Interference in quorum sensing signal transmission amongst microbial species

Zoltán G Varga[1], Mira Ágnes Szabó[1], Monika Kerényi[2] and Joseph Molnár[1]

[1]Institute of Medical Microbiology and Immunobiology, University of Szeged; and [2]Department of Medical Microbiology and Immunology, University of Pécs, Hungary

Correspondence to: Professor Dr. J. Molnar, Institute of Medical Microbiology, University of Szeged, H-6720, Szeged, Dom ter 10, Hungary. Tel: +36 62545114, +36 62545115, Fax: +36 62545113, e-mail: molnar.jozsef@med.u-szeged.hu

Running title: Interference in QS amongst microbial species.

Abstract

Antibiotics are usually studied on pure cultures of a single bacterial strain, whereas multi-species communities that inhabit human niches and the biosphere are generally ignored. The modification of quorum sensing (QS) is investigated in a system involving a co-existing signal producer and sensor bacterial cells. A pure culture of merely one bacterial species is quite rare in any niche. The interactions of different bacterial species may therefore be of special importance in pathogenicity, antibiotic resistance and signal transmission.

In the present study the authors investigated the QS in model experiments involving several Gram-positive and Gram-negative bacterial species isolated from human infections or laboratory strains. The effects of various compounds on QS were studied in mixed bacterial populations during the incubation period of 24-48 h. As the simplest example of co-existing cell populations, the *N*-acyl homoserine lactone producing Ezf 10-17 was applied with *Chromobacterium violaceum* 026 as sensor.

The signal of QS transmission between the co-existing QS system and pathogenic bacteria isolated from various patients was found to be modified by certain bacterial cells. The bacterial-bacterial interactions in a mixed flora can change the classical signal transmission in the microbial community and should therefore be taken into consideration in rational chemotherapy.

Key words: Quorum sensing, *Escherichia coli*, *Chromobacterium violaceum* CV026, coexistence, Quorum quenching

Introduction

The quorum sensing (QS) signal systems regulate a wide spectrum of cell functions in the microbial or bacterial flora. In general, microbes contain numerous genes which are activated only when the bacterial population exceeds a threshold concentration. This population size-dependent gene regulation is known as QS. Models have been proposed for the communication of two different bacterium population, where the cells of one bacterial species produce mediator molecules, e.g. homoserine lactones, which induce pigment production in the sensor *Chromobacterium violaceum*, resulting in the signal transmission responsible for violacein production, antibiotic resistance and biofilm formation in the latter population. This signal transmission can be inhibited by various compounds, with the consequence of beneficial effects. In view of this opportunity to modify the QS, it is of interest to learn what happens after the modification of QS signal transmission in a mixed bacterium population of three or more species living and growing together, as in the habitat of the surface waters, in the human mouth or gut, etc., and whether beneficial effects can attained in signal transmission.

Various natural and synthetic compounds have been shown to exert QS inhibition *in vitro* [1]. The QS inhibitory effects of phenothiazines and trifluoromethyl ketones have been studied in a number of cultures containing only the sensor and producer strains [1-3]. However, there are differences in the antiplasmid effect of promethazine in mixed bacterial cultures [4]. Under natural conditions, bacteria in the environment and in the human body form multi-species communities, such as the densely populated normal and pathogenic flora of the gastrointestinal tract, skin or oral cavity [5-9]. The functions of the normal flora include maintenance of the integrity of metabolic processes, regulation of the rate of growth of

pathogens and ensurance of the persistence of some type of "immunity" at the biological niche trough competition.

Disturbance of the flora by antibiotics, xenobiotics or superinfections leads to functional changes mediated by QS mechanisms in the microbial communities. Those can come about trough changes in virulence, antibiotic resistance, biofilm formation, etc. [10-16]. The normal QS can be modified by xenobiotics and the presence of other micro-organisms. Direct evidence has been found that *N*-acyl homoserine lactones (AHLs) are decomposed by the lactonase produced by some members of the Gram-positive bacterial family Bacillaceae. [17]. The objective of our present experiments was to study the stability of QS in mixed bacterial cultures, through measurement of the QS signal transmission in the presence of various Gram-positive cocci, Gram-positive bacilli and Gram-negative pathogenic and non-pathogenic bacterial species. The possible modification of QS has also been determined in the presence of phenothiazine compounds in mixed bacterial cultures.

Materials and Methods

Microbial strains: The *Chromobacterium violaceum* CV026 sensor strain, which detects AHLs with a short acyl side-chain via development of the purple pigment violacein [18]. Ezf 10-17 (isolated from "ezerfürtű", a traditional Hungarian grape variety), which belongs in the Sphingomonadaceae family and *Enterobacter cloaceae* 31298 (a clinical isolate from a wound) are AHL-producing strains.

Microbes tested for QS modification or inhibition: the *Candida albicans*, *C. tropicalis*, *C. krusei*, *Achromobacter xylosoxidans* 40502, *Acinetobacter baumannii* 32703, *A. baumannii* 32905, *A. baumannii* 42701, *Bacillus cereus*, *B. subtilis*, *B. clausii*, *B. megaterium* PV 361, *B. megaterium* MS 941, *B. megaterium* 216, *Staphylococcus epidermidis*, *S. aureus* and

Escherichia coli clinical isolates were applied from extraintestinal infections numbered 5536, 10902, 10904, 11925, 14525, 14584, 18596, 19579, 19672, 24310, 24409, 24442, 33444, 36446 and 40312.

Media: Blood agar complemented with sheep blood; and a modified LB medium (LB*) containing yeast extract 5 g/l, Tryptone 10 g/l, NaCl 10 g/l, K_2HPO_4 1 g/l, $MgSO_4x7H_2O$ 0.3 g/l and FeNaEDTA 36 mg/l, supplemented with agar (Difco) 20 g/l) (pH 7.2).

QS modification experiments with E. coli strains:

Suspensions of each *E. coli* strain were separately mixed with molten LB* agar medium. One hour later parallel lines of the pair of CV026 sensor and Ezf 10-17, and *E. cloaceae* 31298 AHL-producing strains were inoculated and incubation was performed at room temperature (20 ^{o}C) for 24-48 h.

QS inhibition of bacterial strains:

Each investigated strain was inoculated at right angles through the parallel lines of the pair of CV026 sensor and EZF 10-17 AHL-producing strains, and then incubated at room temperature for further 24-48 h. LB* media were used, for *Candida*, *Acinetobacter*, *Achromobacter*, *Bacillus*, *E. coli*, and *Staphylococcus* species, etc. For *Streptococcus* species, blood agar was used, and the plates were pre-incubated for 5 h at 37 ^{o}C and further incubation being continued at room temperature. QS inhibition was revealed as a decreased level of violacein production by CV026.

Results

Our results, reflecting *ex vivo* interactions, and exemplify various bacterial interactions on QS. We investigated 31 bacteria and 3 yeast strains for their ability to inhibit or modify QS (**Tables 1 and 2**), of which two bacterial genera, *Escherichia* and *Bacillus*, proved to be

effective inhibitors. Of the 6 investigated bacillus strains, *B. cereus* was the best inhibitor, with a clear QS inhibitory effect (**Photo 1**), while *B. subtilis* and *B. clausii* inhibited QS moderately, and the three *B. megaterium* strains (PV361, MS941 and 216) did not exhibit any QS inhibitory activity (**Table 1**).

Surprisingly, 14 of the 15 investigated *Escherichia* clinical isolates were effective inhibitors and only one had no inhibitory effect. We investigated the QS inhibitory activity between *E. cloaceae* 31298 and CV026 and also between Ezf 10-17 and CV026: 10 strains exerted an antibacterial effect on *E. cloaceae*, and 4 of them inhibited the growth of CV026 too (**Table 2**). The antibacterial effects of the *E. coli* isolates on *E. cloaceae* were more pronounced than those on Ezf 10-17. This probably originated from the long co-evolution in the same niche. The 2 strains, with antibacterial activity on Ezf 10-17 also had antibacterial effects on *E. cloaceae* 31298 and CV026. There were 5 strains which had no growth inhibitor activity against either the sensor or the producer strains.

All of the tested isolates except strain 19579 modified the QS (**Table 2**). The most exciting strains were 5539, 24310, 33444 and 40312, which strongly inhibitied the established QS system, but the tested isolates did not affect the growth of the indicator and 2 producer strains (**Photo 2**).

Interestingly, phenothiazines enhanced the QS inhibitory effect of the ineffective *E. coli* 19579 and the 2 bacillus strains, which display moderate QS inhibitory effects without phenothiazines.

Table 1
Effects of various bacterial and *Candida* species on QS signal transmission.

Strain	Medium	QS inhibition
Candida albicans 40502	**LB***	**-**
Candida tropicalis 47402	**LB***	**-**
Candida krusei 47813	**LB***	**-**
Acinetobacter baumannii 32703	**LB***	**-**
Acinetobacter baumannii 32905	**LB***	**-**
Acinetobacter baumannii 42701	**LB***	**-**
Achromobacter xylosoxidans 40502	**LB***	**-**
Staphylococcus aureus	**LB***	**-**
Staphylococcus epidermidis	**LB***	**-**
Bacillus subtilis	**LB***	**moderate**
Bacillus cereus	**LB***	**+**
Bacillus clausii	**LB***	**moderate**
Bacillus megaterium PV 361	**LB***	**-**
Bacillus megaterium MS 941	**LB***	**-**
Bacillus megaterium 216	**LB***	**-**
Streptococcus pneumoniae	**blood agar**	**-**
Streptococcus salivarius	**blood agar**	**-**
Streptococcus agalactiae	**blood agar**	**-**
Streptococcus pyogenes	**blood agar**	**-**

Table 2.

Effects of various *E. coli* strains on the QS signal transmission between CV026 sensor and Ezf 10-17 and *E. cloaceae* 31298 AHL producer strains.

E. coli strain number	Growth inhibition of *CV*026	Growth inhibition of Ezf 10-17	Growth inhibition of *E. cloaceae* 31298	QS inhibition	Origin of isolate (specimen)
5536	-	-	-	+	abscess
10902	+	+	+	+	blood culture
10904	+	+	+	+	blood culture
11925	-	-	+	+ (very low)	blood culture
14525	-	-	+	+	abdominal wound
14584	+ low	-	+	+	conjunctiva
18596	+ low	-	+	+	wound
19579	-	-	-	-	urine
19672	+	-	+	+	urine
24310	-	-	-	+	blood culture
24409	-	-	+	+	blood culture
24442	-	-	+ (very low)	+	blood culture
33444	-	-	-	+	blood culture
36446	-	-	+	+	blood culture
40312	-	-	-	+	blood culture

Discussion

In nature, bacteria live in a complex environment, in which they share their niche with a number of other bacterial and eukaryotic cells. Bacterial cells must select among numerous alternatives to find the most advantageous way to coexist with their neighbours and to maintain their own optimal population level. They may gain benefit from the quorum quenching of other species. The microbial world is very complex with an abundance of social interactions, and bacteria with QS systems can acquire many benefits.

Mixed bacterial infections are common in oropharyngeal, gastrointestinal and urinary tract infections. The presence of different bacterial species can result in difficulties in chemotherapy because the co-existing bacterial population can modify the interspecies communications and horizontal gene transfer [4].

Nature provides numerous examples of bacterial-bacterial and eukaryotic-bacterial interactions [17, 19-26]. As an example a furanosyl borate diester, autoinducer II (AI2), a universal signal molecule that is characteristic in both Gram-negative and Gram-positive bacteria, plays a very important role in bacterial-bacterial interspecies communication. Bacteria also synthesize molecules with special effects on eukaryotic cells. For instance, *Pseudomonas aeruginosa* operates with a signal molecule, N-3-O-dodecanoyl homoserine lactone, which exerts various effects on mammalian cells, induces apoptosis and modulates the expression of immune mediators in murine fibroblast and human vascular epithelial cells [20].

To reduce the advantages of QS, microbes has do their best to reduce the signals of other species. Among the many alternatives available to silence the QS of competitive bacteria, probably the most common way is the production of lactonase, an enzyme which opens the lactone ring of AHLs, this being a characteristic feature of the majority of *Bacillus* species

[17]. Another widely used alternative for inactivating AHLs is AHL-acylase production, which can occur in both Gram-positive and Gram-negative bacteria. These enzymes are more specific than lactonases. The AHL-acylase of *Ralstonia eutropha* is more effective on AHLs with a long acyl side-chain [21], whereas that of *Streptomyces sp*. strain M664 exerts a high AHL-degrading effect on AHLs with a short acyl side-chain [22]. This is why a bacterium which communicates with short-chain AHLs can quench long acyl side-chain AHLs without affecting its own communication, and *vice versa*.

Our results have afforded some evidence of the complexity of bacterial-bacterial interactions. Of the tested isolates, *E. coli* strains proved to be the best inhibitors of the AHL-dependent QS, 14 of the 15 samples exhibiting an inhibitory effect. There are a number of possibilities to explain why *E. coli* strains are such good QS inhibitors in our system. They may produce signals which compete with the AHL signals of CV026, or metabolize the signal molecules, or use systems like the AI2 importers. *E coli* possesses a special strategy to compete with the AI2 signals of other bacteria. *E. coli* strains have AI2 specific importers, which are activated at a high level of the inducer molecules. The import of AI2 eliminates these signals from the extracellular environment [19]. These importers probably play a role in the AHL-dependent QS inhibitory effects too in our system. Another, perhaps the most likely way to reduce AHLs from the environment, is the AHL sensing of *E. coli*. This bacterium cannot produce AHLs but has special LuxR-solo receptors, SdiA, which allow it to detect foreign AHLs. This competitive binding probably reduces the number of signal molecules in the population below the threshold concentration [27, 28].

Further studies are clearly needed to clarify the interactions between the various bacterial species in a biological niche, e.g. in the range from the human gut flora to surface waters in nature.

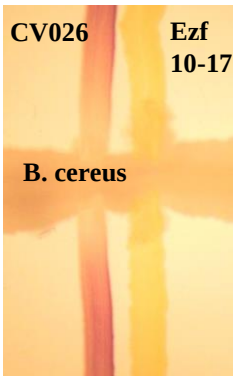

Photo 1: QS inhibitory activity of *B. cereus* in the system containing the CV026 sensor and Ezf 10-17 producer strain.
The inhibition of QS is revealed in the decreased level of violacein production.

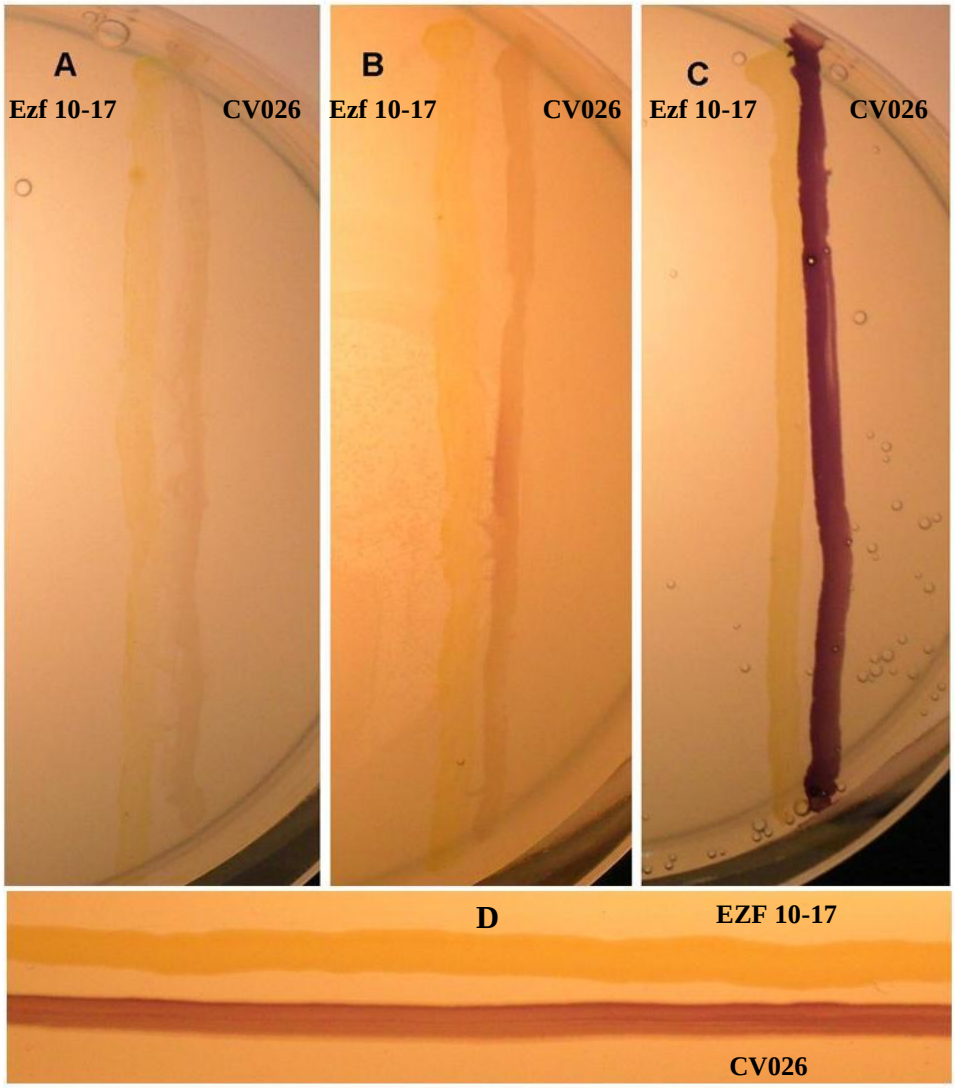

Photo 2. The interference of different *E. coli* strains with the QS signal between the CV026 sensor and the Ezf 10-17 producer strain. The sensor and producer strains are situated on the top of the medium containing different *E. coli* strains.

A: Strain 5536 lacks QS between the sensor CV026 and producer Ezf 10-17 strains.
B: Strain 11925 has high QS inhibitor activity.
C: Strain 19579 has no QS inhibitor activity.
D: Control.

Acknowledgements

The authors acknowledge the skillful technical assistance of Anikó Várady Vigyikán and Marianna Ábrahám, Institute of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary. This work was supported by ÁOK grant KA-34038/10-13, TÁMOP 4.2.2.B and the Szeged Foundation of Anticancer Research.

References

1. Szabó, M. A., Varga, G. Z., Hohmann, J., Schelz, Zs., Szegedi, E., Amaral, L., Molnar, J.: Inhibition of quorum-sensing signals by essential oils. Phytother Res **24,** 782-786 (2010).

2. Varga, G. Z., Szabó, M. A., Hohmann, J., Schelz, ZS., Szegedi, E., Amaral, L., Molnar, J.: Quorum sensing snhibition by phenothiazines and related compounds. Lett Drug Design Discovery **8,** 133-137 (2011).

3. Varga, Z. G., Armada, A., Cerca, P., Amaral, L., Subki, M. A., Savka, M. A., Szegedi, E., Kawase, M., Motohashi, N., Molnar, J.: Inhibition of quorum sensing and efflux pump system by trifluoromethyl ketone proton pump inhibitors. In Vivo **26,** 277-285 (2012).

4. Molnár, A., Amaral, L., Molnár, J.: Antiplasmid effect of promethazine in mixed bacterial cultures. Int J Antimicrob Agents **22,** 217-222 (2003).

5. Rosenthal, M., Goldberg, D., Aiello, A., Larson, E., Foxman, B.: Skin microbiota: microbial community structure and its potential association with health and disease. Infect Genet Evol **11,** 839-848 (2011).

6. Zhu, B., Wang, X., Li, L.: Human gut microbiome: the second genome of human body. Protein Cell **1,** 718-725 (2010).

7. Filoche, S., Wong, L., Sissons, C. H.: Oral biofilms: emerging concepts in microbial ecology. J Dent Res **89,** 8-18 (2010).

8. Hojo, K., Nagaoka, S., Ohshima, T., Maeda, N.: Bacterial interactions in dental biofilm development. J Dent Res **88,** 982-990 (2009).

9. Parahitiyawa, N. B., Scully, C., Leung, W. K., Yam, W. C, Jin, L. J., Samaranayake, L. P.: Exploring the oral bacterial flora: current status and future directions. Oral Dis **16,** 136-145 (2010).

10. Reeser, R. J., Medler, R. T., Billington, S. J., Jost, B. H., Joens, L.A.: Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. Appl Environ Microbiol **73,** 1908-1913 (2007).

11. Ji, G., Beavis, R., Novick, R. P.: Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc Natl Acad Sci **92,** 12055–12059 (1995).

12. Pumbwe, L., Skilbeck, C. A., Wexler, H. M.: Presence of quorum-sensing systems associated with multidrug resistance and biofilm formation in *Bacteroides fragilis*. Microb Ecol **56,** 412-419 (2008).

13. Liu, X., Bimerew, M., Ma, Y., Müller, H., Ovadis, M., Eberl, L., Berg, G., Chernin, L.: Quorum-sensing signaling is required for production of the antibiotic pyrrolnitrin in a rhizospheric biocontrol strain of *Serratia plymuthica*. FEMS Microbiol Lett **270,** 299-305 (2007).

14. Tian, Y., Wang, Q., Liu, Q., Ma, Y., Cao, X., Guan, L., Zhang, Y.: Involvement of LuxS in the regulation of motility and flagella biogenesis in Vibrio alginolyticus. Biosci Biotechnol Biochem **72,** 1063-1071 (2008).

15. Dunny, G. M.: The peptide pheromone-inducible conjugation system of *Enterococcus faecalis* plasmid pCF10: cell-cell signalling, gene transfer, complexity and evolution. Philos Trans R Soc Lond B Biol Sci **29,** 1185-1193 (2007).

16. Tomasz, A.: Control of the competent state in *Pneumococcus* by a hormone-like cell product: an example for a new type of regulatory mechanism in bacteria, *Nature* **9,** 155-159 (1965).

17. Dong, Y. H., Gusti, A. R., Zhang, Q., Xu, J. L., Zhang, L. H.: Identification of quorum-quenching N-acyl homoserine lactonases from Bacillus species. Appl Environ Microbiol **68,** 1754-1759 (2002).

18. McClean, K. H, Winson, M. K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift, S., Bycroft, B.W., Stewart, G. S. A. B., Williams, P.: Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acyl homoserine lactones. Microbiology **143,** 3703-3711 (1997).

19. Xavier, K. B., Bassler, B. L.: Interference with AI-2-mediated bacterial cell-cell communication. Nature **437,** 750-753 (2005).

20. Shiner, E. K., Terentyev, D., Bryan, A., Sennoune, S., Martinez-Zaguilan, R., Li, G., Gyorke, S., Williams, S. C., Rumbaugh, K.P.: *Pseudomonas aeruginosa* autoinducer modulates host cell responses through calcium signalling. Cell Microbiol **8,** 1601-1610 (2006).

21. Lin, Y. H., Xu, J.L., Hu, J., Wang, L.H., Ong, S.L., Leadbetter, J. R., Zhang, L. H.: Acyl-homoserine lactone acylase from Ralstonia strain XJ12B represents a novel and potent class of quorum-quenching enzymes. Mol Microbiol **47,** 849-860 (2003).

22. Park, S. Y., Kang, H. O., Jang, H. S., Lee, J. K., Koo, B. T., Yum, D.Y.: Identification of extracellular N-acylhomoserine lactone acylase from *Streptomyces* sp. and its application to quorum quenching. Appl Environ Microbiol **71,** 2632-2641 (2005).

23. Kaplan, F., Badri, D. V., Zachariah, C., Ajredini, R., Sandoval, F. J., Roje, S., Levine, H. L. F., Robinette, S. L., Alborn, H. T.: Bacterial attraction and quorum sensing inhibition in *Caenorhabditis elegans* exudates. Journal of Chemical Ecology **35,** 878-892 (2009).

24. Draganov, D. I., Teiber, J.F., Speelman, A., Osawa, Y., Sunahara, R., Ladu, B. N.: Human paraoxonases are lactonases with overlapping and distinct substrate specificities. J Lipid Res **46,** 1239-1247 (2005).

25. Leadbetter, J. R., Greenberg, E. P.: Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. J Bacteriol **182,** 6921-6926 (2000).

26. Giskov, M., de Nys, R., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, S., Steinberg, P. D., Kjelleberg, S.: Eukaryotyc interference with homoserine lactone-mediated prokaryotic signalling. J Bacteriol **178,** 6618-6622 (1996).

27. Ahmer, B. M.: Cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica*. Mol Microbiol **52,** 933-945 (2004).

28. Yao, Y., Martinez-Yamount, M. A., Dickerson, T. J., Brogan, A. P.: Structure of the *Escherichia coli* quorum sensing protein SdiA: activation of the following switch by acyl homoserine lactones. J Mol Biol **355,** 262-273 (2006).