Review

Phenotypic Heterogeneity Generated by Histidine Kinase-Based Signaling Networks

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Abstract

A complex relationship exists between environmental factors, signaling networks and phenotypic individuality in bacteria. In this review, we will focus on the organization, function and control points of multiple-input histidine kinase-based signaling cascades as a source of phenotypic heterogeneity. In particular, we will examine the quorum sensing cascade in *Vibrio harveyi* and the pyruvate sensor network in *Escherichia coli*. We will describe and compare these histidine kinase-based signaling networks in terms of robustness, the molecular mechanisms of signal transduction and the role of RNA switches. Finally, we will discuss the biological significance of phenotypic heterogeneity for the respective bacteria in relation to environmental factors.

Introduction

Bacteria as single living organisms are exposed to constant changes in nutrients and communication signals, but also of life-threatening compounds, such as antibiotics [1]. In addition, bacteria exchange information with other cells and adapt to different life styles, such as living in a biofilm, or to the eukaryotic host environment. For all these processes, bacteria have evolved sophisticated signaling systems to sense and respond to environmental alterations, to change their behavior and physiological state, and to protect themselves against stress.

Another excellent strategy for ensuring survival of the whole population in fluctuating environments is phenotypic heterogeneity in isogenic populations wherein one or more subpopulations of cells express different functions to allow growth and stress adaptation [2,3]. Our group and others found individualized cell responses to quorum sensing (QS) signals in the marine bacteria *Vibrio harveyi* [4], *Vibrio fischeri* [5] and *Dinoroseobacter shibae* [6], and in the soil bacteria *Pseudomonas putida* [7] and *Sinorhizobium fredii* [8], but also in the human pathogen *Listeria monocytogenes* [9]. Other examples concern the formation of persister cells and protection against antibiotics and heavy metals in *Staphylococcus* sp. [10], *Salmonella* Typhimurium [11], and *Pseudomonas aeruginosa* [12]. Also, nutrient utilization seems not to be a uniform process, as examples involving the utilization of myo-inositol [13], lactose [14] and pyruvate [15] show. Therefore, switching of phenotypic states and formation of two or more subpopulations have evolved as an efficient survival or adaptation strategy. These observations raise the question about the nature of molecular switches responsible for generating distinct subpopulations.

Bacteria constantly sense all changes in their environment, which typically take the form of chemical and physical stimuli. Therefore, these organisms use numerous transmembrane receptors to detect these environmental cues and transduce the information into an intracellular signal that triggers some form of cellular response. By now, it is widely recognized that the information is not transmitted in a simple linear fashion, but that most signaling pathways are actually highly interconnected networks involved in decision making, in adaptation and ultimately in ensuring survival. Redundancy of signaling pathways, for example, in QS systems, is possibly based on social evolution [16]. Furthermore, signaling pathways play a critical role in the complex regulatory circuits that control cellular

behavior not only because they integrate multiple stimuli but also because they are involved in generating phenotypic heterogeneity, which is our topic in this review.

Several bacterial multi-input signaling cascades that control various important functions have been identified and are well characterized, for example: the LadS/GacS/RetS virulence signaling complex in *Pseudomonas* [17], the KinA/KinB/KinC/KinD/KinE dependent sporulation cascade in *Bacillus* [18–20], the LuxN/LuxQ/CqsS QS complex in *V. harveyi* [21], the LuxQ/CqsS, VqmA QS network in *Vibrio cholerae* [22,23], the bacitracin stress response network (BceA/BceB and BceS) in *Bacillus* [24], the pyruvate-responsive BtsS/YpdA sensing network in *Escherichia coli* [25], the envelope stress-sensing RcsC/RcsB, IgaA network in *Escherichia* and *Salmonella* [26], and the chemotaxis receptor array in *Escherichia* and other genera [27].

Here we discuss how the design of multi-input signaling cascades correlates with phenotypic heterogeneity. We will first focus on the three-input histidine kinase (HK)-based QS cascade in *V. harveyi* in correlation with phenotypic heterogeneity. This signaling cascade is also found in many other *Vibrio* species, for example, in *V. cholerae,* and is responsible for the regulation of not only biofilm formation and virulence, but also bioluminescence in case of *V. harveyi*. The term QS describes an intra- and interspecific cell–to–cell communication process for sensing population densities. In this process, bacteria produce, release, and detect small QS molecules, the so-called autoinducers (AIs) that set the stage for group behavior resulting in coordination and synchronization of distinct phenotypes within a population. As described below, we found that the design of this signaling cascade with three bifunctional HKs and a small RNA (sRNA)-regulated switch is inherently capable of causing phenotypic heterogeneity. The output of this QS cascade can be homogeneous, but only when all three QS molecules are found in sufficiently high concentration in the immediate environment of the bacteria [28].

In the second part, we turn our attention to the HKs pyruvate-sensing BtsS/YpdA network in *E. coli* [25,29]. BtsS (formerly YehU) and YpdA belong to the LytS family of HKs. We discuss how bacterial populations deal with nutrient limitation and employ signaling in combination with individual cellular needs and responses to balance the physiological state of the entire population. This endows the population with a higher metabolic capacity and a lower percentage of spontaneous persister cells [15]. This pyruvate-sensing network is also found in other members of the *Enterobacteriaceae* including many pathogens, for example, *Klebsiella*, *Cronobacter* and *Citrobacter* [30].

Focus on these two signaling networks lets us discuss and compare important design principles of nature that generate phenotypic heterogeneity.

The QS cascade of *V. harveyi* comprises three parallel bifunctional HKs and an sRNA-regulated switch for generating phenotypic heterogeneity

The bioluminescent marine bacterium *V. harveyi* uses a complex signal transduction cascade for regulation of QS-dependent behaviors. Three different AI signals are integrated into one QS cascade. This cascade comprises a phosphorelay involving three hybrid sensor kinases, the histidine transfer protein LuxU and the δ^{54}-dependent transcriptional activator LuxO, an Hfq/sRNAs switch, and the transcriptional regulator LuxR (Fig. 1). *V. harveyi* produces the three AI molecules AI-2 (autoinducer 2, a furanosyl borate diester), CAI-1 ([(*Z*)-3-aminoundec-2-en-4-one, Ea-C_8-CAI-1]) and HAI-1 (*N*-3-(hydroxybutyryl)-homoserine lactone). These three AIs are unevenly distributed in the bacterial world: AI-2 use is widespread and is thought to be an interspecies signaling molecule [31]; CAI-1 is produced by all *Vibrio* spp. albeit with different acyl chain lengths and modifications [32], and HAI-1 is largely specific to *V. harveyi* and its close relatives [33].

When the AI concentration reaches a certain threshold, QS is triggered, and cells produce a phenotypic answer by activating genes for traits such as luminescence [34], biofilm formation [4] or proteolysis [35], gene repression for type III secretion [21] and siderophore production [36]. Each of the three AIs is perceived by a specific membrane-integrated hybrid sensor kinase. AI-2 is sensed by LuxQ in combination with the periplasmic binding-protein LuxP, CAI-1 is sensed by CqsS, and HAI-1 is sensed by LuxN (Fig. 1). Upon sensing these AIs, the hybrid sensor kinases channel the information into a shared regulatory pathway [21,37–39]. At low cell densities and therefore low AI concentrations, the respective sensors act as kinases. Each sensor autophosphorylates, subsequently transferring the phosphoryl group to the histidine phosphotransfer protein LuxU, which in turn phosphorylates the transcriptional regulator LuxO [40]. Phosphorylated LuxO is activated and, together with σ^{54}, induces the expression of five Qrr regulatory sRNAs [36,41]. These Qrrs in association with the RNA chaperone Hfq act to destabilize and degrade *luxR* transcripts, maintaining the QS phenotypes in an OFF state [42]. In addition, expression of the second master regulator AphA in this cascade is induced at low cell densities and acts as a counterpart to LuxR [43,44]. At high cell densities and upon perception of their cognate AI, the kinase activity of the sensors is inhibited resulting in the dephosphorylation of LuxU and draining of the phosphate from the phosphorelay cascade [45]. Non-phosphorylated LuxO is inactive, and no Qrr sRNA is transcribed, starting the production of LuxR, the QS master regulator. LuxR then induces the genes necessary for the expression of the different phenotypes (Fig. 1).

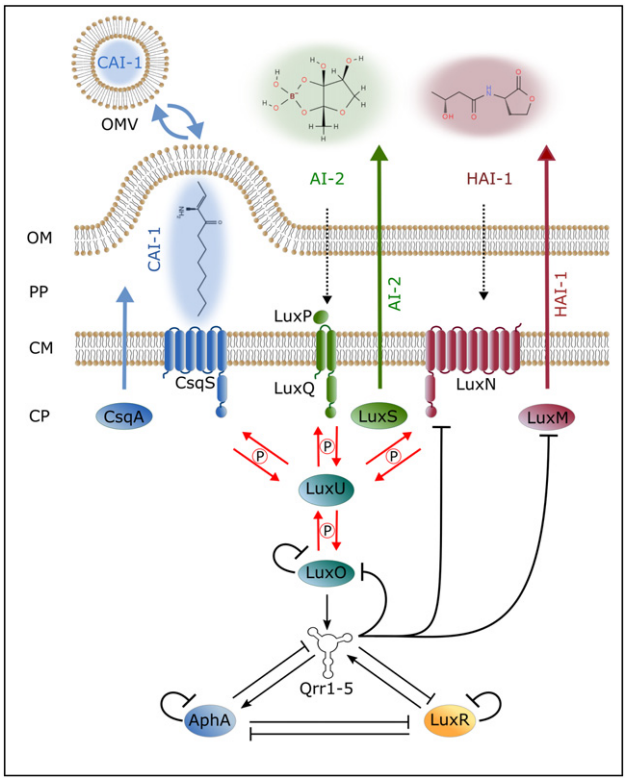

Fig. 1. The QS cascade of *V. harveyi.* The three AIs—HAI-1, AI-2 and CAI-1—are synthesized by the synthases LuxM, LuxS and CqsA, respectively. They are detected by the hybrid HKs (sensors) LuxN and LuxQ (in interplay with LuxP) and CqsS in the periplasmic space. As the AI concentration increases, kinase activity of the sensors decreases, while phosphatase activity increases (coral-colored arrows). Phosphorylated sensors transfer the phosphoryl group to LuxU and then to LuxO. Phosphorylated LuxO activates transcription of five regulatory sRNAs, Qrr1–Qrr5, which destabilize the transcript of the master regulator LuxR. In contrast to LuxR, AphA is induced at low cell density and acts as the counterpart of LuxR. Regulation also includes several feedback loops (black solid lines). Dashed lines indicate diffusion of AIs into the periplasm; red and green solid arrows indicate facilitation of HAI-1 and AI-2 into the environment; the blue solid arrows indicate release and perception of CAI-1 in outer membrane vesicles (OMVs); CM, cytoplasmic membrane; CP, cytoplasm; PP, periplasm; and OM, outer membrane.

In addition, several feedback loops influence the number of molecules of the different components of the QS signaling cascade: LuxR represses its own expression by functioning as an autorepressor and induces the expression of Qrr2–Qrr4 [44,46,47], LuxO represses its own expression, *luxO* mRNA is targeted by the Qrr sRNAs [44,48] and *luxMN* translation is also prevented by the Qrr sRNAs [44,49]. The Qrr sRNAs repress the translation of LuxR and activate the translation of AphA. AphA feeds back to repress the expression of the *qrr* genes and *luxR* and its own expression [43,44,50]. Overall, the Qrr sRNAs play a key role in both the activation and repression of the QS phenotypes in *V. harveyi*.

Activities of the bifunctional kinases in conjunction with a tightly regulated copy number of LuxR determine the degree of heterogeneity of the QS cascade

Because the HKs of the QS cascade are bifunctional sensors comprising kinase and phosphatase activity, the activity of the QS cascade at the population level and the single-cell level was investigated focusing on signal integration and noise levels [28]. The ratios of kinase to phosphatase activities of the three sensors and hence the extent of phosphorylation of LuxU/LuxO are important not only for the signaling output but also for the degree of noise in the system. Moreover, out of the three HKs of the QS system, LuxN has the highest capacity for phosphorylating LuxU, while the phosphatase activity was comparable to LuxQ and CqsS *in vitro* [51].

In the presence of the three AIs, all sensors switch into the phosphatase mode and the population responds homogeneously (Fig. 2, right panel). However, in the absence of one or two AIs, some sensors are in the kinase mode, while others are in the phosphatase mode. This prevents full activation of the QS cascade leading to a highly heterogeneous answer at the single-cell level with cell-to-cell standard deviation reaching 44% of the mean using a P_{luxC}-*mCherry* reporter fusion as readout for luminescence production (Fig. 2, middle panel). A low AI concentration also leads to homogeneous but low QS cascade activity (Fig. 2, left panel) [28].

Moreover, not only are the availability and concentration of AIs critical for heterogeneous activation of the QS in *V. harveyi*, but so is the molecule number of the LuxR protein. The pool of phosphorylated LuxO per cell directly determines the amount of Qrr sRNAs (Fig. 1). A high number of Qrr sRNAs in association with the RNA chaperone Hfq destabilize and degrade *luxR* transcripts, keeping the QS phenotypes in an OFF state. In the presence of all AIs, LuxO remains in a non-phosphorylated state, no Qrr sRNAs are transcribed, the QS master regulator LuxR is produced, and the QS phenotypes are in an ON state. It suggests that tight regulation like this is strongly dependent on the amount of sRNAs and hence is determined by the ratio of kinase/phosphatase activities in the phosphorelay (Fig. 2) [28]. In summary, the ratio of the different modes of the bifunctional HKs in combination with a tightly regulated copy number of LuxR determines the degree to which the QS cascade is heterogeneously activated in a *V. harveyi* population.

One pathogenicity mechanism of *V. harveyi* is the capacity to attach to surfaces and then to induce the formation of biofilms at high cell densities [52].

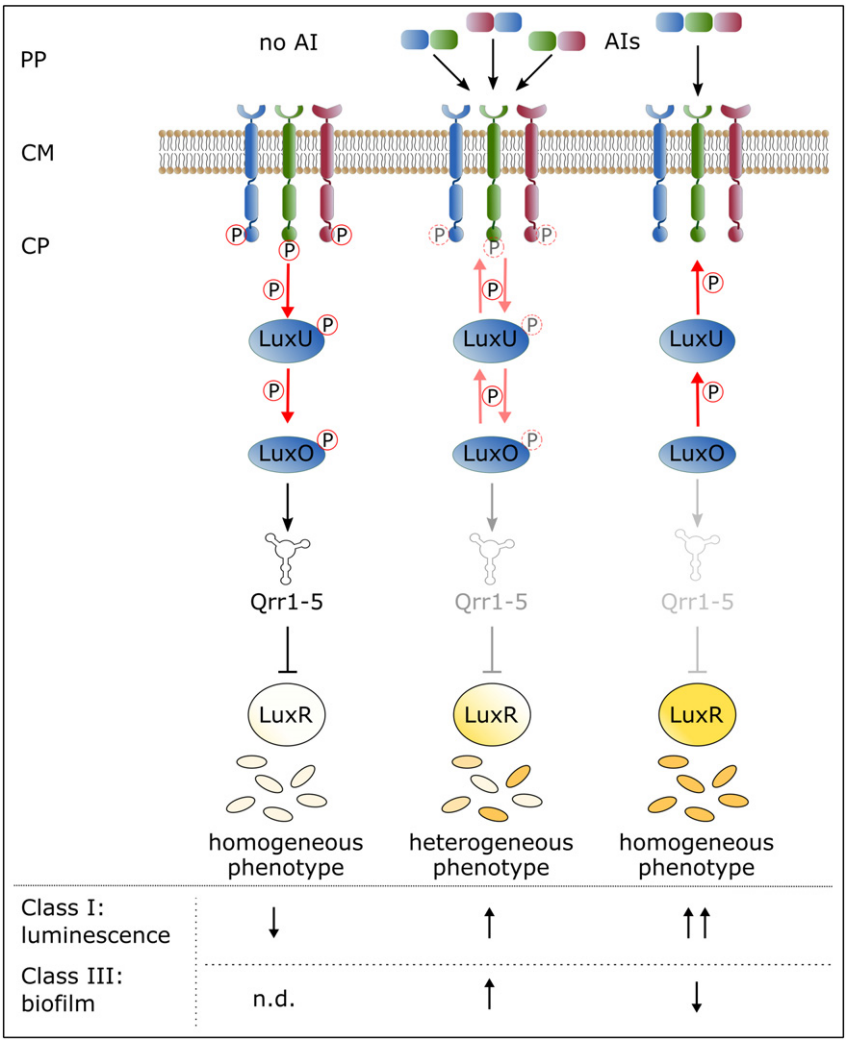

Fig. 2. Impact of different AI concentrations on QS-mediated phenotypic heterogeneity. In the absence of AIs, when all sensors are in the kinase mode, they autophosphorylate and transfer the phosphoryl group via LuxU to LuxO, leading to the expression of Qrr sRNAs and the degradation of *luxR* mRNA. This produces a population that is homogeneously in a QS OFF state (left panel). At high AI concentrations, all sensors are in the phosphatase mode and LuxU gets dephosphorylated. LuxR can be produced and the population is homogeneously in a QS ON state (right panel). Either one AI or a combination of two AIs cause a state in which some sensors are in the kinase mode and some in the phosphatase mode leading to high cell-to-cell variations (heterogeneous phenotype, middle panel). The master regulator LuxR controls the expression of different phenotypic traits such as luminescence (class I genes) and biofilm formation (class III genes). CM, cytoplasmic membrane; CP, cytoplasm; PP, periplasm; n.d., not determined.

When compared to a constitutive QS-active mutant (Δ*luxO*), wild-type cells showed a tendency to form aggregates in the stationary phase of a liquid culture. This phenomenon led our group to closely study biofilm formation. After 14 h of growth, a switch to biofilm formation was observed with wild-type cells and Δ*luxO* cells. Overall, the production of biofilms was significantly reduced in the constitutive QS-active mutant Δ*luxO*. This strain, however, exhibited a higher bioluminescence. The ability of wild type to form biofilm diminished consistently when the culture was grown in the presence of an artificially high concentration of all three AIs. In this state, heterogeneity decreased (Fig. 2), demonstrating that a heterogeneous population produces more biofilm than a homogeneous one [4].

Natural variations in the availability of the three AIs during growth generate phenotypic heterogeneity

Although the three hybrid kinases channel information into one shared regulatory pathway, we showed above that different availabilities of their cognate AIs generate heterogeneous activity of the QS cascade and result in heterogeneous phenotypes. Analyzing the temporal presence of the three AIs revealed that there is no growth phase when all three are present at maximum concentration. The exponential growth phase is characterized by an increase in AI-2 and the induction of bioluminescence. Subsequently, HAI-1 is produced, while CAI-1 reaches its maximum upon entry into stationary phase (Fig. 3) [53]. The external AI-2 concentration decreases in stationary phase; however, to date no transport system has been identified in *V. harveyi*. In *E. coli* and *S. enterica* serovar Typhimurium, AI-2 is rapidly taken up upon entry of cells into stationary phase by a specific AI-2 importer belonging to the ABC-transporter family [54,55]. For the *V. harveyi* system, an AI-2 uptake mechanism is predicted by analyzing the design of QS cascades, which our group verified experimentally [56]. The concentration of HAI-1 seems to be adjusted by metabolism (through the availability of *S*-adenosyl methionine) rather than acyl-homoserine lactone

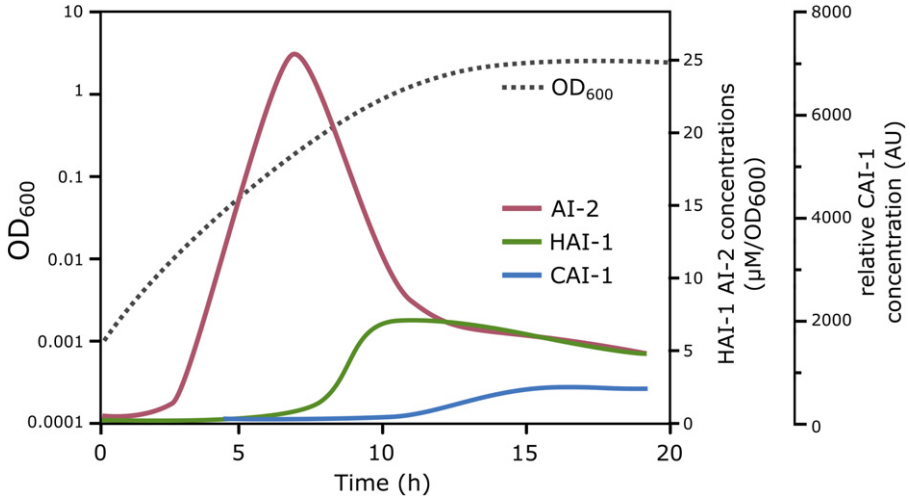

Fig. 3. Schematic diagram on the fluctuation of the level of the three AIs over a typical growth curve of *V. harveyi*. Data taken from Ref. [53].

degrading enzymes [31,53]. Ultimately, CAI-1 is produced upon transition of cells into stationary phase. However, this transition point is not reached at equal cell densities but instead influenced by the nutrient availability in the medium. Thus, a nutrient-rich medium will increase CAI-1 production [21,57]. Overall, a *V. harveyi* population displays distinct AI profiles at different growth stages: the early exponential growth phase is marked by low AI-2, the mid-exponential growth phase by high AI-2, the late exponential and the early stationary phase by a mixture of AI-2 and HAI-1, and the later stationary phase by a combination of AI-2, HAI-1 and CAI-1. Due to these temporal variations of availability and concentration of the three AIs, there is no growth phase when all three are available at maximum concentration. Therefore, heterogeneity in QS regulated phenotypes in *V. harveyi* is the rule rather than the exception [3,53].

Besides these timing differences, the three AIs of *V. harveyi* possess distinct physiochemical properties that influence their spread and stability in aqueous media, for example, the ocean. HAI-1 and AI-2 supposedly cross the cell envelope via diffusion. In contrast, CAI-1 is highly lipophilic and cannot diffuse across the polar lipopolysaccharide layer on the outside of the cell [57,58]. Recently, we could show that CAI-1 is delivered between *Vibrio* cells via outer-membrane vesicles. This stabilizes CAI-1 in an aqueous environment and facilitates its crossing the polar lipopolysaccharide layer of both the producing and targeted cells [57].

In summary, phenotypic heterogeneity through the QS cascade of *V. harveyi* is generated by two design principles: (i) the design of the signaling cascade with three bifunctional HKs and a tightly regulated copy number of the master regulator LuxR, and (ii) variations in the availability of the three AIs during growth.

The pyruvate-sensing network and phenotypic heterogeneity in *E. coli*

E. coli is a ubiquitous bacterium primarily found in the intestinal tract of warm-blooded animals, but also in water, sediment and soil. To monitor environmental changes and adapt its physiology, *E. coli* harbors a multitude of two-component systems (TCSs), composed of a membrane-integrated HK for sensing extracellular signals and a cognate response regulator (RR) for altering gene expression accordingly. In previous works, we characterized two closely related HK/(RR) systems BtsS/BtsR and YpdA/YpdB that both responded to extracellular pyruvate. Each of these systems controls the expression of a transporter [59,60].

Two interconnected pyruvate-sensing kinases that control transporters

The YpdA/YpdB and the BtsS/BtsR TCSs, characterized as low and high affinity pyruvate sensor systems respectively, belong to the LytS/LytTR family. Of the two HKs, YpdA responds to extracellular pyruvate (> 600 μM) and activates its cognate RR YpdB, while BtsS detects extracellular pyruvate concentrations as low as 50 μM and activates the RR BtsR [29]. Both kinases possess a N-terminal Lyt domain with at least five transmembrane helices linked to a cytosolic region comprising the GAF and kinase (DHp and CA)

domains [59,60]. While the role of this GAF domain remains unknown, these domains have been shown as being involved in protein–protein interactions and able to bind ligands such as formate, 2-oxoglutarate, cGMP and aromatic compounds [61–63]. The RRs consist of a CheY-like receiver domain, with a conserved aspartate at position 53 connected to a LytTR-type DNA-binding (effector) domain. BtsS/BtsR and YpdA/YpdB of *E. coli* exhibit remarkable similarity. It should be noted that BtsS/BtsR is the predominant LytS/LytTR-type HK/RR system among γ-proteobacteria, whereas YpdA/YpdB primarily appears in a supplementary role and is missing in many bacteria [30].

The gene *yhjX* is the major target of YpdB and codes for a putative transport protein of the major facilitator superfamily. YhjX is predicted to have 12 transmembrane regions and is produced whenever cells are cultivated in media containing a minimum concentration of 600 μM pyruvate [60]. The gene *btsT* is the sole target of BtsR identified so far. It encodes the membrane protein BtsT, a member of the CstA transporter family, predicted to contain 18 transmembrane domains. In *E. coli, btsT* expression is induced in the presence of pyruvate and under nutrient limitation. BtsT was recently identified as pyruvate/H^+ symporter [59,64]. *btsT* expression is controlled not only by BtsR but also by cAMP-CRP to release catabolite repression and the LysR-type transcriptional repressor LeuO. Moreover, the carbon storage regulator A (CsrA) post-transcriptionally inhibits BtsT synthesis while increasing YhjX synthesis. Finally, both *btsT* and *yhjX* mRNAs are stabilized by the ribosomal protein L4, which protects against RNase E-dependent cleavage (Fig. 4) [25,59].

When *E. coli* is grown in an amino acid-rich medium containing tryptone or casamino acids, both TCS are activated in the post-exponential growth phase and are functionally interconnected. Deleting either TCS component or its target gene influences the level of expression of the target gene regulated by the other TCS and vice versa. The YpdA/YpdB system promotes BtsS/BtsR-mediated induction of *btsT*, while the BtsS/BtsR system has the opposite effect on *yhjX* expression. Remarkably, the deletion of the corresponding transporter genes exerted also feedback regulation on the signaling systems [25,65]. In addition, *in vivo* protein–protein interaction assays suggested that each HK can interact with both transporters forming a single, large signaling unit [25]. This interconnection is reinforced by both systems sharing the same stimulus and both target genes being post-transcriptionally regulated by L4-RNaseE interaction and by CsrA. The interlinked response of the two signaling systems appears to be important for nutrient selection at a stage in the growth phase when carbon becomes a growth limiting factor [25].

Heterogeneous expression of the TCS-regulated transporters influences the metabolic state of cells

Clonal populations showed heterogeneous activation of both promoters (P_{btsT} and P_{yhjX}) when the cells grew in amino acid-rich media; however, expression of *btsT* was more variable than that of *yhjX* [15]. Single-cell analysis showed that when cells reached the end of the exponential growth phase, the majority of the population expressed *yhjX*, with a high degree of cell-to-cell variability. In these experiments, less than 4% of the population did not respond to pyruvate. YpdA/YpdB-mediated activation of P_{yhjX} is dependent on the concentration of pyruvate in the medium and became more homogenous when cells were grown in minimal medium containing high concentration pyruvate (20 mM) as the sole carbon source.

At the single-cell level, BtsS/BtsR-mediated activation of *btsT* was characterized by high cell-to-cell variability, which was virtually unaffected by the pyruvate concentration in the medium. It was shown as well that this activation is not only dependent on the external pyruvate concentration but also influenced by internal nutrient limitation, where the high degree of heterogeneity might reflect variations in the nutritional state of individual cells [15].

Based on a single-cell analysis, our group proposed a model to explain the heterogeneity observed. During growth in amino acid-rich media, cells will excrete pyruvate due to overflow metabolism. Subsequently, cells can detect external pyruvate, and, depending on its concentration and their particular nutritional needs, individual *E. coli* cells will activate either the high-affinity BtsS/BtsR or both the high- and the low-affinity (YpdA/YpdB) systems upon entry into the post-exponential growth phase. An interplay between transporters with different affinities for the same substrate has already been described, and this seems to be a successful strategy under nutrient limitation [66]. The multiple factors known to affect the expression of *yhjX* and *btsT* could explain the heterogeneity observed. These factors include, among others, the binding of the RR to the target, the regulation through CsrA, RNase E and the catabolite repression, which may vary from cell to cell.

To better understand the role of the pyruvate-sensing network in the physiological state of *E. coli* during post-exponential growth phase, the rate of ribosome synthesis was analyzed [15]. Activation of the *rrnB* P1 promoter, controlling 16S ribosomal RNA synthesis, correlates with intracellular ATP levels and is characteristic of growing cells. Wild-type cells showed a unimodal distribution of cells with high ribosomal synthesis, indicating a uniform growth of all cells. On the other hand, in a mutant lacking the network, the population showed a bimodal distribution, suggesting that the population differentiates into two subpopulations, one with normal and the other with reduced ATP levels. This

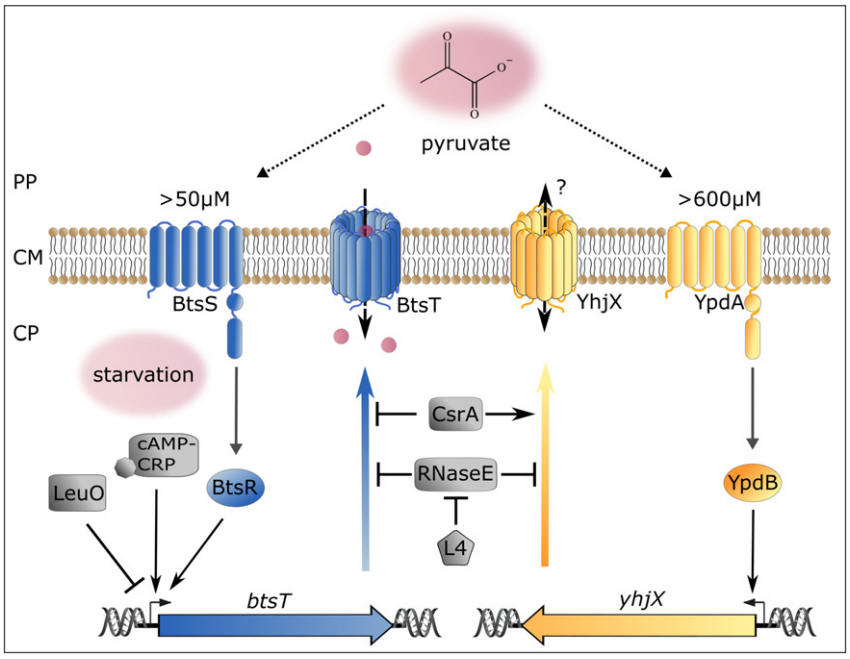

Fig. 4. Model of the pyruvate-sensing network in *E. coli.* The BtsS/BtsR and YpdA/YpdB HK/RR systems respond to pyruvate, albeit with different affinities. YpdB regulates expression of *yhjX* coding for a transporter of unknown function, while BtsR activates *btsT* transcription, coding for a pyruvate/H^+ symporter. *btsT* expression is influenced by the nutritional state, but also controlled by LeuO and activated by the cAMP-CRP complex. Finally, transcript levels of *btsT* and *yhjX* are influenced by CsrA and the L4-RNase E interaction. "?" indicates transport of an unknown substrate. CM, cytoplasmic membrane; CP, cytoplasm; PP, periplasm.

indicates that heterogeneous activation of either P_{yhjX} or P_{btsT} in individual wild-type cells allows uptake of nutrients, for example, pyruvate, according to the individual requirement of the cells.

In addition, when *E. coli* cells were forced to overproduce a cytosolic, or a membrane-integrated, or a periplasmic protein (under the control of an inducible promoter), all wild-type cells were able to do so, while only half of the mutant cells lacking the sensing network overproduced these proteins. This result indicates that only half of the population was sufficiently fit to take the metabolic burden of protein overproduction, which correlates with the ribosomal synthesis pattern. This suggests that BtsS/BtsR, together with YpdA/YpdB, equalizes the physiological state of all cells within the population by uptake of nutrients. In agreement, the frequency of stochastically formed antibiotic-tolerant persisters increased significantly in the mutant [15].

Recently, it could be shown that the pyruvate-sensing network also plays a crucial role in the resuscitation of viable but non-culturable (VBNC) cells. The VBNC state is a deep dormancy in which bacteria retain viability, exhibit a low metabolic activity, and maintain membrane integrity but cannot be cultivated on conventional culture media [67]. Cells usually enter the VBNC state as a survival strategy when facing starvation, long exposure to low temperatures, presence of reactive oxygen species or other natural stresses [68]. Due to their low metabolism, VBNC cells are also less sensitive to antibiotic treatment, which can be problematic when pathogens are involved. These dormant cells can, however, resume cell division and regain culturability. This so-called resuscitation phenomenon remains poorly understood, although several promoting factors have been identified [69]. Interestingly, pyruvate is one of these factors due to its reactive oxygen species scavenger property [70], and it appears that the BtsSR/YpdAB pyruvate-sensing network is also involved in resuscitation. Notably, BtsT is more abundant in VBNC cells than in exponentially growing cells [71]. Moreover, pyruvate was rapidly taken up, suggesting that it is used during the early stage of resuscitation. Interestingly, single-cell analysis showed that about 20% of the VBNC cells resumed cell division, while the rest remained unculturable consistent with the heterogeneous expression of *btsT* [71] (Fig. 5).

Sensing of external pyruvate by the BtsS/BtsR and YpdA/YpdB systems and the tightly regulated synthesis of the two transporters BtsT and YhjX, depending on the needs of the individual cell, ensures that the physiological state within the whole population is optimized to withstand upcoming metabolic stress. These findings are important not only for understanding the host colonization by pathogenic species and their persistence but also for metabolic engineering. Finally, the effects of the pyruvate-sensing network on VBNCs resuscitation, on formation of persisters, and on protein overproduction highlight its relevance for bacterial metabolism and fitness under challenging conditions. A high cell-to-cell variability was observed for all these phenotypes. This heterogeneity could serve a bet-hedging strategy, in which a subset of the cells (persisters) exhibits low metabolic activity that promotes survival under starvation and another, metabolically active subset that facilitates growth (resuscitation) under more favorable conditions.

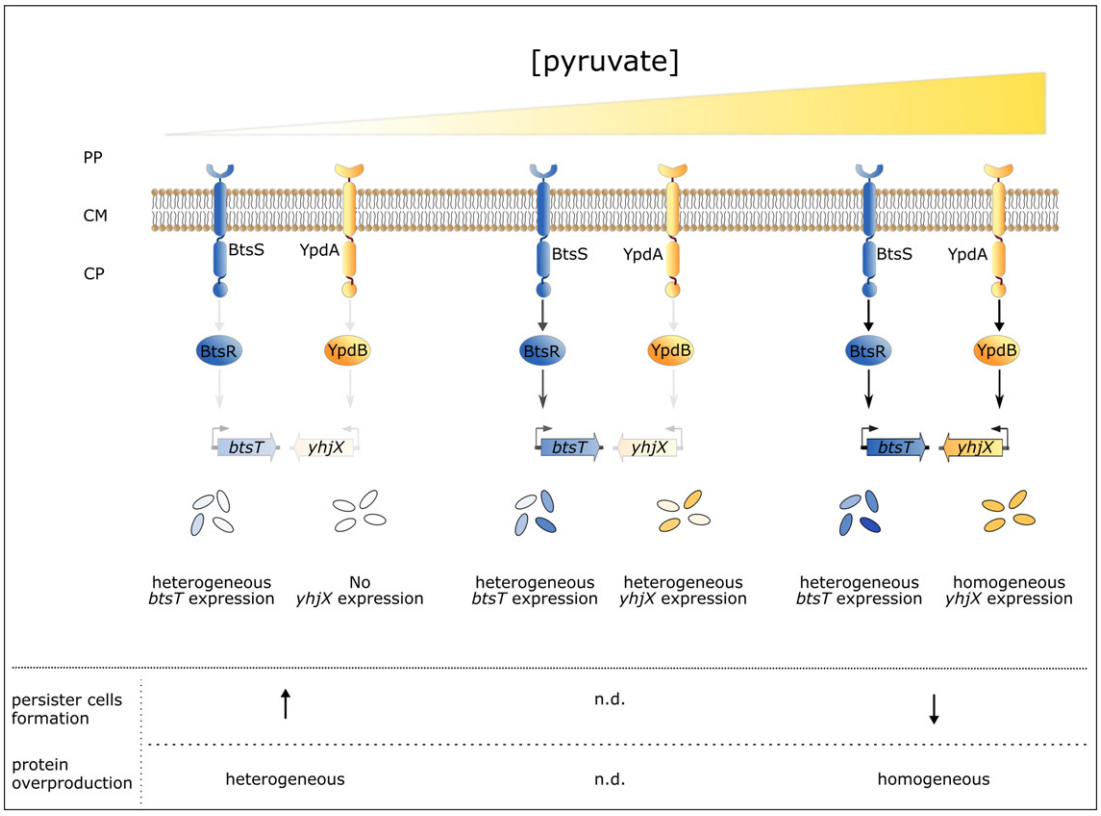

Fig. 5. Impact of the network on physiology and phenotypic heterogeneity. At low concentrations of pyruvate, only BtsS/BtsR is active and *btsT* is expressed at low levels in only a few cells. Under these conditions, the percentage of persisters increases and cells are less likely to overcome metabolic burden such as protein overproduction. With high concentration of pyruvate, YpdA/YpdB is also activated and both target genes are expressed, but the degree of expression varies from cell to cell. Cells are more metabolically active, persister formation is decreased and VBNC cells are poised to resuscitate. CM, cytoplasmic membrane; CP, cytoplasm; PP, periplasm; n.d., not determined.

Comparison of the two signaling systems

The QS network of *V. harveyi* and the pyruvate-sensing system of *E. coli* both use multiple HK-based signaling systems to control heterogeneous phenotypic behavior. The QS network of *V. harveyi* integrates three different signaling molecules via the HKs LuxN, LuxPQ and CqsS into the same signaling cascade, for example, to activate genes for traits such as luminescence or biofilm formation [4,34]. In contrast, the two HKs BtsS and YpdA of the pyruvate-sensing system of *E. coli* respond only to the central metabolite pyruvate, although with different affinities, resulting in a heterogeneous activation of their target genes [15].

Upon perceiving extracellular signal(s), the QS network of *V. harveyi* and the pyruvate-sensing system of *E. coli* transduce the signals resulting in an intracellular response, specifically activation of target gene(s) expression. Signal transduction in the QS cascade of *V. harveyi* relies on a phosphorelay involving the three hybrid sensor kinases, the histidine transfer protein LuxU and the LuxO regulator [21]. In contrast, the LytS-type HKs BtsS and YdpA are characterized by an incomplete G-box and unusual N- and F-boxes in their kinase domains [59,72], which almost prevent autophosphorylation (unpublished information). In this case, the interaction strength between the HKs and their cognitive RRs or with their target gene products, BtsT and YhjX, is exceptionally strong [25].

Both of these HK-based signaling networks use post-transcriptional regulation to establish phenotypic heterogeneity. The QS network of *V. harveyi* involves the Qrr sRNA switches together with Hfq to destabilize and degrade *luxR* transcripts, thereby tightly controlling the copy number of the master regulator LuxR [41,42]. The target gene transcripts of the *E. coli* pyruvate-sensing system are post-transcriptionally regulated by L4-RNaseE interaction and by CsrA [25].

Perspectives

Bacteria are able to communicate within a population, not only with other bacterial species but

also with eukaryotes via low-molecular signaling compounds in the form of AIs and primary or secondary metabolites (e.g., antibiotics, pigments and siderophores) [73–75]. In the process, bacteria rely on sophisticated signaling networks to receive various chemical stimuli and to integrate the information into an intracellular signal that generates an appropriate response. Here, we describe two examples of how these signaling processes can be used to generate phenotypic heterogeneity for division of labor and/or as a bet-hedging strategy. These studies indicate that the potential for a heterogeneous output is already designed into these signaling cascades. Both extrinsic factors, for example, the concentration of one chemical stimulus or a combination of several stimuli, and intrinsic factors are crucial for decision making within an individual cell. The most important among the latter are as follows: (i) more than one receptor channels the information into the signaling cascade, (ii) high- and low-affinity receptors are combined, (iii) the copy number of the subsequent cytosolic signaling components is low, and (iv) there are additional check-points at the post-transcriptional level. Neither of the signaling cascades contain positive-feed forward loops, which are a major driving force of phenotypic heterogeneity, for example, in competence and sporulation in *Bacillus subtilis* [76].

While we have now insights into the design of signaling cascades and the contribution of their receptors, the impact of post-transcriptional regulation for phenotypic heterogeneity is far from clear because of the involvement of global regulators like the carbon storage regulator CsrA or RNA chaperone Hfq. The impact of global regulators on bi- and heterostability still needs to be explored to gain a better understanding of the complete signaling network of bacteria.

Beyond this future research direction, the focus should increasingly turn to two important practical questions: (i) What role does phenotypic heterogeneity play in complex ecosystems, for example, the human microbiota, and (ii) to what extent can phenotypic phenomena be artificially manipulated in natural ecosystems? Phenotypic heterogeneity is fascinating from a biological point of view but adds on the already high complexity of natural ecosystems. Moreover, the formation of subpopulations consisting of growing and non-growing/dormant phenotypes is an important medical problem about which almost nothing is known, either in corresponding ecological niches or in the microscale gradients of biofilms [77]. Finally, randomness and unpredictability in biotech or biomedical environments are undesirable because they significantly affect product yield or treatment efficiency. It should be stressed that the described phenomena are of general importance in biology. For example, it is known that tumor heterogeneity contributes to drug resistance [78] and that eukaryotic cell-to-cell variabilities arise from changes in microecological conditions, signaling networks and intrinsic stochastic fluctuations [79].

In conclusion, further experimental and theoretical approaches, combined with research in synthetic molecular biology, are needed to unravel the complex relationships between environmental influences, signaling networks and phenotypic properties of individual cells.

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Abbreviations used:
QSquorum sensing; AIautoinducer; sRNAsmall RNA; TCStwo-component system; HKhistidine kinase; CsrAcarbon storage regulator A; VBNCviable but non-culturable; RRresponse regulator

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