Bacterial Cell-to-cell Communication (Quorum Sensing)

Michael Givskov[1], Thomas Bovbjerg Rasmussen[2], Dacheng Ren[3], Naomi Balaban[4]

Abstract Within the biofilm, the bacteria use cell-to-cell communication systems to pool their activities and act in a multicellular organized manner. One such activity is to launch their arsenal of virulence factors at the strategically right moment, and hence coordinate the progressive attack on the host. This process is termed quorum sensing (QS), whereby bacteria produce diffusible chemical signals (autoinducers) that interact with specific receptors on itself and on neighboring cells, which in turn regulate the expression of specific target genes. By integrating this with other environmental stimuli, bacteria are capable of exhibiting complex responses and take part in sophisticated interactions, allowing them to survive in most adverse environments. This chapter describes the molecular mechanisms of QS in Gram-negative and gram positive bacteria, and QS in a biofilm, leading to what is described in subsequent chapters that QS is a highly attractive target for therapy against biofilm chronic infections.

Well within the biofilm, the bacteria use cell-to-cell communication systems to pool their activities and act in a multicellular organized manner. One such activity is to launch their arsenal of virulence factors at the strategically right moment and hence coordinate the progressive attack on the host. The view of bacterial biofilms as sanc-

Michael Givskov (✉)
Technical University of Denmark, BioCentrum-DTU, BioScience and Technologie Bldg 227, DK-2800 Kgs Lyngby, Denmark, e-mail: immg@pop.dtu.dk
Thomas Bovbjerg Rasmussen
Chr. Hansen A/S, Innovation, Cultures & Enzymes Division, Bøge Allé 10-12, DK-2970 Hørsholm, Denmark
Dacheng Ren
Syracuse University, Department of Biomedical and Chemical Engineering, Syracuse, NY, USA
Naomi Balaban
Tufts University, Cummings School of Veterinary Medicine, Department of Biomedical Sciences, 200 Westborow Rd, North Grafton, MA, 01536, USA

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tuaries in a hostile environment has gained momentum over the last decade. Bacteria released from these protected areas are then able to spark systemic infections (Costerton et al. 2003). The ability of coordinating gene expression in accordance with population density and hence to act as a group is a process termed **quorum sensing** (QS) (Fuqua et al. 1994; Withers et al. 2001) (see Fig. 1). The amount of bacteria needed to activate QS-controlled genes are known as the "quorum" or the "quorum size," reflecting the number of individual cells needed to make a qualified decision.

The underlying mechanism of QS is the production of diffusible chemical signal molecules by the bacteria (autoinducers) that interact with specific receptors on self and on neighboring cells, which in turn regulate expression of specific target genes. By integrating this with other environmental signals and stimuli, bacteria are capable of exhibiting complex responses and taking part in sophisticated interactions (Gray 1997).

QS is implicated in the regulation of phenotypes that are also involved in interactions with higher organisms. These interactions can be beneficial to the host, or they can be pathogenic. From an evolutionary point of view, it makes sense that the underlying factors are produced only when the bacterial population is sufficiently large to confer a significant effect (Velicer 2003). QS systems form the command line of opportunistic pathogens such as *P. aeruginosa* and *S. aureus* (Winzer and Williams 2001). Expressing the battery of antigenic determinants such as host-damaging virulence factors only when the bacterial population has reached a high level is believed to be a "stealthy strategy"; by the time the host organism realizes it is under attack, it has been left with a poor possibility of mounting an effective defense against the intruder (Donabedian 2003; de Kievit and Iglewski 2000; Parsek and Greenberg 2000).

To date, several types of QS systems are known: one for gram-positive bacteria relying on polypeptides (Abraham 2006; Balaban et al. 1998; Waters and Bassler 2005) and another for gram-negative bacteria mediated by *N*-acyl homoserine lactone (AHL) derivatives (Eberhard et al. 1981; Nasser and Reverchon 2007). A third type of QS system, AI-2, has been proposed as a global signaling system common to all bacteria (Winans and Bassler 2002; Waters and Bassler 2005).

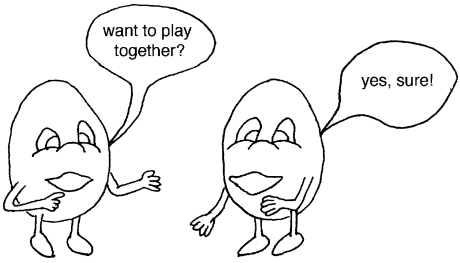

Fig. 1 Bacterial cell-to-cell communication (quorum sensing) (Illustration by Mike Beshiri, Tufts University, Cummings School of Veterinary Medicine, Department of Biomedical Sciences, Division of Infectious Diseases, North Grafton, MA, USA)

1 AHL-mediated Quorum Sensing in Gram-negative Bacteria[1–4]

The core elements of all AHL-based QS systems are a gene termed the *luxI* homolog encoding an AHL synthetase and a *luxR* homolog encoding the signal receptor protein, which also acts as a response regulator. At low cell densities, the *luxI* homolog is expressed constitutively at a low level; hence, AHLs are synthesized in small quantities, which slowly accumulate in the environment around the bacteria, depending on the diffusion restraints (Fuqua and Greenberg 2002). Accordingly, there is a correlation between AHL concentration and population density – more cells results in more signal molecules per volume. Work on the *Vibrio fischeri* QS system has given rise to a "QS dogma," which states that when a sufficient population density has been attained, QS target genes become activated. The signal molecules bind to the LuxR homolog receptor proteins, inducing a conformational change and allowing the proteins to form dimers or multimers. This in turn enables the receptor multimer to bind to DNA and act as a transcriptional regulator. The activity of a QS-controlled gene is determined by the concentration of activated LuxR homolog multimer, which in turn is dependent on the concentration of AHL signal, which again is dependent on the population size/density. In other words, activation of QS-controlled genes relies on both the concentration of AHL signal molecules and the amount of available LuxR homolog receptor protein.

Gram-negative bacteria that are unable to synthesize signal molecules are still able to perceive and respond to the AHL signal molecules. These include *Salmonella typhimurium* and *Escherichia coli*, which both posses a gene, *sdiA*, that is a LuxR homolog. The SdiA receptor is responsive to 3-oxo-C6 and 3-oxo-C8 signal molecules, enabling the bacteria to sense the presence of other AHL-producing bacteria in a mixed community and to respond to the AHL signal molecules.

1.1 Multiple Quorum-sensing Systems Regulate Virulence

The model gram-negative bacterium *P. aeruginosa* produces two QS signals: *N*-(3-oxododecanoyl)-L-homoserine lactone [OdDHL, synthesized by LasI and sensed by LasR (Gambello and Iglewski 1991; Ochsner et al. 1994)] and *N*-butanoyl-L-homoserine lactone [BHL, synthesized by RhlI and sensed by RhlR (Pearson et al. 1994)]. With respect to function, they are organized in a hierarchical manner, the former controlling the expression of the latter. This allows for further fine-tuning of the responses of QS target genes. Sequence analysis suggests that these systems have been acquired independently rather than arising by endogenously gene duplication – the *las* system in *P. aeruginosa* shows no affiliation with the *rhl* system. This indicates that horizontal gene transfer is a mechanism for the spread of, and is important for the prevalence of, QS controllers.

1.2 AHL Signal Generation

The LuxI and homologs direct synthesis of the AHLs. The signal molecules consist of an invariable highly conserved lactone ring and a variable acyl side chain [Fig. 2a,b (AHL or AI-1)]. The side chains differ in length (2–18 carbon atoms), but all side chains contain a keto group on the C1 position and various degrees of substitution on C3. Molecules that carry a keto oxygen on the C3 carbon are referred

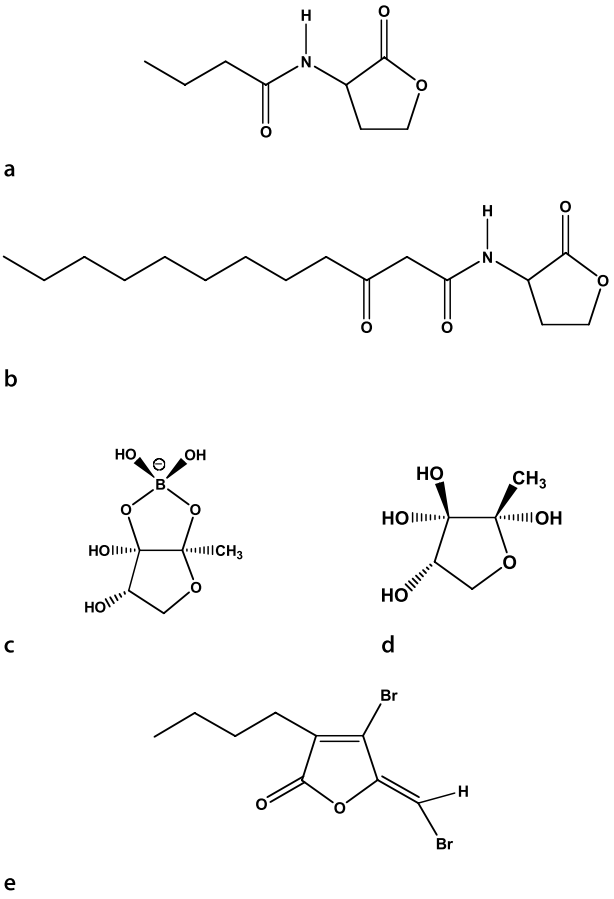

Fig. 2 Structures of representative quorum-sensing signals and brominated furanones. **a** *N*-butanoyl-L-homoserine lactone [encoded by *P. aeruginosa* RhlI (Pearson et al. 1994)). **b** *N*-(3-oxododecanoyl)-L-homoserine lactone [encoded by *P. aeruginosa* LasI (Gambello and Iglewski 1991; Ochsner et al. 1994)]. **c** (2S, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate [AI-2 of *V. harveyi* (Chen et al. 2002)]. **d** (2R, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran [AI-2 of *Salmonella typhinurium* (Miller et al. 2004)]. **e** (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone, natural furanone from *D. pulchra* (de Nys et al. 1993)

to as oxo-HSLs. A few rare AHL species contain a hydroxyl substitution on the C3 position, whereas others posses a double bond in the side chain.

The LuxI synthases use S-adenosylmethionine (SAM) as HSL donor and acyl-acyl carrier protein (acyl-ACP) or acyl-coenzyme A from the fatty acid synthesis complex as acyl donor (Parsek et al. 1999). The different side chains probably reflect variations in acyl-ACP specificity. Conversely, the specificity for SAM must be similar for all LuxI homologs as the HSL ring is invariable (Watson et al. 2002).

After synthesis, the signal molecules enter the surrounding environment, either by passive diffusion in the case of C4 HSL or active efflux in the case of 3-oxo-C12 HSL (Pearson et al. 1999; Waters and Bassler 2005).

1.3 Signal Reception and Response Regulation

Perception of the signal molecules and subsequent gene regulation are performed by the LuxR homologs. LuxR homolog proteins contain two functional domains, the AHL-binding N-terminal and a DNA-binding C-terminal (Hanzelka and Greenberg 1995; Egland and Greenberg 2001; Koch et al. 2005). In LuxR, it has been found that the C-terminal third part of the 250-residue protein has a helix-turn-helix motif able to bind to DNA and is able to activate LuxR-controlled genes independently of AHL (Choi and Greenberg 1991). This has led to the model that the N-terminal two-thirds of the protein quenches the DNA-binding activity. Another truncated LuxR, consisting of the N-terminal part, has been shown to bind AHL and in turn activates the C-terminal part (Hanzelka and Greenberg 1995). Activated LuxR homologs are thought to bind as dimers capable of interacting with the promoter regions of QS-controlled genes (Vannini et al. 2002; Zhang et al. 2002b; Ledgham et al. 2003). The *lux* box, where the dimer binds, is a palindromic sequence centered −42.5 bp upstream of the *luxI* start codon. The dimer overlaps the −35 region and acts as an ambidextrous activator of transcription (Egland and Greenberg 1999). Located at this position, the LuxR dimer interacts with the alpha-subunit C-terminal domain of RNA polymerase, where two residues of the subunit interact directly with the C-terminal part of LuxR (Finney et al. 2002; Johnson et al. 2003). Alanine mutation scanning of the N-termini of LuxR has provided evidence for a direct correlation between the binding of LuxR to the *lux* box and activation of QS-controlled target genes (Trott and Stevens 2001; Egland and Greenberg 2001). It appears that each LuxR homolog protein has its own *lux* box type of binding site, and similar binding sites have been identified for LasR and RhlR in *P. aeruginosa*. These *las* and *rhl* boxes are important for the expression of QS target genes such as *lasB, hcnA*, and others (Whiteley and Greenberg 2001). Two *las* boxes are located upstream of the *lasB* gene; one is located directly upstream of the transcriptional initiation site, whereas the other is placed 102 bp upstream. Both participate in controlling *lasB* expression (Rust et al. 1996; Fukushima et al. 1997).

1.4 AI-2 Signaling

AI-2 was initially identified for its control of the expression of bioluminescence in the marine bacterium *Vibrio harveyi* (Bassler et al. 1993) and was identified as a furanosyl borate diester. The AI-2 (Fig. 2c,d) and its synthase LuxS have been identified in a few bacterial species (Chen et al. 2002; Miller et al. 2004, Schauder et al. 2001; Xavier and Bassler 2005). However, the presence of *luxS* analogs in more than 55 species indicates that it is widely used in cell-to-cell signaling (Vendeville et al. 2005) to regulate genes specifying diverse functions, such as those encoding virulence factors in *Actinobacillus actinomycetemcomitans*, enterohemorrhagic *E. coli* (EHEC) O157:H7, *P. gingivalis*, *Streptococcus pyogenes*, *Vibreio cholerae*, and *V. vulnificus*; motility in *Campylobacter jejuni*, EHEC O157:H7, and enteropathogenic *E. coli* O127:H6; cell division in *E. coli* W3110 and EHEC O157:H7; antibiotic production in *Photorhabdus luminescens*; and biofilm formation and carbohydrate metabolism in *Streptococcus gordonii* (Xavier and Bassler 2005; Gonzalez Barrios et al. 2006; Ren et al. 2004b; Sperandio et al. 1999). In *S. aureus*, functional analysis of *luxS*/AI-2 reveals a role in metabolism but not quorum sensing, in which inactivation of *luxS* did not affect virulence-associated traits such as production of hemolysins and extracellular proteases, biofilm formation, and the *agr* QS signaling system (Doherty et al. 2006).

1.5 Other Quorum-signaling Systems

At least two additional QS systems have been identified in gram-negative bacteria. These include autoinducer 3 (AI-3), which is associated with virulence regulation in EHEC O157:H7 (Sperandio et al. 2003), and the *Pseudomonas* quinolone signal (PQS), which is associated with *P. aeruginosa* (Mashburn and Whiteley 2005). AI-3 is associated with *luxS* homologs in EHEC O157:H7, but the signal itself is hydrophobic and thus chemically distinct from the polar AI-2 signals (Sperandio et al. 2003). AI-3 is also biologically distinct from AI-2. During EHEC pathogenesis, both AI-3 and host epinephrine, but not AI-2, stimulate expression of the locus of enterocyte effacement (LEE) genes and thus provide evidence of bacteria and host cross-talk during this infection (Walters and Sperandio 2006). PQS molecules are quite hydrophobic and have been shown to be transported between cells by outer membrane vesicles. There is also strong evidence that the PQS actually induces the formation of these vesicles through interference with Mg^{2+} and Ca^{2+} ions in the outer membrane (Mashburn and Whiteley 2005). In a recent review (Mashburn-Warren and Whiteley 2006), it was suggested that membrane vesicles may represent a mechanism for interkingdom signaling in the plant rhizosphere.

1.6 AHL QS Interference with the Host Immune System

Eukaryotic cells communicate by means of hormones and prostaglandins that are structurally related to the bacterial AHLs, and it has been investigated whether the signal molecules produced by *P. aeruginosa* are able to interact directly with the human host cells (Smith and Iglewski 2003). Indeed, 3-oxo-C12 HSL was found to inhibit lymphocyte proliferation and tumor necrosis factor alpha (TNF-α) production by macrophages. In addition, IgE production, which is stimulated by interleukin-4, was found to be upregulated by 3-oxo-C12 HSL. Furthermore, the presence of the AHL signal molecule downregulated production of interleukin 12 (a Th-1 response promoting signal). As a consequence, a Th-2 response is encouraged (Telford et al. 1998). In contrast, other researchers found that 3-oxo-C12 HSL activates T-cells to produce interferon-γ, an inflammatory cytokine that promotes a Th-1 environment (Smith et al. 2002a). These discrepancies probably reflect biases in the underlying immune response. In C57B1/6 mice, which are Th-1 biased, 3-oxo-C12 HSL was found to increase interferon-γ, thereby promoting a Th-1-dominated response. Conversely, if BALB/C mice biased for Th-2 were used, the *P. aeruginosa* signal molecules increased production of interleukin-4, favoring a Th-2-dominated response (Moser et al. 1997). In both cases, the underlying immune response bias was accentuated by 3-oxo-C12 HSL (Ritchie et al. 2003). Production of cyclooxygenase 2 was markedly increased in human lung fibroblasts through stimulation of the transcription factor NF-κB. PGE_2, which induces mucus secretion, vasodilatation, and edema, was also produced in higher amounts when the cells were exposed to 3-oxo-C12 HSL (Smith et al. 2002b). AHL signal molecules also inhibit ATP and UTP-induced chloride secretion by submucosal tracheal serous gland cells from cystic fibrosis patients. Normally, the nucleosides lead to relaxation of the bronchia, which, in turn, promotes bacterial clearance. This ability of the 3-oxo-C12 HSL signal molecule to modulate the immune response has promoted research into generating analogs that can be used as treatment for TNF-alpha-driven immunological diseases such as psoriasis, rheumatoid arthritis, and type 1 diabetes (Chhabra et al. 2003).

Other eukaryotes also respond to the presence of AHL signal molecules. The model legume plant *Medicago truncatula*, a close relative of alfalfa, was found to produce elevated amounts of flavonoids in response to 3-oxo-C12 HSL. Interestingly, the plant also begins to secrete compounds that mimic AHL molecules when it encounters QS bacteria such as *P. aeruginosa* (Mathesius et al. 2003).

Similar relationships between QS and infection have been established for several opportunistic pathogens, including *Serratia liquefaciens* (Eberl et al. 1996, 1999), *Chromobacterium violaceum* (Brito et al. 2004), *Burkholderia cepacia* (Wopperer et al. 2006), and *Yersinia* species (Atkinson et al. 2006), all of which cause infections in humans. Other pathogens such as *V. anguliarum*, which causes the deadly infection vibriosis in fish, and the plant pathogens *Agrobacterium tumefaciens* (Sheng and Citovsky 1996) and *E. caratovora* (Whitehead et al. 2002) also employ QS to control infection and virulence. Bacteria such as *S. proteamaculans* B5a and *Enterobacter agglomerans* B6a, which causes food-quality deterioration, utilize QS

to control expression of exoenzymes that are involved in decay (Gram et al. 1999, Christensen et al. 2003).

Inhibition of QS would thus not only be beneficial in a clinical context but could possibly also be applied in aquaculture, agriculture, and food preservation.

2 Quorum Sensing in Gram-positive Bacteria[4]

Quorum sensing in gram-positive bacteria regulates a number of physiological activities, including those involving pathogenesis and biofilm formation. Examples are competence development in *Streptococcus pneumoniae* and *S. mutans*, antibiotic biosynthesis in *Lactococcus lactis* and virulence in staphylococci.

Gram-positive bacteria communicate using polypeptides as autoinducers and two-component or phosphorelay systems for signaling (Hoch and Varughese 2001). The release of the polypeptides from the cells is mediated in many cases by dedicated exporters. In most cases, signal processing and modification are concomitant with signal release. In many peptide QS systems, signals are cleaved from larger precursor peptides, which are then modified to contain lactone or thiolactone rings, lanthionines, or isoprenyl groups (Ansaldi et al. 2002; Mayville et al. 1999; Nakayama et al. 2001). QS in gram-positive bacteria has been well reviewed (e.g., Walters and Bassler 2005, Abraham 2006). Here we will focus on QS in staphylococci, whose inhibitors have extensively been tested in vivo (see the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*). Refer to the chapter *Quorum Sensing in Streptococci* for information on that topic.

2.1 Quorum Sensing in S. aureus

S. aureus pathogenesis is regulated by two QS systems (Balaban et al. 2001; Gov et al. 2004, Korem et al. 2005). As in *P. aeruginosa*, the two QS systems are organized in a hierarchical manner, the former controlling the expression of the latter. This apparently allows for further fine-tuning of the responses of QS target genes.

The two QS systems that have been described to date for *S. aureus* (Balaban et al. 2001) will be referred to herein as staphylococcal quorum-sensing 1 (SQS 1) and staphylococcal quorum-sensing 2 (SQS 2). SQS 1 consists of the autoinducer RNAIII-activating protein (RAP) and its target molecule TRAP (Balaban et al. 1998, 2001). SQS 1 induces the synthesis of the second system, SQS 2, which consists of the components of the *agr* system, including autoinducing peptide (AIP) and its sensor AgrC (Lyon et al. 2000). The two systems interact with one another to collectively regulate the expression of virulence factors (Balaban et al. 2001; Korem et al. 2005).

The notion that more than one QS system regulates virulence in staphylococci had been controversial, and it was suggested that only one QS system regulates

S. aureus pathogenesis – the one encoded by *agr* (Novick 2003). It is now, however, very clear that this is not the case and that SQS 1 in fact regulates SQS 2 (Korem et al. 2005). This phenomenon is not surprising in view of the fact that multiple systems are known to regulate necessary biological functions both in gram-negative and gram-positive bacteria (e.g., Miller and Bassler 2001; March and Bentley 2004; Gambello and Iglewski 1991; Ochsner et al. 1994; Pearson et al. 1994).

2.1.1 Components of SQS 1

RAP is the autoinducer of SQS 1. RAP is a 277AA protein that activates the *agr* by inducing the phosphorylation of TRAP (Balaban et al. 1998, 2001; Korem et al. 2003; Yang et al. 2003). From its sequence, RAP is predicted to be an ortholog of the 50S ribosomal protein L2, which is encoded by the gene *rplB* found in all eubacterial genomes known to date. Recombinant RAP applied to the cells activates the synthesis of RNAIII (which is encoded by the *agr*) like the native RAP molecule that is secreted, confirming that L2 has extraribosomal functions (Korem et al. 2003). Inhibiting RAP by anti-RAP antibodies or by RAP-binding peptides suppresses infections in vivo (Balaban et al. 1998; Yang et al. 2003). (Refer to the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*.)

TRAP is the target protein of RAP and is a master regulator of *S. aureus* pathogenesis. It is a 167-residue-long protein that is histidine-phosphorylated in the presence of RAP (Balaban et al. 2001). TRAP expression is constitutive, but its phosphorylation is regulated by RAP and reaches peak phosphorylation in the mid-exponential phase of growth. Phosphorylated TRAP then leads to *agr* expression, and the components of SQS 2 are made (Gov et al. 2004). (See below.) TRAP is highly conserved among staphylococcal strains and species and contains three conserved histidine residues that are phosphorylated and are essential for its activity (Gov et al. 2004; Korem et al. 2005). TRAP orthologs are found in other gram-positive bacteria including *Bacillus* (Ivanova1 et al. 2003) and *Listeria*. Although the sequence identity between TRAP orthologs is low, their predicted secondary structure is very similar, as is their gene organization (Kiran et al., unpublished data). TRAP-like proteins may thus represent a novel general class of signal transducers in gram-positive bacteria.

Functional genomics studies (Korem et al. 2005) indicate that in the absence of TRAP expression or phosphorylation (TRAP–), multiple regulatory systems are disrupted, such as the global regulatory locus *agr* (*agrABCD* and *hld* [RNAIII]); *sarH2*, otherwise known as *sarU* (Manna and Cheung 2003); and most, if not all, virulence factors known to date. Those include alpha, beta, gamma, and delta hemolysin; triacylglycerol lipase precursor; glycerol ester hydrolase; hyaluronate lyase precursor; staphylococcal serine protease (V8 protease); cysteine protease precursor; cysteine protease; staphopain-cysteine proteinase; 1-phosphatidylinositol phosphodiesterase; zinc metalloproteinase aureolysin precursor; holing-like proteins; and capsular polysaccharide synthesis enzymes (Korem et al. 2005). In the case of genes

involved in bacterial adhesion and consequent biofilm formation, the only overexpressed genes found when TRAP function is disrupted are those encoding for protein A, fibrinogen-binding protein, and Ser-Asp rich fibrinogen-binding bonesialoprotein-binding protein. There is, however, no evidence that these proteins independently contribute to pathogenesis. No upregulation of other known genes encoding for adhesion molecules has been observed, such as fibronectin-binding protein, collagen-binding protein, elastin-binding protein, clumping factor A, extracellular fibrinogen-binding protein, and extracellular adherence protein.

Finally, in the absence of TRAP expression or phosphorylation, the level of expression of genes required for biofilm survival is reduced, such as ArcABC (arginine deaminase, ornithine transcarbamoylase, carbamate kinase), UreABC (urease alpha, beta, gamma subunits), UreDEFG (urease accessory proteins), PyrR (pyrimidine operon repressor), PyrP (uracil permease), PyrB (aspartate transcarbamoylase chain A), PyrC (dihydroorotase), CarA (carbamoyl-phosphate synthase small chain), and CarB (carbamoyl-phosphate synthase large chain) (Korem et al. 2005; Balaban et al. 2005). These proteins are necessary for the persistence of the bacteria within a biofilm, requiring an adaptive response that limits the deleterious effects of the reduced pH associated with anaerobic growth conditions (Beenken et al. 2004).

Functional genomics studies can easily explain that in the absence of TRAP phosphorylation, the ability of the bacteria to produce toxins, to attach to host cells or foreign material, to form a biofilm, and to survive within the host is seriously compromised; therefore, when TRAP is not expressed or phosphorylated (using anti-TRAP antibodies or peptides (see below and the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*), the bacteria do not adhere, do not form a biofilm, do not express toxins, and do not cause disease (Dell'Acqua et al. 2004; Balaban et al. 2000, 2003a,b, 2004, 2005; Cirioni et al. 2003, 2006, 2007; Giacometti et al. 2003, 2005; Gov et al. 2004; Yang et al. 2005; Vieira-da-Motta 2001; Anguita-Alonso et al. 2006).

TRAP represents a novel class of signal transducers in gram-positive bacteria because it does not contain any conserved domains specific for two-component or phosphorelay systems (Han et al. 2005). QS in bacteria typically involves phosphorylation in a two-component system. The classic two-component system is composed of two proteins, the sensor kinase, which is histidine-phosphorylated, and the effector protein, which is aspartic-acid-phosphorylated. The sensor typically contains a transmembrane as well as a kinase domain (Perraud et al. 1999). Like typical sensors, TRAP is histidine-phosphorylated (Balaban et al. 2001; Gov et al. 2004), and studies indicate that it is membrane associated and can be used as a vaccine (Yang et al. 2005). However, TRAP lacks both a typical kinase domain and a predicted transmembrane region. This suggests that TRAP may be associated with the membrane by anchoring of hydrophobic surface residues or by binding to an integral membrane protein. A possible candidate may be OpuCA, discovered by a two-hybrid system (Kiran et al., unpublished data). OpuCA is encoded by the *opuC* operon that is highly conserved, is known as an ABC transporter, and thus is hypothesized to act upstream of TRAP. The type of interaction that OpuCABCD has with TRAP (anchoring?) or with the QS regulators RAP, AIP, and RIP is not yet known.

Interestingly, recombinant RAP or its inhibitor RIP (see the chapter *Quorum-Sensing Inhibitory Compounds*) were added to recombinant TRAP and shown to activate (RAP) or inhibit (RIP) the phosphorylation of TRAP in vitro, in the absence of any other cellular components. This further confirms the working hypothesis that RAP activates and RIP inhibits TRAP phosphorylation and further suggests that TRAP may in fact be a histidine kinase (Kim, personal communications).

One of the regulatory genes that is distinctly regulated by TRAP is *sarH2* (known also as *sarU*) (Balaban et al. 2005). SarU, a positive transcriptional activator of *agr* expression, encodes a 247-residue polypeptide and is a member of the SarA family of proteins. It has conserved basic residues within the helix-turn-helix motif and within the beta hairpin loop, which are two putative DNA-binding domains within this protein family (Manna and Cheung 2003). Of note is that insertions in *sarH2* have diminished the ability of the *S. aureus* strain Newman to kill worms (Bae et al. 2004), suggesting that its role in pathogenesis. SarH2 (SarU) may act as one of the downstream components in the TRAP system, regulating the expression of *agr* (see below). This, however, needs to be confirmed experimentally because *sarH2* is not conserved among strains.

Points of controversy

Lately it has been suggested that TRAP does not regulate the *agr* (Shaw et al. 2007; Tsang et al. 2007) because when the authors deleted *traP*, *agr* activity was still intact. These results will need to be confirmed as the authors have not yet shown that the mutants do not contain an active TRAP molecule (by standard in vivo phosphorylation assays and by western blotting). Interestingly, one of the two strains used, UAMS-1, is non-hemolytic although it is a virulent musculoskeletal isolate, suggesting that perhaps its virulence is not toxin related and maybe virulence of such strains does not involve TRAP. In fact, global transcriptional differences between these clinical isolates and laboratory strains have been documented (Cassat et al. 2006). It is too early to say whether these strains represent the norm or not. Luckily, virulence studies using laboratory strains have in fact resulted in clinical applications using clinical isolates (see the chapters *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs* and *Clinical Wound Healing Using Signal Inhibitors*).

It has also been suggested that the non-virulent *agr*– minus phenotype found in the TRAP– mutant are seen only because of a nonsense mutation in the *agrA* locus (Adhikari et al. 2007). This nonsense mutation was not found in TRAP– strains described in Korem et al. 2005, but other nonsense mutations were found in some of the freezer stocks (Balaban et al., in preparation), suggesting that when TRAP is inactivated, multiple nonsense mutations can occur in *agr* (and possibly other loci) more readily. Interestingly, ClpP is not expressed when TRAP or YhgC (the TRAP-like protein in Bacillus) are mutated (Kiran et al., in preparation). ClpP proteases were shown to be important for expression of various regulons involved in virulence (*agr*), oxidative stress response, autolysis, and DNA repair (Michel et al. 2006). Put

together, these results suggest that in the absence of TRAP, not only virulence is downregulated but also DNA repair is impaired, which can lead to deleterious multiple *agr* mutations (Adhikari et al. 2007). Such mutants cannot survive in vivo because of the lack of expression of virulence factors necessary for in vivo survival. But in vitro, in the absence of adverse conditions, such mutants can survive and be detected. Thus, TRAP not only regulates the production of virulence factors but also other genes necessary for bacterial survival in the host, making TRAP a very attractive target for therapy (see the chapters *Quorum-Sensing Inhibitory Compounds* and *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using QSIs*).

2.1.2 Components of SQS 2

SQS 2 encompasses the products of the *agr* system. The chromosomal *agr* operons, active from the midexponential phase of growth, encode two divergently transcribed transcripts, RNAII and RNAIII (Novick et al. 1993, 1995). RNAII is a polycistronic transcript that encodes AgrB, AgrD, AgrC, and AgrA (Ji et al. 1997; Qiu et al. 2005; Zhang and Ji 2004; Novick et al. 1995; Lina et al. 1998; Koenig et al. 2004). RNAIII is a polycistronic transcript, coding for delta hemolysin and acting as a regulatory RNA molecule that upregulates the expression of multiple exotoxins (Novick et al. 1993).

Agr-autoinducing Peptide (AIP)

AIP is processed from AgrD. AgrD sequences from various staphylococcal species are remarkably divergent, with only four identical amino acids (Qiu et al. 2005). The AIP sequence is in the middle of the AgrD sequence that is preceded by the N-terminal amphipathic helix and followed by a highly hydrophilic C-terminal region. The processing of AgrD to generate mature AIP involves the proteolytic cleavages at two processing sites, the thioester (or ester) bond formation, and the secretion of the mature AIP. The mature AIPs isolated so far from a number of staphylococcal species are seven to nine amino acids in length, and all are thiolactone molecules containing a 5-amino-acid ring linked by a thioester bond formed between the sulfhydryl group of a conserved cysteine residue and the carboxyl group of the C-terminal amino acid, except for the *Staphylococcus intermedius* AIP, in which a lactone molecule contains an ester bond formed between the hydroxyl group of a serine residue (in place of the cysteine residue that is conserved among other AIPs) and the carboxyl group of the C-terminal residue (Qiu et al. 2005; Lyon et al. 2002; Mayville et al. 1999).

A polymorphism in the amino acid sequence of the AIP and its corresponding receptor AgrC divides *S. aureus* strains into four major groups. Within a given group, each strain produces a peptide that can activate the *agr* response in the other strains, whereas the AIPs belonging to different groups are usually mutually inhibitory. Limited in vivo studies have been carried out using inhibitory AIPs (refer to the sec-

tion on inhibitors to SQS 2 in the chapter *Quorum-Sensing Inhibitory Compounds*), but their clinical significance remains unclear.

AgrB

AgrB protein is a putative cysteine endopeptidase and a transporter, facilitating the export of the processed AgrD peptide (Qiu et al. 2005). AgrB is a membrane protein with six transmembrane segments, including four transmembrane helices and two highly hydrophilic regions (Zhang et al. 2002a). Like AgrD and AgrC, AgrB sequenced from various staphylococcal species are also divergent, except for the N-terminal region located in the cytoplasm and the two highly hydrophilic regions that are proposed to be in the membrane (Zhang et al. 2002a). It is likely that all AgrBs are structurally and functionally similar and that the mechanisms of processing AgrD and of secreting the mature AIP by AgrBs are the same or similar, even though the AgrD propeptides are different and the interaction between AgrB and AgrD is specific (Zhang et al. 2004).

AgrC

AgrC is the receptor to AIP. AgrC is a membrane protein with its N-terminal half integrated into the cytoplasmic membrane and is the AIP binding site (Lina et al. 1998; Lyon et al. 2002). Its C-terminal half is located in the cytoplasm and possesses histidine kinase activity (Lina et al. 1998). The N-terminal halves are divergent, and the C-terminal halves are highly conserved. This reflects the fact that the AgrCs are activated only by their cognate AIPs but are inhibited by heterologous AIPs. Based on the AIP cross-activation and cross-inhibition activities, four specificity groups of *S. aureus* and three groups of *S. epidermidis* have been identified. Upon the binding of AIP, AgrC is autophosphorylated (Lina et al. 1998), the phosphoryl group of the phosphorylated AgrC is transferred to AgrA, and phosphorylated AgrA activates the transcription of RNAIII (Koenig et al. 2004) (see below).

AgrA

AgrA is the regulator that is part of the AgrC/AgrA two component system. Once phosphorylated, it shows high-affinity binding to the RNAIII-*agr* intergenic region, where binding is localized to a pair of direct repeats in the P2 and P3 promoter regions of the *agr* locus, consistent with the function of AgrA as a response regulator (Koenig et al. 2004) that activates the production of RNAIII (see below).

RNAIII

RNAIII is the actual regulator that activates the expression of genes encoding secreted virulence factors. RNAIII is a 512-nt-long mRNA, affecting expression of

multiple genes either directly or indirectly. RNAIII also encodes for the toxin δ-hemolysin, once translated at the postexponential phase of growth (Balaban and Novick 1995a). The commonly accepted dogma is that staphylococcal genes encoding secreted proteins are activated by the presence of RNAIII, whereas genes encoding surface proteins are repressed, leading to phase variation (Novick et al. 1993; Lowy 1998). However, this was proven only for alpha-hemolysin and protein A (see below).

The structure of RNAIII suggests that it is able to form 14 different hairpins (Benito et al. 2000). Specific domains of RNAIII control the expression of different targets: The 5′-end of RNAIII positively controls the translation of *hla* (encoding alpha-hemolysin) by competing directly with an inhibitory intramolecular RNA secondary structure that sequesters the *hla* ribosome-binding site. Hybridization of RNAIII to the *hla* mRNA frees the ribosome-binding site and enables translation of *hla* (Novick et al. 1993; Morfeldt et al. 1995).

Complementation analysis suggests that the 3′-end of RNAIII is important for repression of the *spa*-gene that encodes the well-known IgG-binding protein, protein A (Novick et al. 1993). In this case, RNAIII is believed to function either directly or indirectly at the transcriptional level, although it is possible that RNAIII affects the stability of the *spa* transcript.

RNAIII levels are evident from the midexponential phase of growth and reach a maximum in late logarithmic- and stationary-phase cultures. As mentioned above, RNAIII also encodes the small peptide δ-hemolysin in its 5′-end. Intriguingly, translation of the RNAIII transcript into δ-hemolysin is delayed 1 h after the appearance of RNAIII in the midexponential phase. This inhibitory mechanism seems to involve the 3′-end of RNAIII, possibly by blocking access of the ribosome to the ribosome-binding region (Balaban and Novick 1995a).

The mechanism by which RNAIII activates or inhibits expression of the other virulence factors remains unknown. It has been shown that transcriptional activation or repression preferentially occurs at the level of transcriptional initiation rather than by affecting transcript stability. A possible scenario would be that RNAIII functions as an antirepressor by directly binding global transcriptional regulators and then sequestering them, thereby regulating the initiation of transcription at target promoters (Arvidson and Tegmark 2001; Johansson and Cossart 2003).

agr in other Bacteria

An operon termed Fas (fibronectin/fibrinogen binding/hemolytic activity/streptokinase regulator) that shows similarity to the two-component system of *agr* was found in *Streptococcus pyogenes* (Kreikemeyer et al. 2001). As with *agr*, the effector molecule for virulence gene expression is a small, untranslated RNA molecule (fasX), although little is known of its mode of action (Kreikemeyer et al. 2001). In addition, an analogous case was found in *Clostridium perfringens*, where a small, untranslated RNA [VirR-regulated-RNA (VR-RNA)] is the effector molecule of a two-component system shown to be involved in virulence gene ex-

pression (Shimizu et al. 2002). In this case, the 3′-end of VR-RNA appears to be important in mediating virulence gene regulation (Johansson and Cossart 2003).

2.1.3 Interaction Between the two QS Systems in Staphylococci

The two QS systems in staphylococci SQS 1 and SQS 2 interact with one another (Fig. 3) as follows: As the cells multiply and the colony grows, the cells secrete RAP, inducing the histidine-phosphorylation of its target molecule TRAP (possibly via ClpP and OpuC). The phosphorylation of TRAP leads (possibly via SarH2) to the activation of the *agr* (Balaban et al. 2001; Gov et al. 2004; Korem et al. 2005) in the midexponential phase of growth and thus to the synthesis of RNAII and consequently to the production of AIP and AgrC (Novick et al. 1995). AIP downregulates TRAP phosphorylation in an unknown mechanism and upregulates the phosphorylation of its receptor, AgrC (Lina et al. 1998; Balaban et al. 2001).

Phosphorylation of AgrC causes the phosphorylation of AgrA, which together with SarA and SigB (Koenig et al. 2004; Manna and Cheung 2003; Chien et al. 1999; Ziebandt et al. 2001) results in the production of RNAIII (Novick et al. 1993; Lina et al. 1998). RNAIII leads to the expression of toxic exomolecules, resulting in dissemination and disease (Lowy 1998).

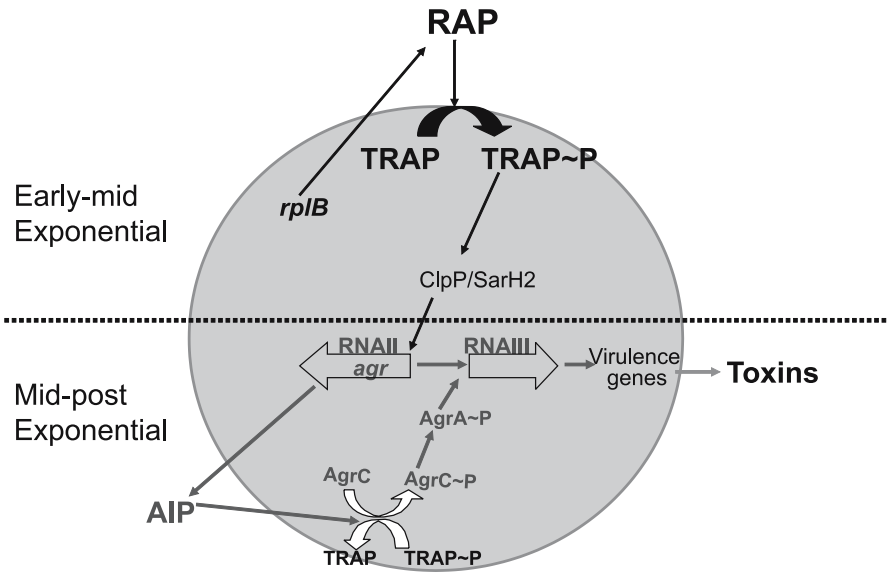

Fig. 3 Diagram showing regulation of toxin production in *S. aureus* via the quorum-sensing systems TRAP and *agr*

3 Quorum Sensing and Biofilm[1–4]

As stated above, QS refers to the ability of the bacteria to sense the density of the surrounding bacterial population. This is done by measuring and responding to the concentration of signal molecules. In order to build up a sufficient concentration of QS autoinducers (QSA), diffusion barriers are required. A dense, mature biofilm is not completely sealed off from the surroundings, but diffusion is certainly lowered compared with the situation in a planktonic culture (Hentzer et al. 2002). The timing of induction of QS-controlled genes probably depends on several factors. The diffusion rate will be dependent on the volume of the surrounding nonbiofilm phase, the flow rate (if any) of bulk fluid outside the biofilm. Furthermore, the chemical composition of the extrapolymeric substances will influence diffusion rates. As the cell density varies in the biofilm, different sets of QS-controlled genes may be expressed in different positions or niches of the biofilm. Hence, QS-controlled genes in biofilms also exhibit a spatial expression pattern (de Kievit et al. 2001).

Indeed, QS signals can be detected in diverse environments. For example, biofilms grown on rocks in the San Marcos River in Texas have been shown to produce AHL signals (McLean et al. 1997). In a completely different setting, in the lungs of cystic fibrosis patients, signal molecules have also been found (Collier et al. 2002, Singh et al. 2000).

In the protected biofilm environment, bacteria are free to produce and secrete a battery of virulence factors. In *S. aureus* and *P. aeruginosa*, for example, many of these virulence factors are controlled by QS (Korem et al. 2005; Dunman et al. 2001; Mittal et al. 2006; Joyce et al. 2004; Wagner et al. 2003). Virulence factors, in conjunction with immune complexes and phagocytic enzymes released by the immune system, cause extensive tissue destruction and inflammation. In the case of cystic fibrosis, this tissue destruction contributes significantly to the loss of pulmonary function (Costerton et al. 1999; Donlan 2002; Donlan and Costerton 2002; Parsek and Singh 2003).

Whether QS is involved in control of the developmental pattern of *P. aeruginosa* biofilms is still controversial (Kjelleberg and Molin 2002), but most of these studies were done in vitro and should be taken with caution. As reported by Charlton et al. (2000a), the concentration of QS signal OdDHL is significantly higher in *P. aeruginosa* biofilm (632 μM) than the effluent (14 nM). Consistent with this observation, QS has been found to play a critical role in the development of *P. aeruginosa* biofilms (Davies et al. 1998), showing that a *lasI* mutant formed flat, undifferentiated biofilms. In striking contrast, Heydorn et al. (2002) demonstrated, using Comstat-assisted image analysis, that a wild-type biofilm is indistinguishable from a biofilm formed by a *lasI* mutant. Again, these differences may be attributed to various strains and the experimental setups employed. When a biofilm of a *P. aeruginosa* QS mutant was grown on glucose as the carbon source, a difference in biofilm architecture could be found using image analysis. If the carbon source was changed to citrate, no difference could be detected (Heydorn et al. 2002).

In addition to AHL, AI-2 also plays a role in biofilm formation. Deletion of *luxS* has been found to influence the biofilm formation of *Streptococcus gordonii* (Blehert

et al. 2003) and *S. mutans* (Merritt et al. 2003), in which the bacteria no longer produced AI-2 and the biofilm had a more granular appearance. Direct addition of AI-2 induces biofilm formation in *E. coli* through a motility QS regulator (MqsR), which in turn regulates the two-component motility regulatory system (QseBC) and motility (Gonzalez Barrios et al. 2006).

In staphylococci, disruption of quorum sensing by mutagenesis or by inhibitory peptides (refer to section 2 in the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using SQIs* and to Fig. 4 in the chapter *Quorum-Sensing Inhibitory Compounds*) leads to suppression of biofilm formation in vivo. As shown in Fig. 4 in the chapter *Quorum-Sensing Inhibitory Compounds*, essentially no biofilm is formed in vivo by the TRAP– mutant (SQS 1 mutant). In comparison, only reduced biofilm is formed by *agr*– mutants (SQS 2 mutant), suggesting that TRAP, which acts upstream of *agr*, regulates multiple genes necessary for biofilm formation in vivo in addition to those regulated by *agr*. These studies are important because for years it had been suggested that *S. aureus* exists in two phases and that the switch between the two phases is regulated by RNAIII (*agr*). It has been suggested that in one phase, in low cell density, before *agr* is expressed, there is high expression of adhesion molecule, whereas in the other phase, in high cell density, after *agr* is expressed, there is reduced expression of adhesion molecules and, instead, increased expression of exotoxins (Lowy 1998; Novick et al. 1993). This meant that if quorum systems TRAP or *agr* are repressed, toxins will be repressed, but adhesion molecules will be expressed. This in turn was expected to enhance biofilm formation, thus making QS inhibitors inadequate for inhibiting biofilm-related infections in vivo and for future clinical use (Otto 2004; Vuong et al. 2000, 2003). However, functional genomics studies show that when TRAP or *agr* is mutated, multiple tox-

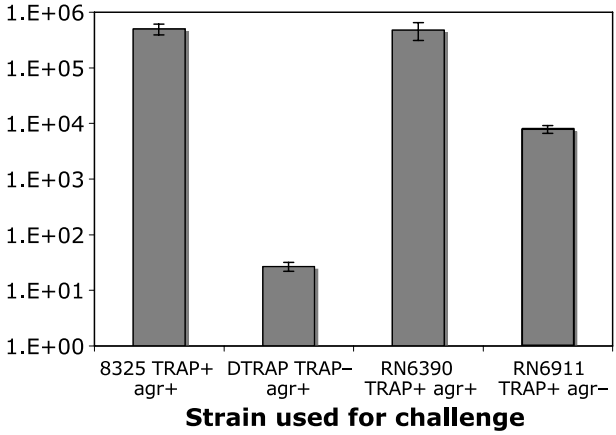

Fig. 4 Formation of a biofilm in vivo by TRAP– or *agr*– mutants. Under the rat graft model (see the chapter *Animal Models Commonly Used To Study Quorum-Sensing Inhibitors*), rats were challenged with *S. aureus* RN6390 (WT), RN6911 (*agr*–), 8325-4 (WT) or TRAP– strains. Grafts were removed 10 days later, and the bacterial loads on grafts were determined and expressed as colony-forming units (CFU)/ml

ins are repressed, but no significant increase in expression of adhesion molecules is observed (Beenken et al. 2004; Dunman et al. 2001; Korem et al. 2005). This is in accordance with the fact that TRAP mutants that do not express *agr* do have reduced ability to form a biofim or infection (Gov et al. 2004) in vivo (Fig. 4). The components of SQS 1 (RAP/TRAP) are very conserved, making them especially attractive as target sites for therapy (e.g., Balaban et al. 2005 and section 2 in the chapter *In Vivo Studies: Inhibiting Biofilm-Associated Bacterial Infections Using SQIs*).

Whether QS is involved in forming one type of biofilm or another is probably of less importance. More interesting are the properties or function of the biofilm. Davies et al. (1998), Hentzer et al. (2003), and Bjarnsholt et al. (2005a) found a link between biofilm tolerance against various antibiotics, biocides, peroxide, and QS. Biofilms formed by QS mutants or biofilms treated with inhibitors of QS were much more susceptible to the actions of these compounds (Davies et al. 1998; Hentzer et al. 2003; Rasmussen et al. 2005a,b; Bjarnsholt et al. 2005a; Dell'Acqua et al. 2004; Balaban et al. 2003a, 2004, 2005; Cirioni et al. 2003, 2006; Giacometti et al. 2003, 2005). These findings, in conjunction with the QS control of virulence factors, point out QS as a highly attractive target for chemotherapy against biofilm chronic infections!

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