Microbial Cell Factories

BioMed Central

Review

Bacterial autoinduction: looking outside the cell for new metabolic engineering targets

Matthew P DeLisa[1,4] and William E Bentley*[2,3]

Address: [1]Department of Chemical Engineering, University of Texas, Austin, Texas, USA 78712, [2]Center for Biosystems Research, University of Maryland Biotechnology Institute, Baltimore, Maryland, USA 20742, [3]Department of Chemical Engineering, University of Maryland, College Park, Maryland, USA 20742 and [4]new address: School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, New York, USA 14853

Email: Matthew P DeLisa - delisa@che.utexas.edu; William E Bentley* - bentley@eng.umd.edu

* Corresponding author

Published: 20 December 2002

Microbial Cell Factories 2002, **1**:5

Received: 22 October 2002
Accepted: 20 December 2002

This article is available from: http://www.microbialcellfactories.com/content/1/1/5

Abstract

Recent evidence has demonstrated that cell-to-cell signaling is a fundamental activity carried out by numerous microorganisms. A number of specialized processes are reported to be regulated by density-dependent signaling molecules including antibiotic production, bioluminescence, biofilm formation, genetic competence, sporulation, swarming motility and virulence. However, a more centralized role for quorum sensing is emerging where quorum signaling pathways overlap with stress and starvation circuits to regulate cellular adaptation to changing environmental conditions. The interplay of these phenomena is especially critical in the expression of recombinant proteins where elicitation of stress responses can dramatically impact cellular productivity.

Review

Gene expression in bacteria can be regulated by a wide variety of intra- and extracellular signals. In fact, numerous morphological and physiological changes are induced by chemical and physical changes in the local environment. Such adaptive responses involve chemical perception and information processing that transiently alter gene expression patterns so as to protect against environmental threats. The discovery that bacteria themselves can produce extracellular chemical signals for intercellular communication has evoked a new paradigm for gene regulation. Now generally termed 'quorum sensing' or autoinduction, bacterial cell-to-cell communication enables population density-based control of gene transcription via the production, release and sensing of low-molecular weight compounds. In the majority of cases, the concentration of extracellular autoinducer increases concomitantly with the bacterial cell density. Upon reaching a 'critical' autoinducer concentration, a signal transduction cascade is triggered that results in expression of a target gene(s). Based on this general theme, quorum sensing systems have evolved as a means for improving a microbe's access to complex nutrients or environmental niches or for collectively enhancing its defense capabilities against other microorganisms or eukaryotic host defense mechanisms.

First described in two species of marine bioluminescent bacteria, *Vibrio harveyi* and *Vibrio fischeri* [1,2], quorum sensing is now known to be widespread among both Gram-positive and Gram-negative bacteria (for detailed reviews the reader is referred to refs [3–7]). For instance, Gram-positive bacteria utilize post-translationally modified peptides as quorum signals. These signals are typically exported to the extracellular milieu via specific ATP-binding cassette (ABC) transporters and are transduced by two-component signal transduction systems [8]. The concentration of secreted peptide autoinducers increases as a function of cell density. Eventually, a sensor kinase detects the peptide quorum signal thereby initiating a phosphoryl

cascade that ends with the phosphorylation (and thus activation) of the cognate response regulator protein. Active regulator protein is capable of binding target promoter DNA which, in turn, instigates a change in the expression patterns of quorum-controlled genes (for a detailed review of peptide-mediated quorum signaling the reader is referred to refs [9,10]). Examples of Gram-positive quorum signaling include the *Streptococcus pneumoniae* ComD/ComE competence system, the *Bacillus subtilis* ComP/ComA competence/sporulation system, and the *Staphylococcus aureus* AgrC/AgrA virulence system [11–13].

In contrast to Gram-positive bacteria, Gram-negative bacteria typically utilize diffusible acylated homoserine lactone (AHL) molecules. This mode of quorum sensing is mediated by proteins belonging to the LuxI-LuxR families (for review see ref [14]): LuxI-type proteins direct AHL synthesis while LuxR-type proteins function as transcriptional regulators that are capable of binding AHL signal molecules. Once formed, LuxR-AHL complexes bind to target promoters of quorum-regulated genes. Preferential binding of an AHL by its cognate LuxR-type protein guarantees a high degree of selectivity (AHLs are produced by over 50 species of bacteria and each differs only in the acyl side chain moiety) and complexity (a single bacterium can employ multiple AHL signals and LuxR-type proteins). Examples include the *V. fischeri* LuxI/LuxR bioluminescence system, the *Pseudomonas aeruginosa* LasI/LasR-RhlI/RhlR virulence system and the *Agrobacterium tumefaciens* TraI/TraR virulence system [14–16].

A unique hybrid quorum sensing circuit is exemplified by *V. harveyi's* ability to regulate bioluminescence. *V. harveyi* signalling is built upon two parallel sensory pathways that channel information to the LuxO response regulator [17–20]: the first responds to *N*-(3-oxohexanoyl)-L-homoserine lactone (autoinducer-1 or AI-1), and the second responds to a recently characterized furanosyl borate diester (autoinducer-2 or AI-2) which bears no resemblance to previously identified autoinducers. Synthesis of these autoinducers is known to require the *luxM* and *luxS* genes for AI-1 and AI-2, respectively, and LuxS is known to play a key role in the enzymatic conversion of precursor to AI-2 [21]. In a manner analogous to Gram-positive bacteria, *V. harveyi* employs a two-component circuit whereby these signal molecules are received by a cognate sensor kinase: LuxN is the sensor for AI-1, while LuxPQ is the sensor for AI-2 [17,18,22]. Of note, LuxP is homologous to the ribose binding protein (RbsB) of *E. coli*. A series of intra- and intermolecular phospho-transfers results in the transmittance of phosphate to the intermediate LuxU phosphotransferase protein [23]. In turn, the signal is relayed to the response regulator, LuxO, which, upon interaction with sigma-54 (σ^{54}), indirectly represses the *lux* operon [24]. In addition to regulating light production, LuxO and σ^{54} regulate siderophore production and colony morphology, demonstrating that multiple processes are regulated by quorum sensing in *V. harveyi* [24]. Given that *V. fischeri* was also found to possess a *luxO*-based phosphorelay regulatory system [25] is suggestive that a general regulatory mechanism may exist among bacteria based on the *V. harveyi* quorum circuit.

Complexity of quorum systems

The remarkable complexity of quorum-based systems is exemplified in the variety of different mechanisms for signal production, signal detection, signal relay and signal response. For example, while both *Vibrio* species utilize quorum sensing to regulate an identical task (i.e. regulation of the luciferase operon controlling luminescence), each use different signals and signaling mechanisms to achieve this goal. As outlined above, several unique modes of sensing are used by different bacteria such as direct autoinducer binding by a response regulator (e.g. *V. fischeri* LuxI-LuxR system), two-component signal transduction (e.g. *B. subtilis*) and even hybrid signaling circuitry (e.g. *V. harveyi*).

The presence of a number of structurally diverse families of autoinducers combined with the ability of a single bacterium to employ multiple cell-to-cell signaling molecules results in hierarchical cascades capable of linking quorum sensing to important cellular processes. Genetic analysis of *V. harveyi* has led to the discovery of two autoinducers (AI-1, *N*-3-hydroxybutanoyl-L-homoserine lactone and AI-2, a furanoysl borate diester) comprising a multichannel two-component phosphorelay used to regulate light production [18]. In the human pathogen *P. aeruginosa* at least four chemically distinct quorum signals have been discovered including *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), *N*-(butanoyl)-L-homoserine lactone, 2-heptyl-3-hydroxy-4-quinolone (PQS) and diketopiperazine (DKP) [15].

Evidence for the existence of quorum sensing in E. coli

Thus far, only a modest body of evidence exists regarding quorum-regulated gene expression in *E. coli*. Some of the earliest indications that *E. coli* use quorum sensing arose from studies using 'conditioned medium' or 'culture filtrate' [26]. Conditioned medium (CM) refers to medium that has previously supported bacterial growth and thus contains numerous secreted metabolites and signaling molecules endogenous to the particular strain of bacteria. One of the first such reports demonstrated that an extracellular factor was capable of regulating cell division by activating transcription of the major cell division gene cluster, *ftsQAZ* from the upstream *ftsQ2p* promoter [27]. Several other groups have reported that this activation involves the SdiA protein, a homologue of quorum regulatory LuxR-type proteins [28,29]. Using CM from late

exponential and stationary phase cultures, it was shown that initiation of chromosomal replication in *E. coli* was inhibited via a quorum-sensing mechanism [30]. Baca-Delancey and coworkers used a *lacZ* reporter screen to identify four genes (*cysK*,*astD*,*tnaB*, and *gabT*) that responded to an unidentified extracellular factor present in CM [31]. Subsequently, indole was identified as one of the extracellular factors at least partially responsible for activating three of these previously isolated genes.

Extracellular factors present in CM have even been reported to promote the growth of *E. coli* cells. For instance, Weichart and Kell recently described an autostimulatory effect of *E. coli* supernatants which was attributed to a small, heat-, acid- and alkali-stable, non-proteinaceous, non-ionic organic molecule [32]. Similarly, a growth-inducing factor(s) was discovered in CM generated from *E. coli* O157:H7 which had been cultured in the presence of an exogenously supplied eukaryotic hormone, norepinephrine (NE) [33]. Analysis of the production kinetics for this small, heat-stabile autoinducer suggested that it differs from other well-characterized bacterial autoinducers. Some doubt on the role of NE and cell-cell signaling is shed by Winzer *et al*. who point out that NE is capable of facilitating the release of the essential nutrient, iron, from the serum iron-binding glycoprotein, transferrin and therefore NE may be promoting growth in the serum-containing medium simply by facilitating the sequestration of iron from transferrin and/or inducing siderophore-based iron-uptake systems [34]. These authors also note that one drawback of CM experiments is the presence of a large number of bacterial products (e.g. secondary metabolites) which are released to the extracellular milieu [34]. The action of exoenzymes used by the cell to degrade media components for nutrient generation can also affect the composition of CM. A typical experiment is the addition of spent culture medium to low-density cells while the addition of uninoculated or fresh medium serves as the control. However, since uninoculated medium will certainly differ quite drastically from spent culture medium, more appropriate controls must be introduced. For instance, DeLisa *et al*. used CM generated from wildtype cells and compared its effect to CM generated from isogenic *luxS* mutant cells to determine the effect of *luxS* on transcription [35]. The observation that hundreds of genes were differentially regulated seems to support (i) the notion that LuxS plays a central metabolic role and (ii) if so, then CM of a LuxS-deficient strain will likely differ in many aspects from that of the parent ruling out AI-2 as the sole effector of the transcriptional changes. Most importantly, follow-up experiments using purified components or appropriate genetic constructs are necessary to isolate the possible effect of extracellular autoinducers.

LuxS-mediated quorum sensing

Discovery of the LuxS/AI-2 signaling system

Perhaps the most important discovery regarding *E. coli* quorum sensing was the observation that CM from spent *E. coli* (and *Salmonella typhimurium*) cultures could activate the AI-2-responsive system of *V. harveyi* [36]. Shortly thereafter, the production of the AI-2 quorum molecule was linked to the *luxS* gene [21] and eventually the biosynthetic pathway for AI-2 synthesis was elucidated [37,38]. In the cell, *S*-adenosylmethionine (SAM) is consumed to form *S*-adenosylhomocysteine (SAH), which in turn is hydrolysed by the nucleosidase, Pfs, yielding adenine and *S*-ribosylhomocysteine (SRH). Subsequently, the LuxS protein, a zinc metalloenzyme recently crystallized by several groups [39–41], converts *S*-ribosylhomocysteine (SRH) to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), a precursor probably requiring further rearrangement for AI-2 signal activity [37,38]. Both *luxS* and *pfs* are required for AI-2 activity, however expression of *luxS* is constitutive while the transcription of *pfs* is tightly correlated to AI-2 production and neither is regulated directly by AI-2 [42].

The occurrence of *luxS*-dependent AI-2 signaling is widespread among both Gram-negative and Gram-positive bacteria including *E. coli* (pathogenic and non-pathogenic varieties), *S. typhimurium*, *Shigella flexneri*, *Helicobacter pylori*, *Streptococcus pyrogenes*, *Neisseria meningitides*, *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. Moreover, 30 of 136 bacterial species contain a highly conserved *luxS* homologue [42] and, not surprisingly, a role for AI-2 in interspecies communication has been proposed [21]. Certainly extensive information charting the existence of AI-2 signaling in numerous bacterial species has come forth. However the chemical structure of the signal itself has until recently remained an enigma. Several lines of evidence suggested that AI-2 might be a cyclized carbohydrate resembling ribose. First, the *V. harveyi* extracytoplasmic receptor of AI-2, LuxP, is homologous to the ribose binding proteins found in *E. coli* and *S. typhimurium*. Second, the *S. typhimurium* LsrB protein (a LuxP homolog) binds AI-2 [43]. Accordingly, in an effort to crystallize LuxP, Chen *et al*. discovered a bound AI-2 molecule which was proposed to be a furanosyl borate diester [44]. In addition, the structural studies confirmed that LuxP bore similarity to the ribose binding protein.

Genes and phenotypes controlled by LuxS/AI-2 signaling system

Although the role of AI-2 in *V. harveyi* as a density-dependent signal for regulating bioluminescence has been well established, the function of AI-2 in other bacteria has yet to be clarified. Extensive screens for AI-2 controlled genes in *S. typhimurium* uncovered a single, previously unidentified operon (*lysRACDBFGE* operon) whose products include an ATP binding cassette (ABC)-type

transporter proposed to uptake AI-2 into the cells [43]. It was recently demonstrated that the type III secretion system is activated at the transcriptional level by CM containing AI-2 [45]. Subsequently, transcriptional profiling experiments demonstrated that large numbers of genes were up-regulated and down-regulated by CM containing AI-2 in enterohemorrhagic (EHEC) *E. coli* O157:H7 and the same has been confirmed in *E. coli* K12 [35,46]. Follow-up experiments validated that expression of a LysR-type regulator (QseA) and a two-component system (QseBC) were controlled by CM containing AI-2 [47,48]. QseA was found to be part of the regulatory cascade that controls EHEC and enteropathogenic (EPEC) virulence genes while QseBC was shown to be involved in the regulation of flagella and motility genes in both EHEC and K12. Although additional experiments are needed (such as using purified AI-2 instead of CM or demonstration that QseABC can bind AI-2 directly), these findings are noteworthy as QseA, B and C are potentially the first members of a signal transduction pathway responding to AI-2 in an organism other than *V. harveyi*.

While a number of genes targeted by AI-2 have emerged, the identification of an obvious phenotype(s) controlled by AI-2 has proven much more enigmatic. For instance, Sperandio *et al*. reported two distinct phenotypes for *E. coli* O157:H7 *luxS* mutants: 1) faster growth rate for the *luxS* mutant; and 2) decreased motility in the *luxS* mutant relative to the wild-type strain [45]. In apparent contrast, *luxS* mutants of *E. coli* K12, *H. pylori* and *Campylobacter jejuni* all grow at a rate indiscernible from the wild-type strain while *S. pyogenes luxS* mutants grow slower than wild-type (DeLisa and Bentley, unpublished observations; [49–51]). In the case of motility, *E. coli* K-12 and *C. jejuni luxS* mutants display decreased motility on semi-solid agar while *H. pylori* motility is unaffected by the *luxS* lesion (L. Wang and Bentley, unpublished observations; [49,50]).

Similar discrepancies exist regarding the role of *luxS* in the pathogenesis of various bacterial species. For example, *P. gingivalis luxS* mutants display decreased levels of virulence determinants including two cysteine proteases and haemagglutin, but loss of *luxS* did not attenuate virulence in a murine lesion model of *P. gingivalis* infection [52]. Similarly, both *C. jejuni* and *S. flexneri luxS* mutants show no attenuation of virulence using *in vivo* models [49,53]. However, *N. meningitidis* requires a functional copy of *luxS* for full meningococcal virulence; strains with a *luxS* deletion are defective for bacteremic infection, a prerequisite of meningococcal pathogenesis [54]. With so many incongruous findings, one is left to ask how AI-2 could be involved in so many diverse phenotypes across many different bacterial species? One possibility put forth by Steve Winans is that perhaps AI-2 was originally a secreted metabolite in numerous bacteria that was co-opted by individual species to be used as an intercellular signal [34,38,55]. Thus, AI-2 has evolved several diverse, species-specific roles while simultaneously remaining a 'universal' signal recognizable across numerous species of bacteria.

Density-dependent signaling or metabolic barometer?

It is clear that AI-2 signaling regulates the expression of numerous genes and is involved in determining phenotypes, but exactly *what* AI-2 is signaling is a murky subject. Emerging evidence indicates that AI-2 may not be a density-dependent signal, but rather a metabolic gauge or waste product [34,38,55]. When first reporting on the discovery of AI-2, Bassler and colleagues reported that AI-2 activity was nonexistent late in the growth phase when the density of the culture was presumably highest. It was also noted that AI-2 release to the medium was dependent on the presence of glucose in the culture medium and could be affected by factors that are known to impact cellular metabolism. Contrarily, very little AI-2 was released by carbon-limited bacteria leading the authors to postulate, for the first time, that AI-2 may provide a signal of the metabolic potential of the growth environment. It was later demonstrated that carbon-limited *S. typhimurium* can scavenge AI-2 from the growth medium raising the possibility that AI-2 may be used as a nutrient [43]. Winzer *et al*. observed that *P. aeruginosa* is capable of removing AI-2 activity, further evidence that AI-2 may be a metabolized nutrient under certain conditions [38]. Thus, akin to acetate, AI-2 is produced and released to the medium in the presence of preferred carbohydrates and is metabolized when primary nutrients are depleted. Intriguingly, a direct overlap between AI-2 and acetate has been observed recently by Kirkpatrick *et al*., who reported that the LuxS protein is upregulated when high levels of acetate are supplied exogenously to the growth medium [56].

Strong evidence supporting the metabolic gauge nature of AI-2 comes from chemostat experiments where AI-2 accumulation was shown to accumulate linearly with growth rate and was independent of culture density [57]. In other words, as *E. coli* cells grow, they produce AI-2 and the faster they grow (via glucose and additional medium components) the faster the rate of AI-2 production on a per cell basis. This can be explained by the fact that AI-2 synthesis is stoichiometrically linked to SAM utilization. As SAM is primarily utilized when the choline moiety of phospholipids is synthesized, AI-2 is indirectly linked to membrane biosynthesis and thus to cellular growth rate. In fact, AI-2 was seen to upregulate a number of genes specifically involved in membrane and cell-surface architecture [35]. Additionally, the AI-2 responses to culture perturbations (e.g. heat shock, osmotic shock, amino acid starvation and expression of heterologous protein) were described as indicative of a shift in metabolic activity [57]. The need for

bacteria to communicate their metabolic activity, especially given the abundance of specific receptors which have evolved to probe the environment, perhaps lies in the intercellular nature of AI-2 signalling. There may be an inherent advantage for one species to detect the emergence of another. Also, and more importantly in the context of exploiting microbes as cell factories, the stress placed on host cells by heterologous production of numerous protein products resulted in significant loss of AI-2 signaling [57,58]. That is, the production of proteins was perhaps 'signalled' to the population by a dramatic reduction in AI-2.

Hierarchy of quorum cascades: overlap with cellular stress responsive circuits

Quorum sensing is a high cell density phenomenon in most microorganisms. However, recent evidence is shedding light on a more central role for quorum sensing in the adaptation of cells to stress and starvation. In certain contexts, quorum signals may even directly signal entry into stationary phase [59]. One of the first indications that quorum sensing systems overlap with stress-related circuits in *E. coli* came from the experiments of Huisman and Kolter [60]. At the heart of this work was the demonstration that expression of the stationary phase sigma factor, σ^S, in *E. coli* was induced by homoserine lactone (HSL), a metabolite synthesized from intermediates in threonine biosynthesis. Recall also that, although not found in *E. coli*, acylated homoserine lactones (AHLs) are one of the primary signals used in density-dependent expression systems of numerous bacteria. The authors suggest that synthesis of HSL may serve as a general signal of starvation. In fact, σ^S is regulated by a number of proteins and small metabolites, including cAMP, UDP-glucose, ppGpp as well as HSL, which provide a link between the nutritional status of the cell and the induction of stationary phase [61].

Along similar lines, several groups have reported that a substance present in conditioned medium was capable of upregulating expression of *rpoS* [29,62]. Sitnikov *et al.* further observed that conditioned medium and several exogenous autoinducers could upregulate the cell division genes *ftsQA* in an SdiA-dependent fashion [29]. Note that SdiA (suppression of division inhibitor A) is homologous to the LuxR transcriptional activator of *V. fischeri*. The upregulation of *ftsQA* in this context resulted in the acquisition of a more resistant spherical shape [27] and would presumably serve to increase the cells resistance to stress in general. Importantly, whether there is an interaction between *sdiA* and *luxS* (or any other quorum component for that matter)in the cell remains an open question. Surrette and Bassler found that the *sdiA* and *luxS* systems operate independently, while DeLisa *et al*. observed that AI-2 could stimulate a two or threefold increase in *sdiA* using microarray technology and continuous culture, respectively [35,57,63]. Thus, while it seems that there is an interaction between the two systems, it is relatively weak and possibly indirect [64]. It has even been suggested that SdiA does not respond to any endogenous signal but rather senses AHLs produced by other bacteria in mixed bacterial populations [65]. The work of Kanamaru *et al*. [66] is partially in agreement with this hypothesis as they reported that SdiA from enterohaemorrhagic *E. coli* could bind non-native AHLs *in vitro* but did not appear to bind endogenous AI-2.

In addition to σ^s-dependent responses, emerging evidence also suggests that autoinduction may also overlap with σ^{32} and σ^{54}-mediated responses in bacteria. For example, studies on the regulation of the *V. fischeri lux* genes in *E. coli* have demonstrated a link between LuxR-mediated quorum sensing and the σ^{32}-mediated stress circuit. The luminescence response of *E. coli* containing the *lux* genes to exogenously added autoinducer is highly dependent on the level of the LuxR protein. In turn, expression of the LuxR protein increases at the onset of late exponential phase and is dependent on the expression of *rpoH* and the *groE* system [67]. Several σ^{32} stress genes also appear to play a role in AI-2 signaling. For instance, DeLisa *et al*. reported that *groEL* and*groES* mutants of *E. coli* produced considerably higher levels of AI-2 while *dnaK*, *dnaJ*, and *grpE* mutations resulted in a significant attenuation in AI-2 activity [57]. Whether the role of these genes in the AI-2 pathway is direct or indirect remains to be determined.

In the case of σ^{54}, the linkage appears much more direct. Based on studies of luminescence regulation in *V. harveyi* it was demonstrated that LuxO, in response to AHL or AI-2, functions as an activator protein via interaction with the alternative sigma factor, σ^{54} (encoded by *rpoN*) [24]. It was suggested that LuxO, together with σ^{54}, activates the expression of a negative regulator of luminescence. In *E. coli*, significant AI-2 triggered induction of *ygeV*, a putative σ^{54}-dependent transcriptional activator, and *yhbH*, a σ^{54} modulating protein, suggesting that σ^{54} may be involved in autoinduction in this bacterium [35]. Although, to date it is not known whether *E. coli* possess the same pathway described above for *V. harveyi*. Interestingly, increased levels of *Bacillus cereus* YhbH, which is strongly induced in response to environmental stresses and energy depletion via both σ^B and σ^H, could be observed within 2 h in both attached cells and planktonic cultures growing in the presence of glass wool, indicating that this protein plays an important role in regulation of the biofilm phenotype [68]. Since biofilm formation is commonly regulated by quorum sensing, it is intriguing that *E. coli* YhbH responds to AI-2 and one possible role for this interaction might be to regulate the phenotypic transition from planktonic growth to biofilm formation.

Quorum sensing as a target for metabolic engineering

The emerging link between quorum sensing, cellular metabolism and stress-responsive circuits raises the possibility of targeting quorum pathways for improving cellular productivity. Huisman and Kolter identified RspA (regulator of stationary phase A) as a mulitcopy repressor of *rpoS* expression and σ^S-dependent gene expression [60]. They hypothesized that the effect of RspA on *rpoS* expression could result from the degradation of a metabolite that signals starvation. They further supposed that the metabolite was a lactone, as the RspA protein resembled a lactonizing enzyme (chloromuconate cycloisomerase). Although *E. coli* do not appear to produce an extracellular AHL quorum signal, it is possible that HSL serves as a metabolic signal or even toxic by-product used to regulate *rpoS* expression. In seemingly unrelated work, Weikert *et al*. engineered a strain of bacteria via directed evolution that exhibited improved physiological properties such as higher specific growth rate, increased biomass yields and improved resistance to a variety of stresses [69]. Unexpectedly, they found that *rspA* was upregulated in the mutant strain and that overexpression of *rspAB* in wild-type cells could partially mimic the complex mutant phenotype [70]. Most importantly, they demonstrated that multicopy expression of *rspAB* resulted in a nearly 3-fold improvement in recombinant β-galactosidase production. The authors suggest that RspA-dependent reduction of lactone byproducts of amino acid metabolism such as HSL might be responsible for the improved phenotype of the mutant strain. Whether these findings are attributable to HSL acting as a specific signal with a corresponding receptor or simply as a toxic by-product of metabolism is still unclear.

In another study, DeLisa *et al*. hypothesized that AI-2 signaling in *E. coli* was an important molecular determinant in recombinant cultures of bacteria [58]. It was seen that recombinant protein expression resulted in decreased AI-2 activity, which is likely a result of two factors. First, depressed AI-2 levels could result from the decline in culture growth rate typically observed during heterologous protein production [71,72], as AI-2 and culture growth rate are linearly correlated. Second, the ppGpp-mediated stringent response, elicited by a failure of tRNA aminoacylation to keep up with the demands of protein synthesis, induces several biosynthetic operons, including those for threonine and methionine [73]. This induction, an outcome of the stringent response triggered by heterologous protein production, would be expected to divert intermediates away from AI-2 synthesis. The finding that AI-2 activity is significantly attenuated following amino acid starvation [57] is consistent with this proposal. Based on this logic, we reasoned that exogenous AI-2 addition would change the 'apparent' environmental robustness (or growth rate potential) experienced by the cells to significantly higher levels earlier in the growth phase. Remarkably, restoration of AI-2 activity levels using either conditioned medium or co-overexpression of *luxS* (in a *luxS* mutant) resulted in a 3–4-fold increase in soluble accumulation of a recombinant protein [DeLisa, Valdes and Bentley, submitted for publication].

Additional roles for quorum sensing in the production of biotechnologically relevant molecules

In addition to the synthesis of recombinant proteins, quorum sensing systems play a critical role in the production of many secondary microbial metabolites which are commercially and industrially significant. In fact, it has been shown that quorum sensing serves as the key regulatory mechanism that controls the production of a wide range of molecules possessing antimicrobial activity including: (i) the polyketide antibiotic mupirocin produced by *Pseudomonas fluorescens* NCIMB and used clinically as a topical treatment for staphylococcal infections [74]; (ii) the antimicrobial metabolite bacteriocin produced by lactic acid bacteria and used as a food additive [75]; and (iii) the potent beta-lactam carbapenems with a broad spectrum of activity against both Gram-positive and Gram-negative bacteria [76]. In the latter case, these types of antiobiotics are naturally produced metabolites and have been isolated from species of *Streptomyces, Erwinia* and*Serratia*. In *Erwinia* and more recently in *Photorhabdus luminescens*, carbapenem biosynthesis is transcriptionally controlled by a quorum sensing mechanism. Specifically, *Erwinia* use the signal 3-oxo-C6-HSL to regulate the expression of the *carABCDEFGH* gene cluster responsible for carbapenum production [76]. Interestingly, a halogenated furanone produced by *Delisea pulchra* was found to inhibit this process by disrupting 3-oxo-C6-HSL quorum sensing [77]. *P. luminescens* use a similar strategy to regulate expression of the *cpm* operon. However, this operon and thus carbapenum biosynthesis was regulated by a *luxS*-like signaling mechanism whereby *luxS* could repress *cpm* gene expression at the end of exponential growth [78]. As quorum sensing allows synchronized, population-wide production of antimicrobial peptides as a function of cell density, production strategies capitalizing on the regulatory effects of quorum signals (both protagonistic and antagonistic) should prove to be quite rewarding.

Conclusions

Metabolic engineering has been used to introduce novel biochemical pathways and realign metabolic fluxes in microorganisms to improve product yields [79–81]. Unfortunately, concurrent host-specific physiological perturbations, such as altered specific growth rate, and pleiotropic metabolic effects derived from the desired genotypic changes can limit overall productivity. Therefore, alternative approaches to "cell condition" or dynamically change intracellular architecture in response to

environmental cues have been explored. One of the first such approaches was genetic knockout of loci (e.g. *rpoH*, *degP*) known to affect heterologous product stability [82]. More recently, transient down-regulation of a target gene (*rpoH*) using antisense technology was used to increase activity of a recombinant enzyme [83]. Alternately, strategies that promote a host response (as opposed to deletion or inhibition) have been implemented. For instance, external induction of heat shock or cold shock in a manner designed to favorably change intracellular conditions (i.e. increased chaperone activity or decreased protease activity) has been utilized for improved product yields [84,85]. Likewise, altering the protein folding environment by addition of reducing (or oxidizing) reagents such as dithiothreitol (DTT) [86,87] or by coexpressing redox regulating proteins, such as DsbC [88] has been demonstrated.

To date, there have been no reports on the use of quorum-sensing or autoinducers for improving recombinant protein yield in any expression system. In related work, Lee and Shuler utilized *Catharanthus roseus* conditioned medium (CM) to improve the production of two secondary metabolites, ajmalicine and catharanthine, and further attributed the enhancing effects of CM to an unidentified factor produced and secreted by *C. roseus* suspension cultures [89]. Further, Nozawa *et al*., showed that CM can enhance lipopolysaccharide production in *E. coli* [90]. However, the direct demonstration of a quorum-specific strategy had until recently not been shown.

Since it appears that AI-2 communicates both cell density and metabolic potential of the growth environment [36], AI-2 mediated quorum sensing will likely have a role in biotechnological processes. For example, we showed that the SdiA activated ftsQp2 promoter could be used to drive protein expression without addition of exogenous inducers [DeLisa, Valdes and Bentley, submitted for publication]. We also demonstrated that production of AI-2 was highly dependent on *groEL* and *groES*, as well as the σ^{32} sequestering complex *dnaK/dnaJ/grpE*. Based on this evidence, it was postulated that AI-2 production and degradation overlaps chaperone-assisted folding pathways within the cell [57]. In fact, we found that altering AI-2-mediated autoinduction could enhance the yield of several heterologous proteins. Improvement in protein yield was achieved by repeated or continuous conditioning of protein-expressing cultures through addition of conditioned medium containing high levels of AI-2 *or* through co-expression of plasmid-encoded *luxS* [DeLisa, Valdes and Bentley, submitted for publication]. In the absence of a specific mechanism, we propose that AI-2-stimulated enhancement of protein yield is perhaps a consequence of a link to chaperone-assisted folding pathways and/or proteolytic pathways.

Determination of the chemical structure of bacterial AI-2 is a milestone for researchers studying both mechanistic and applied aspects of bacterial quorum sensing. In the context of cellular productivity, extracellular signalling cascades are likely to play an important role in the cellular productivity of microorganisms. Since AI-2 appears to 'communicate' the metabolic potential of the bacterial growth environment, strategies designed to alter the pattern of communication can be used to alter the physiology of each individual cell. Determining how the signalling patterns should be altered to obtain a desired cellular output is not a trivial matter. However, the explosion of data (and modelling efforts) regarding the genetic and biochemical details of autoinduction in *E. coli* and numerous other bacterial species should promote improved understanding.

In particular, the development of signal transduction pathways as targets for metabolic engineering is likely to find widespread appeal as the importance of global regulators becomes more developed. That is, effective metabolic engineering strategies that define and manipulate control points must begin to incorporate a hierarchical structure corresponding to the same structure already present in microorganisms. In the absence of such a hierarchical structure, both near and distant pleiotropic effects that plagued early metabolic engineering studies [79] will likely continue to confound researchers. Among the attractive targets that build on this objective are sigma factors in bacteria [83] and molecular regulators or associated complexes (e.g., chaperones) that control macromolecular synthetic processes. Also attractive are signal transduction pathways that converge on global transcriptional regulators (e.g., quorum circuitry, as reviewed here), which thereby indirectly control the assembly of holoenzymes and other key metabolic controllers. These targets first enable a favourably altered global landscape that can then be fine-tuned by manipulating specific metabolic enzymes and pathways of enzymes. Finally, increased knowledge of these regulatory circuits (e.g. quorum) and the role of the individual proteins within the circuit should help to enable additional strategies for cross-wiring or inverse metabolic engineering [91] of the quorum circuit for improved cellular function.

References

1. Nealson KH, Platt T and Hastings JW **Cellular control of the synthesis and activity of the bacterial luminescent system.** *J Bacteriol* 1970, **104:**313-322
2. Nealson KH and Hastings JW **Bacterial bioluminescence: its control and ecological significance.** *Microbiol Rev* 1979, **43:**496-518
3. Fuqua C and Greenberg EP **Self perception in bacteria: quorum sensing with acylated homoserine lactones.** *Curr Opin Microbiol* 1998, **1:**183-189
4. Miller MB and Bassler BL **Quorum sensing in bacteria.** *Annu Rev Microbiol* 2001, **55:**165-199
5. Williams P, Camara M, Hardman A, Swift S, Milton D, Hope VJ, Winzer K, Middleton B, Pritchard DI and Bycroft BW **Quorum**

sensing and the population-dependent control of virulence. *Philos Trans R Soc Lond B Biol Sci* 2000, **355:**667-680
6. Withers H, Swift S and Williams P **Quorum sensing as an integral component of gene regulatory networks in Gram-negative bacteria.** *Curr Opin Microbiol* 2001, **4:**186-193
7. Bassler BL **How bacteria talk to each other: regulation of gene expression by quorum sensing.** *Curr Opin Microbiol* 1999, **2:**582-587
8. Lazazzera BA, Solomon JM and Grossman AD **An exported peptide functions intracellularly to contribute to cell density signaling in *B. subtilis*.** *Cell* 1997, **89:**917-925
9. Kleerebezem M, Quadri LE, Kuipers OP and de Vos WM **Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria.** *Mol Microbiol* 1997, **24:**895-904
10. Lazazzera BA and Grossman AD **The ins and outs of peptide signaling.** *Trends Microbiol* 1998, **6:**288-294
11. Haverstein L and Morrison DA **Quorum sensing and peptide pheromones in *Streptococcal* competence for genetic transformation.** *In Cell-Cell Signaling in Bacteria (Edited by: Dunney GaW) SC: ASM Press* 1999, 9-26
12. Solomon JM, Magnuson R, Srivastava A and Grossman AD **Convergent sensing pathways mediate response to two extracellular competence factors in *Bacillus subtilis*.** *Genes Dev* 1995, **9:**547-558
13. Norvick R **Regulation of pathogenicity in *Staphylococcus aureus* by a peptide-based density-sensing system.** *In Cell-Cell Signaling in Bacteria (Edited by: Dunney GaW) SC: ASM Press* 1999, 129-146
14. Fuqua C, Winans SC and Greenberg EP **Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators.** *Annu Rev Microbiol* 1996, **50:**727-751
15. Parsek MR and Greenberg EP **Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms.** *Proc Natl Acad Sci U S A* 2000, **97:**8789-8793
16. Piper KR, Beck von Bodman S and Farrand SK **Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction.** *Nature* 1993, **362:**448-450
17. Bassler BL, Wright M, Showalter RE and Silverman MR **Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence.** *Mol Microbiol* 1993, **9:**773-786
18. Bassler BL, Wright M and Silverman MR **Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway.** *Mol Microbiol* 1994, **13:**273-286
19. Bassler BL, Greenberg EP and Stevens AM **Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*.** *J Bacteriol* 1997, **179:**4043-4045
20. Cao JG and Meighen EA **Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*.** *J Biol Chem* 1989, **264:**21670-21676
21. Surette MG, Miller MB and Bassler BL **Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production.** *Proc Natl Acad Sci U S A* 1999, **96:**1639-1644
22. Freeman JA, Lilley BN and Bassler BL **A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*.** *Mol Microbiol* 2000, **35:**139-149
23. Freeman JA and Bassler BL **Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*.** *J Bacteriol* 1999, **181:**899-906
24. Lilley BN and Bassler BL **Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54.** *Mol Microbiol* 2000, **36:**940-954
25. Miyamoto CM, Lin YH and Meighen EA **Control of bioluminescence in *Vibrio fischeri* by the LuxO signal response regulator.** *Mol Microbiol* 2000, **36:**594-607
26. Lodge RM and Hinshelwood CN **Physiochemical aspects of bacterial growth. Part IX. The lag phase of *Bacteriol lactis aerogenes*.** *J Chem Soc* 1943, 213-219
27. Wang XD, de Boer PA and Rothfield LI **A factor that positively regulates cell division by activating transcription of the major cluster of essential cell division genes of *Escherichia coli*.** *EMBO J* 1991, **10:**3363-3372
28. Garcia-Lara J, Shang LH and Rothfield LI **An extracellular factor regulates expression of *sdiA*, a transcriptional activator of cell division genes in *Escherichia coli*.** *J Bacteriol* 1996, **178:**2742-2748
29. Sitnikov DM, Schineller JB and Baldwin TO **Control of cell division in *Escherichia coli*: regulation of transcription of *ftsQA* involves both *rpoS* and SdiA-mediated autoinduction.** *Proc Natl Acad Sci U S A* 1996, **93:**336-341
30. Withers HL and Nordstrom K **Quorum-sensing acts at initiation of chromosomal replication in *Escherichia coli*.** *Proc Natl Acad Sci U S A* 1998, **95:**15694-15699
31. Baca-DeLancey RR, South MM, Ding X and Rather PN ***Escherichia coli* genes regulated by cell-to-cell signaling.** *Proc Natl Acad Sci U S A* 1999, **96:**4610-4614
32. Weichart DH and Kell DB **Characterization of an autostimulatory substance produced by *Escherichia coli*.** *Microbiology* 2001, **147:**1875-1885
33. Lyte M, Frank CD and Green BT **Production of an autoinducer of growth by norepinephrine cultured *Escherichia coli* O157:H7.** *FEMS Microbiol Lett* 1996, **139:**155-159
34. Winzer K, Hardie KR and Williams P **Bacterial cell-to-cell communication: sorry, can't talk now – gone to lunch!** *Curr Opin Microbiol* 2002, **5:**216-222
35. DeLisa MP, Wu CF, Wang L, Valdes JJ and Bentley WE **DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*.** *J Bacteriol* 2001, **183:**5239-5247
36. Surette MG and Bassler BL **Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*.** *Proc Natl Acad Sci U S A* 1998, **95:**7046-7050
37. Schauder S, Shokat K, Surette MG and Bassler BL **The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule.** *Mol Microbiol* 2001, **41:**463-476
38. Winzer K, Hardie KR, Burgess N, Doherty N, Kirke D, Holden MT, Linforth R, Cornell KA, Taylor AJ and Hill PJ **LuxS: its role in central metabolism and the in vitro synthesis of 4-hydroxy-5-methyl-3(2H)-furanone.** *Microbiology* 2002, **148:**909-922
39. Ruzheinikov SN, Das SK, Sedelnikova SE, Hartley A, Foster SJ, Horsburgh MJ, Cox AG, McCleod CW, Mekhalfia A and Blackburn GM **The 1.2 A structure of a novel quorum-sensing protein, *Bacillus subtilis* LuxS.** *J Mol Biol* 2001, **313:**111-122
40. Hilgers MT and Ludwig ML **Crystal structure of the quorum-sensing protein LuxS reveals a catalytic metal site.** *Proc Natl Acad Sci U S A* 2001, **98:**11169-11174
41. Lewis HA, Furlong EB, Laubert B, Eroshkina GA, Batiyenko Y, Adams JM, Bergseid MG, Marsh CD, Peat TS and Sanderson WE **A structural genomics approach to the study of quorum sensing: crystal structures of three LuxS orthologs.** *Structure (Camb)* 2001, **9:**527-537
42. Beeston AL and Surette MG ***pfs*-Dependent Regulation of Autoinducer 2 Production in *Salmonella enterica* Serovar Typhimurium.** *J Bacteriol* 2002, **184:**3450-3456
43. Taga ME, Semmelhack JL and Bassler BL **The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium*.** *Mol Microbiol* 2001, **42:**777-793
44. Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL and Hughson FM **Structural identification of a bacterial quorum-sensing signal containing boron.** *Nature* 2002, **415:**545-549
45. Sperandio V, Mellies JL, Nguyen W, Shin S and Kaper JB **Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*.** *Proc Natl Acad Sci U S A* 1999, **96:**15196-15201
46. Sperandio V, Torres AG, Giron JA and Kaper JB **Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7.** *J Bacteriol* 2001, **183:**5187-5197
47. Sperandio V, Torres AG and Kaper JB **Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*.** *Mol Microbiol* 2002, **43:**809-821
48. Sperandio V, Li CC and Kaper JB **Quorum-sensing *Escherichia coli* regulator A: a regulator of the LysR family involved in the regulation of the locus of enterocyte effacement pathogenic-**

ity island in enterohemorrhagic *E. coli*. *Infect Immun* 2002, **70:**3085-3093
49. Elvers KT and Park SF **Quorum sensing in *Campylobacter jejuni*: detection of a *luxS* encoded signalling molecule.** *Microbiology* 2002, **148:**1475-1481
50. Joyce EA, Bassler BL and Wright A **Evidence for a signaling system in *Helicobacter pylori*: detection of a *luxS*-encoded autoinducer.** *J Bacteriol* 2000, **182:**3638-3643
51. Lyon WR, Madden JC, Levin JC, Stein JL and Caparon MG **Mutation of *luxS* affects growth and virulence factor expression in *Streptococcus pyogenes*.** *Mol Microbiol* 2001, **42:**145-157
52. Burgess NA, Kirke DF, Williams P, Winzer K, Hardie KR, Meyers NL, Aduse-Opoku J, Curtis MA and Camara M **LuxS-dependent quorum sensing in *Porphyromonas gingivalis* modulates protease and haemagglutinin activities but is not essential for virulence.** *Microbiology* 2002, **148:**763-772
53. Day WA Jr and Maurelli AT ***Shigella flexneri* LuxS quorum-sensing system modulates *virB* expression but is not essential for virulence.** *Infect Immun* 2001, **69:**15-23
54. Winzer K, Sun YH, Green A, Delory M, Blackley D, Hardie KR, Baldwin TJ and Tang CM **Role of *Neisseria meningitidis luxS* in cell-to-cell signaling and bacteremic infection.** *Infect Immun* 2002, **70:**2245-2248
55. Winans SC **Bacterial esperanto.** *Nat Struct Biol* 2002, **9:**83-84
56. Kirkpatrick C, Maurer LM, Oyelakin NE, Yoncheva YN, Maurer R and Slonczewski JL **Acetate and formate stress: opposite responses in the proteome of *Escherichia coli*.** *J Bacteriol* 2001, **183:**6466-6477
57. DeLisa MP, Valdes JJ and Bentley WE **Mapping stress-induced changes in autoinducer AI-2 production in chemostat-cultivated *Escherichia coli* K-12.** *J Bacteriol* 2001, **183:**2918-2928
58. DeLisa MP, Valdes JJ and Bentley WE **Quorum signaling via AI-2 communicates the "Metabolic Burden" associated with heterologous protein production in *Escherichia coli*.** *Biotechnol Bioeng* 2001, **75:**439-450
59. Lazazzera BA **Quorum sensing and starvation: signals for entry into stationary phase.** *Curr Opin Microbiol* 2000, **3:**177-182
60. Huisman GW and Kolter R **Sensing starvation: a homoserine lactone – dependent signaling pathway in *Escherichia coli*.** *Science* 1994, **265:**537-539
61. Hengge-Aronis R **Recent insights into the general stress response regulatory network in *Escherichia coli*.** *J Mol Microbiol Biotechnol* 2002, **4:**341-346
62. Mulvey MR, Switala J, Borys A and Loewen PC **Regulation of transcription of *katE* and *katF* in *Escherichia coli*.** *J Bacteriol* 1990, **172:**6713-6720
63. Surette MG and Bassler BL **Regulation of autoinducer production in *Salmonella typhimurium*.** *Mol Microbiol* 1999, **31:**585-595
64. Volf J, Sevcik M, Havlickova H, Sisak F, Damborsky J and Rychlik I **Role of SdiA in *Salmonella enterica* serovar Typhimurium physiology and virulence.** *Arch Microbiol* 2002, **178:**94-101
65. Michael B, Smith JN, Swift S, Heffron F and Ahmer BM **SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities.** *J Bacteriol* 2001, **183:**5733-5742
66. Kanamaru K, Tatsuno I, Tobe T and Sasakawa C **SdiA, an *Escherichia coli* homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7.** *Mol Microbiol* 2000, **38:**805-816
67. Adar YY and Ulitzur S **GroESL proteins facilitate binding of externally added inducer by LuxR protein-containing *E. coli* cells.** *J Biolumin Chemilumin* 1993, **8:**261-266
68. Pragai Z and Harwood CR **Regulatory interactions between the Pho and sigma(B)-dependent general stress regulons of *Bacillus subtilis*.** *Microbiology* 2002, **148:**1593-1602
69. Weikert C, Sauer U and Bailey JE **An *Escherichia coli* host strain useful for efficient overproduction of secreted recombinant protein.** *Biotechnol Bioeng* 1998, **59:**386-391
70. Weikert C, Canonaco F, Sauer U and Bailey JE **Co-overexpression of RspAB improves recombinant protein production in *Escherichia coli*.** *Metab Eng* 2000, **2:**293-299
71. Bentley WE, Mirjalili N, Andersen DC, Kompala DS and Davis RH **Plasmid encoded protein: The principal factor in the "metabolic burden" associated with recombinant bacteria.** *Biotechnol Bioeng* 1990, **35:**668-681
72. Georgiou G **Optimizing the production of recombinant proteins in microorganisms.** *AIChEJ* 1988, **34:**1233-1248
73. Cashel M and Rudd KE **The stringent stress response.** *In Escherichia coli and Salmonella typhimurium (Edited by: Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Schaechter M, Umbarger HE) American Society for Microbiology* 1987, **2:**
74. El-Sayed AK, Hothersall J and Thomas CM **Quorum-sensing-dependent regulation of biosynthesis of the polyketide antibiotic mupirocin in *Pseudomonas fluorescens* NCIMB 10586.** *Microbiology* 2001, **147:**2127-2139
75. Quadri LE **Regulation of antimicrobial peptide production by autoinducer-mediated quorum sensing in lactic acid bacteria.** *Antonie Van Leeuwenhoek* 2002, **82:**133-145
76. McGowan SJ, Holden MT, Bycroft BW and Salmond GP **Molecular genetics of carbapenem antibiotic biosynthesis.** *Antonie Van Leeuwenhoek* 1999, **75:**135-141
77. Manefield M, Welch M, Givskov M, Salmond GP and Kjelleberg S **Halogenated furanones from the red alga, *Delisea pulchra*, inhibit carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen *Erwinia carotovora*.** *FEMS Microbiol Lett* 2001, **205:**131-138
78. Derzelle S, Duchaud E, Kunst F, Danchin A and Bertin P **Identification, characterization, and regulation of a cluster of genes involved in carbapenem biosynthesis in *Photorhabdus luminescens*.** *Appl Environ Microbiol* 2002, **68:**3780-3789
79. Stephanopoulos G and Vallino JJ **Network rigidity and metabolic engineering in metabolite overproduction.** *Science* 1991, **252:**1675-1681
80. Liao JC, Hou S-Y and Chao Y-P **Pathway analysis, engineering, and physiological considerations for redirecting central metabolism.** *Biotechnol Bioeng* 1996, **52:**129-140
81. Bailey JE **Toward a science of metabolic engineering.** *Science* 1991, **252:**1668-1675
82. Meerman HJ and Georgiou G **Construction and characterization of a set of *E. coli* strains deficient in all known loci affecting the proteolytic stability of secreted recombinant proteins.** *Biotechnology (N Y)* 1994, **12:**1107-1110
83. Srivastava R, Cha HJ, Peterson MS and Bentley WE **Antisense downregulation of sigma(32) as a transient metabolic controller in *Escherichia coli*: effects on yield of active organophosphorus hydrolase.** *Appl Environ Microbiol* 2000, **66:**4366-4371
84. Kusano K, Waterman MR, Sakaguchi M, Omura T and Kagawa N **Protein synthesis inhibitors and ethanol selectively enhance heterologous expression of P450s and related proteins in *Escherichia coli*.** *Arch Biochem Biophys* 1999, **367:**129-136
85. Mujacic M, Cooper KW and Baneyx F **Cold-inducible cloning vectors for low-temperature protein expression in *Escherichia coli*: application to the production of a toxic and proteolytically sensitive fusion protein.** *Gene* 1999, **238:**325-332
86. Gill R, DeLisa M, Valdes J and Bentley W **Genomic analysis of high-cell-density recombinant *Escherichia coli* fermentation and "cell conditioning" for improved recombinant protein yield.** *Biotechnol Bioeng* 2000, **72:**85-95
87. Gill RT, Cha HJ, Jain A, Rao G and Bentley WE **Generating controlled reducing environments in aerobic recombinant *Escherichia coli* fermentations: effects on cell growth, oxygen uptake, heat shock protein expression, and *in vivo* CAT activity.** *Biotechnol Bioeng* 1998, **59:**248-259
88. Bessette PH, Aslund F, Beckwith J and Georgiou G **Efficient folding of proteins with multiple disulfide bonds in the *Escherichia coli* cytoplasm.** *Proc Natl Acad Sci U S A* 1999, **96:**13703-13708
89. Lee CW and Shuler ML **The effect of inoculum density and conditioned medium on the production of ajmalicine and catharanthine from immobilized *Catharanthus roseus* cells.** *Biotechnol Bioeng* 2000, **67:**61-71
90. Nozawa RT, Sekiguchi R and Yokota T **Stimulation by conditioned medium of L-929 fibroblasts, *E. coli* lipopolysaccharide, and muramyl dipeptide of candidacidal activity of mouse macrophages.** *Cell Immunol* 1980, **53:**116-124
91. Bailey JE, Sburlati A, Hatzimanikatis V, Lee K, Renner WA and Tsai PS **Inverse metabolic engineering: A strategy for directed genetic engineering of useful phenotypes.** *Biotechnol Bioeng* 1996, **52:**109-121