native eukaryotic proteins may be transcribed through this mechanism [17**]. The *luxS* gene, which is responsible for AI-2 synthesis, is perhaps one of the most interesting quorum-sensing genes because of its wide-ranging functionality between numerous species, both prokaryotic and eukaryotic [15*,16]. LuxS plays a key role in determining virulence, but is also involved in toxin synthesis, DNA processing, motility, cell division, iron acquisition, and light production in bacteria [15*]. LuxS controls a subset of genes in the Lyme disease spirochete *Borrelia burgdorferi*, possibly the genes required for transmission from ticks to hosts [18]. New work using DNA microarrays suggests that AI-2 mutant strains of *E. coli* produce extracellular quorum signals other than AI-2, that have a negative effect on AI-2 synthesis in wild-type (AI-2-synthesizing) *E. coli* [19].

Measuring quorum sensing

One of the most widely used methods for measuring quorum sensing or, more specifically, bacterial autoinduction, is based on the bioluminescent response of *V. harveyi*, which was developed by Bassler and others [20]. In this method a cell-free conditioned medium from a culture of interest is incubated with a culture of *V. harveyi* and the bioluminescent response is recorded. There is inherent error in relying on an indicator organism for sensing culture conditions, but the reactive nature of species such as AI-2 make direct assessment challenging. Limitations in measuring quorum sensing are starting to be addressed in the literature. There have been several new techniques intended to more accurately quantify the amount of signal and the level of response exhibited in a quorum system.

Frommberger and co-workers [21] describe a liquid chromatography-based concentration and separation method with mass spectrometer determination of various AHLs in bacterial culture. Also, a colourimetric method for determining salicylic acid carboxyl methyltransferase (SAMT) activity has been reported [22]. In this assay, SAMT converts *S*-adenosylmethionine to *S*-adenosylhomocysteine, which is converted sequentially to homocysteine by nucleosidase, and LuxS *in vitro*. Furthermore, a method for measuring bacterial proteins expressed during pathogenic infection was used to isolate infection-phase-specific proteins from *V. cholerae*. Sera from patients infected with *V. cholerae* were adsorbed against *in vitro* *V. cholerae*, and probed with a genomic expression library of *E. coli* constructed from a variant of the infecting strain [23]. The researchers detected the quorum-related protein LuxP among others.

PCR techniques have greatly simplified quorum data gathering and differentiation between pathogenic and non-pathogenic strains of bacteria. Hernandez and Olmos [24] used PCR probes and the random amplified polymorphic DNA (RAPD) method for distinguishing

V. harveyi pathogenic to shrimp [24]. The researchers were able to positively identify *V. harveyi* by the quorum-sensing transcript *luxN*. Furthermore, by building a consensus quorum gene cassette consisting of an autoinducing peptide, a receptor kinase, and a response regulator, Nakayama *et al.* [25] PCR amplified quorum-sensing regions from *Enterococcus*, *Clostridium*, and *Lactobacillus* species. The putative gene sequences were cloned by inverse PCR from *Lactobacillus paracasei* E93490 and *Lactobacillus plantarum* WCFS6, and grouped by phylogenetic analysis. This high-throughput method could help assign functionality to groups of quorum signals that show interspecies expression. In another PCR-based method, *P. aeruginosa* mutants were screened for infectivity in a rat model using signature-tagged mutagenesis (STM) and high-throughput screening [26]. In the late stationary phase, 450 genes were upregulated and 222 genes were found to be quorum-sensing repressed.

From a biotechnological standpoint, it might be desirable to isolate and characterise AHL-degrading organisms, to potentially develop a bio-based antimicrobial agent. Recent work by Jafra and van der Wolf [28] used a rapid-screening method to assess degradation of AHLs from *E. coli* expressing a previously reported LuxR/LuxI-GFP fusion [27] in potato rhizospheres. The screen isolated numerous strains capable of degrading synthetic and naturally derived AHLs. A new method has been reported that allows rapid screening of inhibitors of quorum signalling in Gram-positive bacteria [29]. Kinetic and mechanistic data can now be obtained to facilitate more rapid and effective screening for quorum-interrupting compounds.

Applications

Pathogen/pest management

Pathogen and pest (i.e. any organism whose presence in a defined environment is undesirable) management comprise most of the current applications of quorum-sensing technology. Inhibition of quorum signalling is the most obvious and, in practice, most ubiquitous application of quorum-sensing knowledge. Table 1 lists organisms that either have the potential for control via quorum disruption (proposed) or that have been proven susceptible to such an approach (realised). The topic of targeting quorum sensing for the treatment of bacterial infections has been reviewed several times [30–33]. The limiting factor for many applications is delivery of a quorum-functional (either stimulating or repressing) compound to the organism to be controlled. Some notable examples using various aspects of quorum sensing are discussed briefly below.

Staphylococcal subspecies, such as methicillin-resistant *S. aureus*, remain one of the most opportunistic pathogens found in hospital settings. Work published by Balaban and co-workers capitalised on quorum sensing to inhibit

Table 1

Management of microorganisms using quorum sensing as a target.^a

Microorganisms	Host/substrate	Quorum system target	Proposed (P) or realised (R)	References
<i>Staphylococcus</i> spp.	Human catheters/medical tubing	RNAIII inhibitor against AI-2 synthesis	R	[34,35*,36]
	Biofilm	Accessory gene regulator (<i>agr</i>)	R	[58]
<i>Pseudomonas</i> spp.	Cystic fibrosis patients	AI-2, PQS	P	[37,59]
	Ground beef, food	Several ^b	P	[42,60,61]
	Review of antagonists blocking quorum synthesis/signalling	Several ^b	P, R	[62]
	<i>In vitro</i>	3-oxo-C ₁₂ -HSL, C ₄ -HSL	R	[63,64*]
Enterobacteriaceae (other than <i>Pseudomonas</i>)	Smoked salmon	AHL	P	[65]
<i>Borrelia burgdorferi</i>	Ticks carrying Lyme disease	AI-2	P	[18]
<i>Salmonella</i> spp.	Poultry	yhjH	P	[66]
<i>Burkholderia cepacia</i>	Onion	C ₆ -HSL, C ₈ -HSL	R	[40]
<i>Ceratocystis ulmi</i>	Elm tree	Farnesol	P	[41]
Wine grape consortia	Wine grapes	Several ^b	P	[43]
<i>Serratia liquefaciens</i>	<i>In vitro</i>	HSL	R	[67]
<i>Chromobacterium violaceum</i>	<i>In vitro</i>	Violacein	R	[68]
<i>Pectobacterium carotovorum</i>	Potato	AHL	R	[28,68]
<i>Erwinia carotovora</i>	Potato	AHL	R	[45,46]
<i>Agrobacterium tumefaciens</i>	Tomato	AHL	R	[46]
Aquatic consortia	Aquarium water	AHL	R	[47]

^aApplications listed by target organism are either proposed in the references or have been realised experimentally. ^bInstances of several targets. PQS, *Pseudomonas* quinolone signal.

the development of *S. aureus* [34] and *Staphylococcus epidermidis* [35**] biofilms. Both strains were susceptible to an RNA III inhibiting peptide (RIP), a specific inhibitor of quorum sensing that interferes with the gene locus *agr*, which is responsible for staphylococcal toxicity. *S. aureus* was cultured on various surfaces typically found in dialysis catheters [34*]. When RIP was added to the culture, the cells were less able to form a biofilm. Expanding this approach to include antibiotics, the authors determined the effect of combining RIP with seven different combinations of antibiotics and found that in every case the combination of RIP and an antibiotic greatly improved the performance of the antibiotic [35**]. This work was carried out on RIP and antibiotic-coated Dacron grafts that had been inserted in rat hosts.

Looking to Gram-negative bacterial infections, characterisation of *P. aeruginosa* from intubated patients revealed quorum-signalling targets that could be potentially inhibited to prevent the formation of biofilms [36]. Most of the quorum genes expressed were classified as transcription factors. In other work, high concentrations of AI-2 were detected in sputum from cystic fibrosis patients [37]. These researchers found that *P. aeruginosa* transcriptional virulence factors could be stimulated by oral pharyngeal microflora and that high concentrations of AI-2 were present in *in vitro* cultures of both *P. aeruginosa* and the host microflora, indicating that the host's own bacteria might be stimulating the growth of *P. aeruginosa* [37].

Plants have long been known to interact with symbiont bacteria through quorum signalling, and plant pathogens

use quorum signalling to colonise their hosts. A review of quorum signalling in plant-pathogen symbiont interactions discusses some of the potential applications that could arise from these relationships [38]. Newton and Fray [39] focus more specifically on *Erwinia carotovora* and *Agrobacterium tumefaciens* in their review of AHL expression and repression in the plant rhizosphere. Plant-microbe relationships with potential for pathogen control are described in other work as well: *Burkholderia cepacia* in onions [40], *Ceratocystis ulmi* (a dimorphic fungus that causes Dutch elm disease) [41], *Sinorhizobium melilot* (symbiont) and *P. aeruginosa* (pathogen) in legumes [42] (reviewed in [38]), and wine grape consortia [43].

Part of the biocontrol activity of *Bacillus thuringiensis* is through AHL lactonase, an AHL-degrading enzyme [44]. Biocontrol efficacy against *E. carotovora* was reduced in *B. thuringiensis* mutated for AHL lactonase. Also, strains of *E. coli* and *Bacillus fusiformis* lacking AHL lactonase showed a similar lack of anti-pathogenic capability when cultivated on potatoes [45]. Interestingly, *B. thuringiensis* did not inhibit the growth of *E. carotovora*, but rather, inhibited its virulence and ability to cause soft rot disease in potatoes. The authors [45] speculate that the virulence inhibition capability of *B. thuringiensis* might make it a candidate for fighting bacterial infections. In earlier work, Molina and co-workers [46] demonstrated the effect of recombinant AHL lactonase in transforming strains incapable of biocontrol into biocontrol agents. *P. fluorescens* was transformed with the *ahhA* gene encoding AHL lactonase under a constitutive promoter. Another enzyme (porcine kidney acylase I) was shown to have

AHL-degrading capability *in vitro* and the ability to inhibit growth of aquatic biofilms in an aquarium water sample [47].

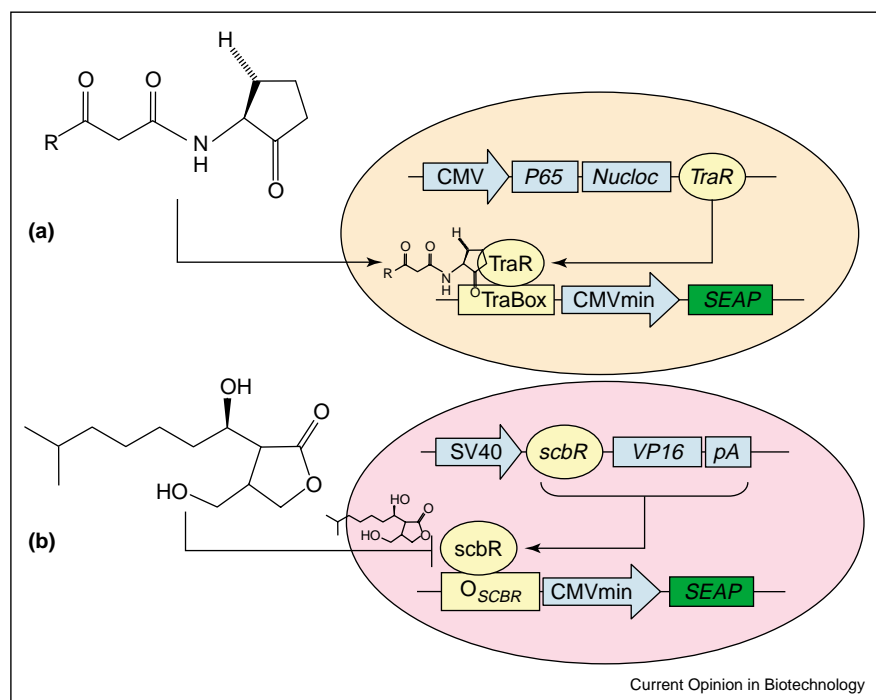
Inhibiting growth in aquatic environments is another potential application of quorum inhibition. A useful review of this topic is provided by Pasmore and Costerton [48]. In a screen for fungicidal bacteria in a Tasmanian estuary, researchers reported finding numerous strains capable of degrading invasive *Gymnodinium catenatum* [49]. Included in the fungicidal strains were *Pseudomonas* ssp. expressing AI-2; however, the occurrence of AI-2 did not correlate with fungicidal activity. In a far different aquatic environment, activated sewage sludge, quorum sensing has been shown to change the characteristics of waste water [50]. Valle and co-workers [50] added AHL compounds to activated sewage sludge and found an increase in the phenol degradative capacity. In a non-aquatic study of food spoilage, neither AI-2 nor AHL signalling correlated with biofilm formation of Gram-negative bacteria [51]. These two studies further demon-

strate the complicated, and at times controversial, role of quorum signalling, especially with respect to its applicability to biological control.

Recombinant gene expression

Perhaps one of the most exciting areas for investigation in quorum sensing is the synthesis of recombinant gene products and in metabolic engineering. Quorum sensing has been used to regulate gene expression and control cellular growth. A review by Toniatti *et al.* [52] discusses some of the advances in control of gene expression through the perspective of potential gene therapy applications. Earlier reviews by members of our group [53], and later by Bulter and co-workers [54], examine the potential of regulatory networks for controlling recombinant gene expression. In the area of biological engineering, quorum-sensing approaches to protein synthesis are developing in both prokaryotic and eukaryotic models. Working in *E. coli*, Bulter and co-workers have developed an artificial gene expression system controlled by quorum sensing [55*]. Furthermore, within eukaryotic cells, two

Figure 2



Quorum-driven protein expression in eukaryotic hosts. Two systems for recombinant protein expression using bacterial quorum sensing in mammalian cells are shown. **(a)** Nedderman and coworkers drove expression of recombinant secreted alkaline phosphatase (SEAP) in HeLa cells using a eukaryotic transactivator composed of ligand- and DNA-binding domains of the bacterial quorum sensor TraR [56]. Expression of TraR was driven by a cytomegalovirus (CMV) promoter upstream of a p65 transcriptional activator (p65) and a nuclear localization site (Nucloc). HeLa cells were incubated in the presence of 3-oxo-C₈-HSL (chemical structure shown). The presence of 3-oxo-C₈-HSL enabled TraR to bind to the TraBox on the reporter construct and to drive expression of SEAP through the CMV minimal promoter (CMVmin). **(b)** Quorum signaling was used to control protein expression in CHO cells and rats [57]. An SV40 promoter drove expression of the *scbR* transactivator, consisting of the *Streptomyces coelicolor* quorum-sensing receptor fused to a eukaryotic transactivation domain (VP16) and a polyadenylation site (pA). The reporter construct was composed of the *scbR* operon (*O_{SCBR}*) upstream of the CMV minimal promoter (CMVmin), which drove expression of SEAP. The *scbR* transactivator was released from the *scbR* operon in the presence of butyrolactones (chemical structure shown), and expression of the target gene halted.

groups have developed different manifestations of a system controlled with bacterial autoinducers [56*,57].

In *E. coli*, an artificial quorum-signalling system was used to induce synthesis of recombinant green fluorescent protein (GFP) [55*]. To ensure that acetate could behave as a quorum signal, the authors set and satisfied four criteria: that the molecule is constantly produced in cells, that it diffuses across the cell membrane, that the concentration of the molecule correlates with cell density, and that transcription is activated only when a threshold density is reached. An *E. coli* mutant for *pta*, encoding phosphotransacetylase, expressed GFP under the *glnAp₂* promoter, which is sensitive to acetyl phosphate, a product of amino acid synthesis. When threshold levels of acetate reached approximately 0.4 mM, GFP synthesis was induced. The construct can also work as a cell–cell communicator when *pta* expression is not inhibited. In this case, the authors suggested that the circuit no longer reflects cell density but rather the cell's metabolic state, presumably by way of reporting a bottleneck at pyruvate caused by influx of carbon through glycolysis. One could imagine that such a system would be beneficial to large-scale recombinant fermentation, especially if the promoter was modified to react at much higher concentrations of acetate (closer to that of growth inhibition), and the induction process reversed the pH gradient across the cell membrane.

Nedderman *et al.* [56*] constructed a eukaryotic transactivator composed of the ligand- and DNA-binding domains of the bacterial quorum sensor TraR (Figure 2). When transfected in HeLa cells supplemented with 3-oxo-C₈-HSL, the system induced expression of the reporter molecule SEAP (secreted alkaline phosphatase). The system was three orders of magnitude less sensitive than the TraR system in its bacterial host (*Agrobacterium tumefaciens*), but the expression was tightly controlled. In other, related work, the *Streptomyces coelicolor* quorum-sensing receptor (ScbR) was fused to the mammalian transactivation domain (VP16) to control expression of SEAP and erythropoietin in recombinant mammalian cells [57**] (Figure 2). The construct proved effective in controlling the expression of SEAP to near that of control constructs expressing SEAP under control of the SV40 promoter. By adding butyrolactones to the cells, the transactivator was released from the SEAP promoter and synthesis declined. Thus, the authors were able to demonstrate tuneable expression of mammalian proteins using bacterial quorum regulation.

Conclusions

Applications for quorum sensing are presently limited by our understanding of quorum-sensing mechanisms. Although there have been advances made in the use of quorum sensing, further understanding of quorum functionality is required before the power of this tool can be

fully realised. Thus far we have been able to block quorum sensing and make use of isolated components for driving protein expression. However, the full-scale manipulation of the bacterial quorum circuit in a biotechnological application remains an unfulfilled goal. How can we use the wide-reaching and sensitive quorum circuit to tailor the full range of bacterial physiological phenomena to meet specific performance goals, such as bio-based computers or controllers? What aspects of prokaryotic–eukaryotic interaction are essential for bacterial infection or symbiotic relationships? What are the limits to bacterial group behaviour, and are there levels of amplitude for such behaviour that can be characterised and predicted from environmental conditions? These questions will require more basic scientific inquiry into quorum sensing and, equally importantly, more efforts in applying this basic knowledge; exemplified by those reported in this review.

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