MODIFICATION OF QUORUM SENSING IN BACTERIAL CULTURES

Ph.D. Thesis

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Publications related to the thesis

I. Szabó MA, Varga GZ, Hohmann J, Schelz Z, Szegedi E, Amaral L, Molnár J. Inhibition of quorum-sensing signals by essential oils. Phytother. Res. 2010; 24(5):782-6.

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II. Varga GZ, Szabo MA, Schelz Zs, Szegedi E, Amaral L, Molnár J. Quorum Sensing Inhibition by Phenothiazines and Related Compounds. Lett Drug Design Discovery. 2011; *8*: 133-137.

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III. Varga GZ, Armada A, Cerca P, Amaral L, Subki AM, Savka M, Szegedi E, Kawase M, Motohashi N, Molnár J. Inhibition of Quorum Sensing and Efflux Pump System by Trifluoromethyl Ketone Proton Pump Inhibitors. In Vivo. 2012; 26:277-286

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IV. Varga GZ, Szabó AM, Kerényi M, Molnár J. Interference in quorum sensing signal amongst microbial species. Acta Microbiol Immunol Hung. 2012; 9(4):477-486

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Abbreviations

5-FU 5-fluorouracil

ACP acyl carrier protein

AHK Alpha-hydroxyketone

AHL acyl homoserine lactone

AI autoinducer

AIP autoinducer peptide

AO acridine orange

A. tumefaciens *Agrobacterium tumefaciens*

C. albicans *Candida albicans*

CAI cholera autoinducer

CSP competence stimulating peptide

CV026 *Chromobacterium violaceum* 026

C. violaceum *Chromobacterium violaceum*

DMSO dimethyl sulfoxide

DPD 4,5-dihydroxy-2,3-pentadione

DSF diffusible signal factor

EB Ethidium bromide

E. cloaceae *Enterobacter cloaceae*

E. coli *Escherichia coli*

EPS extracellular polymeric substances

HHQ 2-heptyl-4-(1H)-hydroxyquinolone

H. pylori *Helicobacter pylori*

IDU iodine deoxyuridine

LB Luria-Bertani

LB* modified Luria-Bertani

MHA Mueller-Hinton agar

MHB Mueller-Hinton broth

MIC minimum inhibitory concentration

OD optical density

P. aeruginosa *Pseudomonas aeruginosa*

PBS phosphate-buffered saline

PDA potato dextrose agar

PMF proton motive force

PQS Pseudomonas quinolone signal

P. syringae *Pseudomonas syringae*

QQ quorum quenching

QS quorum sensing

RFF relative final fluorescence

SAH S-adenosylhomocysteine

SAM S-adenosylmethionine

S. aureus *Staphyllococcus aureus*

S. pneumoniae *Streptococcus pneumoniae*

SRH S-ribosylhomocysteine

S. typhimurium *Salmonella typhimurium*

THMF methyl-2,3,3,4-tetrahydroxytetrahidrofuran

TI tumour inducing

TLC thin layer chromatography

TZ thioridazine

V. cholerae *Vibrio cholerae*

V. fischeri *Vibrio fischeri*

V. harveyi *Vibrio harveyi*

V. paradoxus *Variovorax paradoxus*

X. campestris *Xanthomonas campestris*

X. fastidiosa *Xilella fastidiosa*

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1. Introduction

Quorum is a Greek word. In the ancient Hellenic and Roman forums, it was necessary for the members of the senate to form at least a minimal majority in order for them to make a decision. The group of persons whose number was sufficient for them to be able to accept or reject an act was the quorum.

In general, microbes have numerous genes which become activated only when the bacterial population owersteps a threshold concentration. The term used to describe this population size-dependent gene regulation is quorum sensing (QS), involving communication between bacteria.

In spite of the ancient origins of the word "quorum", QS is a relatively new area of science. When Leuweenhoek first glimpsed inside the bacterial world, it was thought that microbes live in a blind and deaf world, where all cells live their own lives without any influence from others. The first signs of the collapse of this theory, was the discovery of the competence stimulating factor of *Streptococcus pneumoniae* (*S. pneumoniae*) in 1965 (1). It was oberwed that this bacterium needs an extracellular peptide to reach the competent state, and this molecule can be produced only at a high population size density. This is very useful, because there is no reason for a bacterium to waste energy reaching the competent state if there are insufficient cells in the near environment. The first direct evidence and the birth of QS emerged 5 years after this interesting discovery. This was the light production of *Vibrio fischeri* (*V. fischery*), (2). This bacterium can colonize the light organ of several species of deepwater animals, includeing squids and fish. In this special organ, all nutrients are given to reach a high cell density. The wall of this organ is usually very flexible and fish can press water out of it. When this happens, the cell density of the vibrios increases, and light is produced. The first-described QS system was the *V. fischeri* LuxI/LuxR system (3), which is a characteristic feature in other G- bacterial species too.

After these initial discoveries, it seemed that QS is very rare and almost unique in the bacterial world, influencing merely a few and not too important bacterial features. Nowadays, this has completely changed. As more and more QS systems have been discovered, it has become clear that bacterial communication does not occur in only a few isolated cases.

QS is an essential part of bacterial life. The bacterium, with its QS system, has leverage, which is why QS is widely prevalent in the microbial world. With communication systems, microbes can accommodate to their habitat like multicellular organisms, and can fight as a unit against environmental impacts, other bacteria or the host immune system. Group

behaviour is probably the most developed bacterial lifestyle. In a complex multispecies biofilm, every bacterium knows its place, and what is the best to do for the community and for itself. Division of labour is an evident thing in multicellular eukaryotes, but in prokaryotic biofilms too. QS has a special role not only in prokaryotic-prokaryotic, but also in eukaryotic-prokaryotic communication. The human body contains 10 times more bacterial cells than human cells, and most bacteria generally live in full harmony with us. It is clear that, it is virtually impossible to maintain this symbiosis without communication. We have hormones which affect our flora, and our symbionts also deploy QS signals which have effects on our cells. QS is also a very important phenomenon for the pathogenic bacteria. Signal molecules contribute directly to pathogenesis via the organization of biofilms or the synchronized production of virulence factors.

Antibiotic resistance is the most important problem in modern microbiology. Bactericidal or bacteriostatic compounds exert strong selective pressure on microbes. Because of the irresponsible use of antibiotics, pathogenic bacteria nowadays often display resistance to at least a few medicines. The appearance of multidrug-resistant strains which tolerate the latest antibiotics too enhances this problem. There is an urgent need to discover new antimicrobial, antipathogenic and antivirulence drugs. QS inhibitors may be a good alternative to solve this situation.

1.1. QS

QS is a widely prevalent feature of both Gram-negative and Gram-positive bacteria. There are many different types of QS systems and signal molecules in the bacterial world, but each system relies on the same basics. Each bacterium synthesizes signal molecules in low amounts. When the population density is low, the number of signal molecules is also low, when the number of bacteria increases, the concentration of QS molecules increases. When the concentration of signal molecules exceeds a threshold concentration, a positive feedback starts, and the bacteria increase the production of signal molecules. At this very high concentration of QS molecules, they bind their receptors. The nature of the receptors can differ considerably in each case, but in the end of the process is the same: the initiation of target gene transcription.
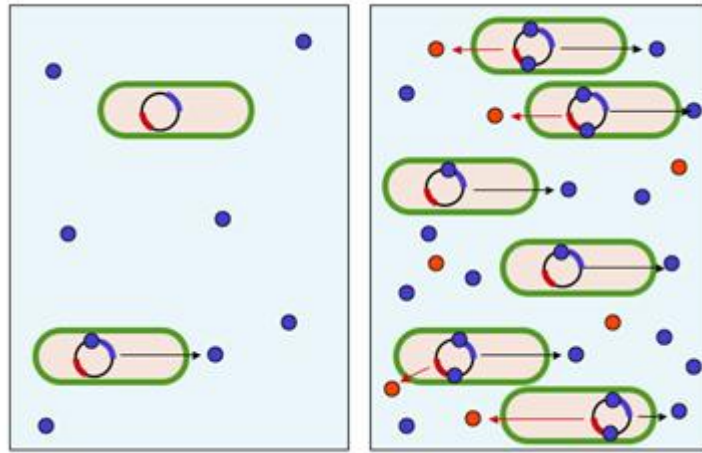
Figure 1.
Schematic outline of QS.
Left side: Low concentrations of bacteria and signal molecules (blue circles).
Right side: When the concentration of bacteria increases, the amount of signal molecules increases too. when it reaches the threshold concentration, binding to the receptors occurs and activates the transcription of the target genes (4).

1.2. QS in Gram-negative bacteria

1.2.1. LuxI/LuxR system of *V. fischeri*

The first described QS was the LuxI/LuxR system of *V fischeri* (3). Two proteins, LuxI and LuxR, control the synthesis of the enzyme luciferase. The bacterium synthesizes the signal molecules with the acyl homoserine lactone (AHL) synthase LuxI. When the AHL reaches the threshold concentration, it can bind to LuxR, the cytoplasmic receptor (5,6). The LuxR-AHL complex activates the transcription of the luciferase operon. The LuxR-AHL complex also induces the expression of luxI because it is encoded in the luciferase operon. This positive feedback leads to a flood of more and more AHL molecules to the environment, which can diffuse freely to the receptor and activate the transcription of the structure genes. The end of this process is the light production of the bacteria. LuxI/LuxR-type AHL-dependent QS systems are highly prevalent in G-bacteria. It mainly takes place in intraspecies communication too.

1.2.2. LasI/LAsR-RhlI/RhlR system of *Pseudomonas aeruginosa* (*P. aeruginosa*)

P. aeruginosa is an abundant, opportunistic pathogenic bacterium which lives in soil and natural waters, and frequently colonizes and organizes biofilms in taps, air conditioners and

other water-associated equipment. Although this bacterium rarely causes infections in healthy adults, it involves a great health risk. This bacterium is responsible for most nosocomial infections, and it exhibits very strong antibiotic resistance.

P. aeruginosa has two LuxI/LuxR homologues, LasI/LasR and RhlI/RhlR (7). LasI and RhlI are autoinducer (AI) synthases that produce the signal molecules 3OC12HSL and C4HSL (8, 9). LasR and RhlR are specific AHL receptors. The LasR-AHL complex activates the expression of lasI and a variety of other target genes, and also activates the RhlI/RhlR system. There are hundreds of genes which are under QS control in *P. aeruginosa*. Some of them are induced by only one AI, while some need both 3OC12HSL and C4HSL to activate. There are also genes which are activated by either C4HSL or 3OC12HSL (10, 11). *P. aeruginosa* has a LuxR "solo" receptor which does not have a cognate synthase pair. This is called QscR, and is activated by 3OC12HSL (12). The QS system of *Pseudomonas* species is very complex. Besides the two LuxI/LuxR type synthase/receptor pairs and the LuxR solo, it uses other types of AIs for the stringent control of QS. The *Pseudomonas* quinolone signal (2-heptyl-3-hydroxy-4(1H)-quinolone; PQS) is used only by *P. aeruginosa*, its precursor 2-heptyl-4(1H)-hydroxyquinolone (HHQ), is used by other *Pseudomonas* species and *Burkholderia* spp. as well. Both PQS and HHQ act as AIs via the interaction of the transcriptional regulator PqsR (13).
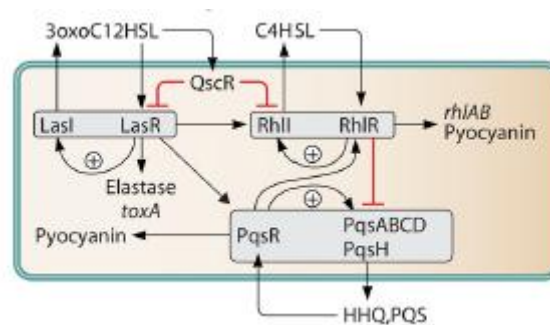
Figure 2.
The QS system of *P. aeruginosa* (14)

1.2.3. The TraI/TraR QS system of *Agrobacterium tumefaciens* (*A. tumefaciens*)

A. tumefaciens is a tumour-inducing (TI) phytopathogenic bacterium which causes infections mainly in arboreal dicotyledonous plants. The bacterium penetrates into the host through injured tissues, and causes crown gall tumours via the intercalated T-DNA (located in the TI plasmid). These tumours produce opines which the bacteria use as carbon source. Besides the opine genes, TI plasmid encodes phytohormones, which are necessary for the tumour growth, and the genes traI and traR, which are responsible for the QS of the bacteria (15, 16).

The QS system of *A. tumefaciens* is quite interesting, because this bacterium needs an opine signal from the infected plant to activate the QS system responsible for the mobilization of the TI plasmid. These molecules are detected by the cytoplasmic receptor OccR (15). The OccR-opine complex induces the expression of traR. After the binding of their AHL, the TraR receptors induce the expression of structure genes, which are responsible for the TI plasmid replication. These genes also include traM, which is responsible for the down-regulation of the process.

1.3. QS in Gram-positive bacteria

The Gram-positive bacteria do not have any LuxI/LuxR-type homologues; they mainly use different peptide molecules as autoinducers. Usually, these oligopeptides cannot penetrate through the membrane; specialized active transporters are needed to deliver them into the external space. The AI peptides (AIPs) are subject to various modification events, such as processing or cyclization. The detection of the AIPs can be extra- or intracellular, but most of the receptors are localized extracellularly. When the receptors binds the AIPs, a phosphorylation-dephosphorylation cascade starts which reaches the response regulator and activates or represses the QS target genes (17).
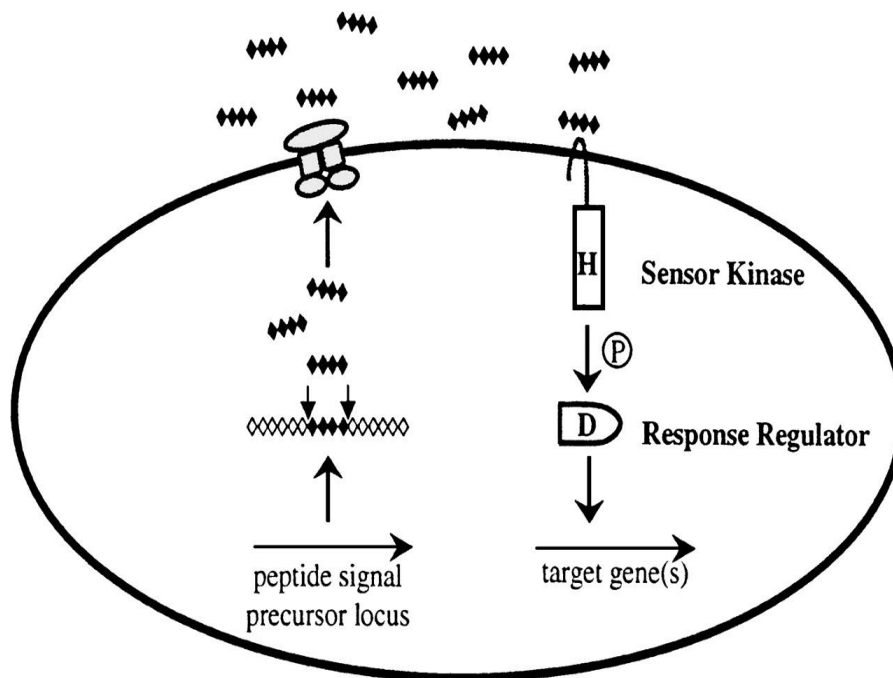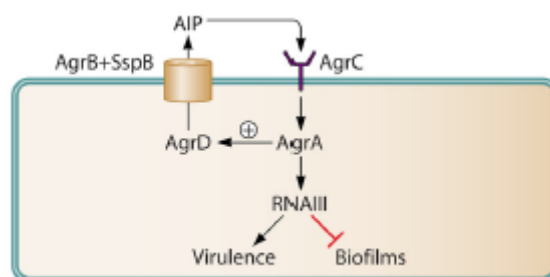

Figure 3.
The mechanism of peptide based QS (18).

1.3.1. The QS system of *Staphylococcus aureus* (*S. aureus*)

S. aureus is a widely prevalent bacterium which causes numerous human diseases. It can nearly infect all kinds of tissues e.g. skin, bone, the lungs and the nervous system. It has great resistance to most antibiotics, and has evolved multiresistant strains, that tolerate all kinds of antibiotics. *S. aureus* strains use interesting QS-mediated strategies to cause diseases. At low cell density they express factors which promote attachment, biofilm formation and colonization, while at high cell density they produce proteases and toxins, which promote dissemination and infection. This density-dependent pathogenicity in *S. aureus* is regulated by the RNAIII molecule, the level of which is mediated through the AgrC/AgrA QS system. The AIP of *S. aureus* is encoded by agrD. The AgrD molecule is transported and modified with a thiolactone ring by the AgrB transporter. The AgrC receptor binds the AIP extracellularly, this complex leads to the phosphorylation of AgrA and the phosphorylated AgrA then induces the expression of RNAIII. This regulatory RNA represes the expression of biofilm formation factors and promotes the expression of toxins and proteases. RNAIII also induces the expression of the agrBDCA locus, which increases the levels of AIPs (19).

 Interestingly, these AIPs and therefore the *S. aureus*-es belong in 4 different groups. Each type of signal molecule competitively blocks the receptors of the others. The bacteria which colonize first, therefore kill or inhibit the colonization of the bacteria from the other groups (20).

4. Figure
The QS system of *S. aureus* (14)

1.3.2. The QS system of *S. pneumoniae*

The QS system of *S. pneumoniae* was the earliest investigated QS system, (although when Tomas carried out his research, this area of science did not exist). It was revealed that *S. pneumoniae* needs an extracellular peptide, called a competence stimulating pheromone, to reach the competence state (1,21). Nearly 30 years later Pestova described the QS system of this bacterium. ComC is the precursor peptide of the CSP (competence stimulating peptide) AI. This molecule is secreted out of the cell through the ComAB ABC transporter. When the

CSP molecules reach the threshold concentration, they bind their receptors, called ComD. This AIP-receptor complex launces a phosphorylation-dephosphorylation cascade, which finally activates the transcription of the comX gene. The ComX protein is essential for the transcription of the genes which take part in the competence (22).

1.4. QS signal molecules

QS is population size-dependent gene regulation, mediated by small signal molecules called AIs. The natures of these molecules are very different, but most belong in 3 groups: AHLs are mainly used by Gram-negative bacteria, autoinducer II (AI-2) is used by both Gram-negatives and Gram-positives, and AIPs are used by Gram-positives. Some bacteria use AIs which do not fit these groups, e.g. the PQS diffusible signal factor (DSF) or autoinducer III (AI-3).

1.4.1. AHLs

AHL-s are the most common signal molecules of Gram-negative bacteria. The first discovered signal molecule was the *V. fischeri* 3OC6-HSL, which catalyses luciferase production. Many other bacteria use the same AHL to communicate, e.g. *Pseudomonas syringae*, *Erwinia carotovora* and *Pseudomonas stewarti*. In these species, these compounds influence the virulence and plant colonization. The length of the acyl side-chain in the AHL molecules can vary in between 4 and 18 carbon atoms. The lactone ring is conserved, but they can also differ in the saturation state of the acyl side-chain and the oxidation state at carbon 3. These molecules are usually synthesized by LuxI-type AHL synthase, using acylated acyl carrier proteins (acyl-ACPs) and S-adenosylmethionine as substrate (23).

Gram-negative bacteria can use only one AHL molecule, e.g. *V. fischeri*, or can use a multiple signal system, e.g. *P. aeruginosa*. This microbe uses two types of AHLs, 3oxoC12-HSL and C4-HSL, supplemented with the PQS and HHQ.

The AHLs mainly takes part in intraspecies communication. The receptors of these molecules are usually AHL-specific, but there are other examples. In *Chromobacterium violaceum* (*C. violaceum*), the bacterium which uses C6-HSLs to supervise violacein production, the AHL receptor can bind other types of AHLs with a short acyl side-chain.

The AHL receptors are usually cytoplasmic receptors for short-chain AHLs, and membrane-bound sensor kinases for long-chain AHLs. The short-chain AHLs can freely diffuse in and out of the cell, but long-chain AHLs need active transport to move to the external space.

Most species use signal/receptor pairs to produce and respond to the AHL molecules. In some cases, there are also orphan receptors, or LuxR solos which can detect distinct AHL signals. These receptors do not have cognate LuxI synthase, e.g. SidA in *Escherichia coli* (*E. coli*) (12).
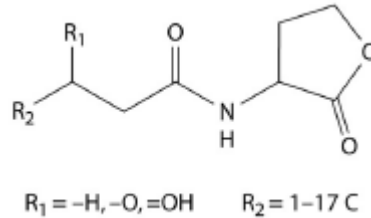
Figure 5.
The AHL molecule (14)

1.4.2. Peptide-type autoinducers

AIPs are mainly used by Gram-positive bacteria. These signals are synthesized ribosomally. A large precursor peptide is usually synthesized first, and after the cleavage can be modified with lactone or thiolactone rings, lanthionines or isoprenyl groups. (24-26). In all cases, specialized active transporters are needed to move the AIPs out of the cell. The transport proteins usually ensure the modification events too. The modified oligopeptides are detected extracellularly, by a two-component membrane-bound sensor histidine kinase. After the binding a phosphorylation-dephosphorylation cascade starts, which reaches the response regulator, and the activated response regulator promotes or represses the target genes.

In some cases, small linear AIPs are transported back into the cell, where they directly influence the response regulation, e.g the PrgX system in *Enterococcus faecalis*, or the PlcR and NprR systems in *Bacillus thuringiensis* (27-29).
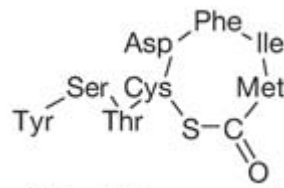
Figure 6.
AIP-1 in *S. aureus* (14)

1.4.3. AI-2

AI-2 is the universal signal molecule in bacteria. It is widely prevalent in both Gram-positive and Gram-negative species. More than a third of the currently investigated bacteria use AI-2 molecules for interspecies communication. The AI-2 systems are usually complemented with AHL (in Gram-negatives) or AIP (in Gram-positives) -based QS.

AI-2 is generated from S-adenosylhomocysteine (SAH), which is formed when S-adenosylmethionine (SAM) releases the activated methyl group. SAH is converted by Psf (5'-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase) to S-ribosylhomocysteine (SRH) (30, 31). LuxS catalyses the cleavage of SRH to homocysteine and 4,5-dihydroxy-2,3-pentadione (DPD). This molecule spontaneously cyclizes into two isomers, S-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (S-THMF-borate) and non-borated R-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF) (32). Both enantiomers have AI-2 activity, and are detected by different bacterial species. The AI-2 molecules are relatively membrane-impermeable; active transport is needed to move them in and out of the cell. The YdgG protein in *E. coli* is the only AI-2 exporter so far known, but other mechanisms of AI-2 transport must exist (33). There are also some black spots in the receptor binding of AI-2 molecules. Three kinds of AI-2 receptors are known. The LuxP family is found only in *Vibrios* spp.. This periplasmic binding protein is typically associated with ATP binding casettes (ABC) (34). The ligand of this receptor is S-THMF-borate (32, 35).

Other well-known AI-2 receptors belong in the LsrB family. This high-affinity substrate-binding periplasmic protein also interacts with the ABC transport system, called Lsr (36). The LsrB family is typically present in enteric bacteria and the members of the *Rhizobiaceae* and *Bacillaceae* and have high affinity for the R-THMF isomer of DPD (35, 37).

Agregatinobacter actinomycetemcomitans and *Haemophilus influenzae* use other type of receptors as well for the sensing of AI-2s, the RbsB proteins, which have high homology (above 70%) with the ribose ABC transporter (38, 39).
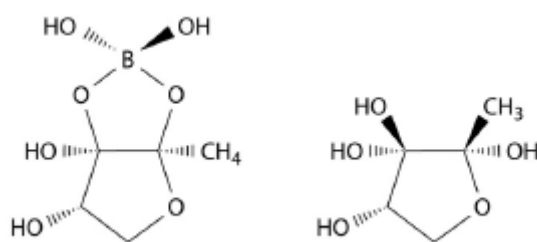
Figure 7
The AI-2 of *V. harvei* (left) and *S. enterica* (right) (14)

1.4.4 Pseudomonas quinolone signal PQS

The PQS and its precursor HHQ both act as QS signal molecules via the PqsR transcriptional regulator. The PQS is found only in *P. aeruginosa*, but HHQ appears in many *Pseudomonas* and *Burkholderia* spp. (40,13).
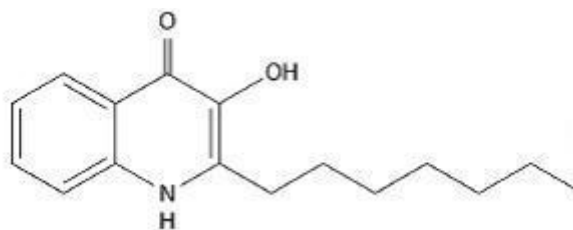
Figure 8.
The PQS molecule.

1.4.5. The DSF

DSF (*cis*-11-methyl-2-dodecenoic acid) is present in many plant pathogenic bacteria e.g. *Xanthomonas campestris (X. campestris)*, *Burkholderia cenocepacia* and *Xilella fastidiosa (X. fastidiosa)*. The mechanism of signal detection differs between species, but after the binding they share a general feature: all influence the intracellular level of cyclic di-GMP (41,42).

Figure 9.
The DSF signal of *X. campestris* (14)

1.4.6. AI-3

Although the structure of AI-3 is not yet known, it must be similar to the human hormones epinephrine and norepinephrine. This QS molecule produced is by the members of the commensal enteric flora and intestinal pathogens. In *E. coli* and *Salmonella typhimurium (S. typhimurium)*, the detection of AI-3 and also the hormones epinephrine and norepinephrine occurs via the two-component QseC/QseB system (43,44). This is an interesting example how we can influence the flora via hormones and how the bacteria can affect their host via QS molecules.

1.4.7. Alpha-hydroxyketones (AHKs)

One of the firstly identified AHK was the 3-hydroxytridecan-4-one (Cholera autoinducer I (CAI-1)) in *Vibrio cholerae* (*V. cholerae*). It produced by the enzyme CqsA from the substrates (*S*)-2-aminobutyrate and decanoyl coenzyme A. The receptor of CAI-1 is CqsS sensor kinase, which have effect on LuxU and LuxO. These proteins also influenced by AI-2 and AHL dependent QS systems in *V. cholerae* and *Vibrio harveyi*. Above *Vibrios*, *Legionella pneumoniae* also use AHKs as QS molecules (45).

Figure 10.
The CAI-1 molecule (14)

1.5. Quorum quenching

Bacteria with different QS systems gain numerous benefits. They can monitor the density of their own population and those of other bacteria and eukaryotes too. They can coordinate and optimize their gene expression in a density-dependent manner. The cost of signal molecule production is very low and is usually associated with necessary cell metabolic pathways (46). For example, a bacterium does not gain leverage and waste much e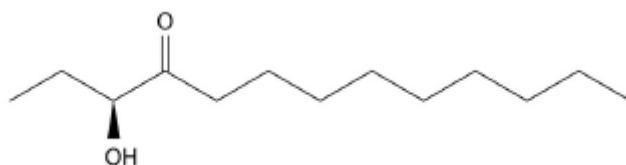nergy if it produces antibiotics when the cell number is not large enough to produce it in sufficient concentration. Hence it is necessary to produce signal molecules to optimize the production of difficult and expensive molecules. QS is essential in bacterial life, and many strategies have therefore been developed to disrupt it in bacterial-bacterial, and also prokaryotic-eukaryotic interactions. The mode of interaction with other QS systems can be very different, but the 3 attack points are the same: the synthesis of the signal molecule, the signal molecule and the signal detection.

1.5.1. Inhibition of signal production

The first step of QS is the biosynthesis of signal molecules, and numerous inhibitors therefore have the aim of this target. Evidence has been found in the inhibition of AHL, AI-2, peptide-type autoinducer and PQS production. AHL signals are produced from SAM and acyl-ACPs by acyl-HSL synthases, e.g. LuxI or AinS-type synthases. Some antimicrobials, e.g. diazobromines and triclosan, inhibit FabI (NADH-dependent enoyl-ACP reductase), the enzyme which catalyses the production of acyl-ACPs (47). Another example is found in the phytopathogenic bacterium *Burkholderia glumae*. The J8C8 molecule is competitivein inhibiting acyl-ACP utilization, and thereby inhibits the production of C8HSL (48). The SAM analogues, S-adenosylcysteine, S-adenosylhomocysteine and sinefungin also inhibit AHL production (22). 5'-Methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) is involved in both the AHL and AI-2 production, and is also necessary in cell metabolic pathways (22). Inhibition of MTAN results in aberrant grown, and not only in decreased signal production. Even so, some tested MTAN inhibitors, immucillin A and DADMe-ImmA

derivatives, have proved to be good inhibitors (49, 50). Besides these molecules, AI-2 production is also impaired by different LuxS inhibitors. Enzyme LuxS is probably the best target to reduce AI-2 production. S-Anhydroribosyl-L-homocysteyl and S-homoribosyl-L-cysteine were the first molecules identified as this type of inhibitors. Both are competitive inhibitors, though unfortunately with very weak effects (51). The most potent LuxS inhibitors were synthetized by the Shen's group: compounds 10 and 11 had the greatest effects (52). Halogenated furanones also inhibit AI-2 production to various degrees (53).

The synthesis of the PQS and HHQ, the signal molecules produced by *Pseudomonas* species, can be inhibited by anthranilate analogues. Methyl anthranilate influences the PQS production in *P. aeruginosa* (54). Farnesol, the signal molecule of *Candida albicans* (*C.* albicans), also inhibits the PQS production of *P. aeruginosa*. Interestingly, this opportunistic fungal pathogen frequently causes mixed infections with this bacterium (55).

The targets involved in the synthesis of AIPs are usually essential for the growth of cells too. Most QSI compounds also have bactericidal activity. There have been only a few studies on this theme. One of them related to the fungal secondary metabolite product ambuic acid, which can inhibit cyclic AIP biosynthesis in many bacterial species (56).

1.5.2. QS signal degradation and inactivation

Most QSI compounds use signal degradation as the mode of action. This is therefore the most studied field of QQ. There are numerous examples in AHL, AI-2, PQS, AIP and DSF signal degradation.

AHL molecules recruit a homoserine lactone ring and a 4-18 carbon atom acyl side-chain. The lactone ring is spontaneously hydrolysed at alkaline pH, and is also spontaneous restored at acidic pH. Through modification of the pH level, bacteria and higher organisms can interact with microbes and communicate with AHL molecules. In most cases the point of attack is more direct, and specific enzymes are used to degrade AHLs. There are 3 main categories: lactonases, acylases and oxidoreductases. AHL lactonases hydrolyse the ester bond on the homoserine lactone ring. The structure of the lactone ring is conserved, and most of lactonases therefore have very wide substrate specificity. Gram-positive microbes and higher organisms usually use this type of QQ. The first examples were found in *Bacillus* species and the aiiA (AI inactivation) gene in them was identified (57). The purified lactonases inactivate numerous types of AHLs. To produce enzymes which attack the lactone ring is not confined to microbes. For example, human paraxonase 1,2 and 3 (PON1-3), mainly produced by airway epithelial cells, also have lactonase activity (58, 59). Studies have revealed the

differences between these 3 proteins. PON1 and PON3 have very wide substrate specificities, but both have lactonase activity. PON2 does not have such a wide substrate specificity but has the greatest activity against AHLs (60-62). AHL acylases hydrolyse the acyl-amide bond between the lactone ring and acyl tail. The substrate specificity of this molecule is much stricter, than in lactonases. The first acylase enzyme was inentified in *Variovorax paradoxus* (*V. paradoxus*). This bacterium is able to use various kinds of AHL as energy source (63). AHL acylases are found in various other bacteria too e.g. AhlM in *Streptomices sp.* strain M664 (64), HacA and HacB in *P. syringae* (65 Shepherd 2009) and PvdQ in *P. aeruginosa* (66). The third and probably the least known enzyme family which can disrupt AHL molecules are the oxidoreductases. This was first discovered in *Rhodococcus erythropolis*. Like *V. paradoxus*, this bacterium uses the AHLs as carbon and nitrogen sources (67). The effectiveness of oxidoreductases is usually lower than those of then AHL acylases and lactonases (68,69), but there have been only a few studies of these molecules. AHL molecules can also be inactivated by different antibodies. For example, MAbs (monoclonal antibodies) inactivate QS-mediated pyocyanin production in *P. aeruginosa* (70).

The QQ of AI-2 molecules is not as well known as in the case of AHLs. The enzyme Lsrk adds a phosphate group to the signal molecule, createing P-AI-2, with a decreased receptor binding ability. LsrK added externally to *S. typhimurium*, *E. coli*, and *V. harvei* cultures causes QQ in each case (71). DSF signals are used by many plant pathogenic bacteria, as in *Xanthomonas* species (causing black root) and *X. fastidiosa*, the causative agent of Pierce disease. Co-cultivation experiments have shown that numerous bacteria interfere with DSF signalling. *Pseudomonas*, *Bacillus* and *Staphylococcus* strains also have the carA and carB genes, responsible for the QQ activity (72). Co-infection with bacteria, harbouring high level of DSF degrading activity results in decreased pathogenicity in both black root and Pierce disease as well. The PQS-mediated QS of *P. aeruginosa* can be decreased by the enzyme Hod. A lower pathogenicity has been demonstrated in a lettuce leaf model (73). The AIPs can be inactivated by different kinds of antibodies. For example, Mabs, like AP4-24H11, decreases the production of virulence factors and increases biofilm production (which is down-regulated by QS) in *S. aureus* (74).

1.5.3. Inhibition of signal detection

The inhibition of signal detection is based on different QS signal analogues. These molecules can bind the receptor of the given QS molecule, but do not cause the same effect in gene expression. This competitive binding is a very good alternative for QQ. Analogues can exhibit

different activities, e.g. no activity, pure antagonism, pure agonism, partial antagonism, partial agonism or synergistic agonism (75-77). Inhibition of signal receptors is found in many cases, including AHL, AIP, AI-2, AI-3, PQS and HHQ-based systems.

A wide range of AHL analogues have been tested, including thiolactones (78), lactams (79), urea (80) and triazolyldihydrofuranone (81) based analogues. Many AHL receptors are known, e.g. QscR, LasR, TraR and LuxR. The Mattmann laboratory has tested a plethora of analogues and found molecules which cause antagonism on these receptors (82). The most interesting molecule is S7, which is active against all 4 investigated AHL receptors (**Fig 11**).

Figure 11.
Different AHL receptor antagonists (82)
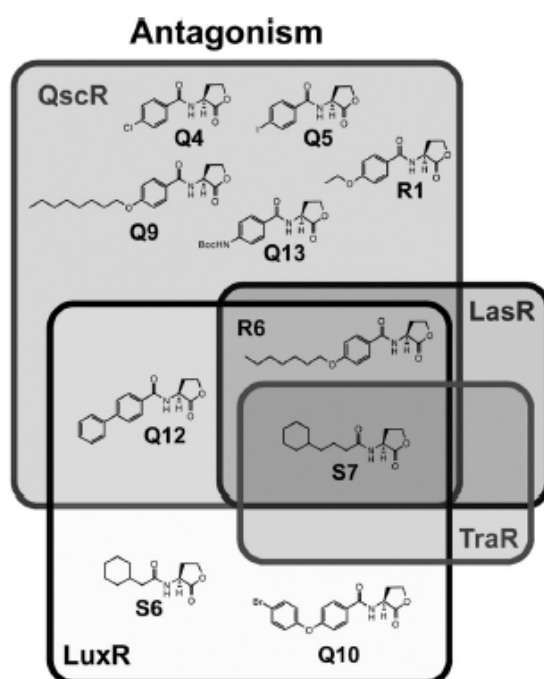
Different halogenated furanones are good inhibitors of both AHL and AI-2 receptors (83-85). Besides brominated and chlorinated furanones, numerous AI-2 receptor inhibitors are known. Pyrogallol is probably the best inhibitor, but different DPD analogues also have a great future (86-88).

The usage of peptide-type AI analogues is a good alternative to quench the QS system of Gram-positive bacteria. The most marked evidence of this is found in *S. aureus*. At least 4 types of *S. aureus* exist as concerns their AIPs. The AgrC receptor responds to its own AIP, but binds the signal molecules from the other groups without modifying the gene expression. This competitive binding ensures the stability of the first colonizing strain (89). One type of

AIP of *S. aureus* can block the QS of the other strains. trAIP-II is a very good inhibitor for all 4 strains (90). Naturally, the AIP antagonists based on Gram-positive QS inhibition are not restricted to *S. aureus*, AIP analogues synthesized against other species as well e.g. *S. pneumoniae* (91).

1.6. Biofilms and QS

Biofilms are complex bacterial communities embedded in extracellular polymeric substances (EPSs). This multicellular lifestyle provides numerous benefits for the bacteria. The EPS matrix protects the bacteria from chemicals, the immune system, predation, desiccation, oxidizing molecules and other damaging agents (92). Division of labour is also evident in multispecies biofilm. In spite of the developed aqueduct structures, there are spaces where oxygen, water and nutrient availability is relatively low. Here, metabolically inactive cells exist, which can recolonize the biofilm after antibacterial attack. The biofilm also prove a good place for genetic information exchange. The EPS holds the microbes close proximity to each other, helping the bacterial-bacterial interactions. The persistence of drug resistance markers and other virulence factors is therefore promoted. Because of these beneficial features, biofilms cause most problematic bacterial infections. The antibiotic resistance inside the biofilm is nearly 1000-fold higher, than in planktonic bacteria. Biofilms have the greatest significance in the following cases: catheter and other device-associated infections (93, 94), urinary tract infections (95), periodontitis (96) and upper respiratory tract infections (97, 98). QS is essential for normal biofilm development in numerous pathogens. The life of a biofilm can be categorized by 3 stages. The first is the attachment, which is followed by the maturation and finally the dispersion. There are examples of the influence of QS in all 3 stages. In the attachment phase, probably the most interesting examples are found in *S. aureus* and *Helicobacter pylori* (*H. pylori*). The AgrC/AgrA QS system of *S. aureus* regulates the level of surface adhesins, which influence the attachment to the host (99). The luxS homologue of *H. pylori* is also implicated in the biofilm attachment. The luxS mutants adhere twice as effectively (100). There is also considerable evidence of the influence of QS-mediated processes in biofilm maturation: in *Serratia liquefaciens* (101), *Burkholderia cepacia* (102), *Aeromonas hydrophila* (103), *Streptococcus mutans* (104). There are also examples of QS mediation of the dispersion; for example, a plant pathogen bacterium, *X. campestris*, uses DSF signals to regulate the biofilm dispersal (105).

1.7. Aims

Antibiotic-resistant and pathogenic bacteria often cause life-threatening infections. To overcome these infections, we wished to know the different mechanisms of various bacterial species and host interactions. In the present thesis, my attention focused on *in vitro* models of interbacterial communication called QS, i.e. the communication system dependent on the size of the population in an *in vitro* model basically related to antibiotic resistance and biofilm formation. The main points studied in the thesis are as follows:

1. First of all, the antibacterial effects of essential oils and other compounds were studied, before studies of the effects of QS signal transmission.

2. As known inhibitors of ABC transporters, the phenothiazines were included in structure-activity studies of QS.

3. The structure-activity-dependent effects of trifluoromethyl ketone (TF) proton pump inhibitors on QS signal transmission were investigated *in vitro*.

4 The interaction with some QS inhibitors was investigated with AHLs.

5. The effect of interference between bacterial population on the QS signal were investigated by using different bacterial strains, including clinical isolates and laboratory strains.

6. The EZF 10-17 grapevine isolate, and its AHL profile were identified.

2. Materials and Methods

2.1. Tricyclic compounds used: promethazine (Pipolphen, EGIS, Hungary), amitriptyline (Teperin, EGIS, Hungary), acridine orange (AO) (Reanal, Hungary), imipramine (Melipramin, EGIS, Hungary), desipramine, chlorprothixene (Truxal, Lundbeck, Denmark), promazine, diethazine (Parkazin, Rhone-Poulenc, France) and desertomycin. Stock solutions of these tricyclic compounds were prepared in distilled water at 25.0 mg/ml before use. Thioridazine (TZ) (Sigma, Madrid, Spain) was used as positive control for the efflux pump inhibition.

2.2. Other chemicals

5-Fluorouracil (5-FU) (Sigma-Aldrich, Budapest, Hungary)

Iodine deoxyuridine (IDU)

Ethidium bromide (EB) (Sigma, Madrid, Spain)

Dimethyl sulfoxide (DMSO)

N-Hexanoyl-DL-homoserine lactone (SIGMA, Budapest).
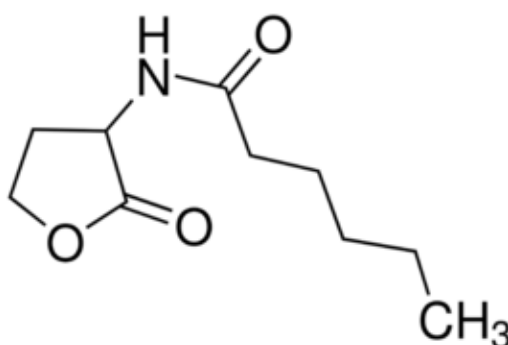
Figure 12.
The N-hexanoil-DL-homoserine lactone molecule (106)

2.3. Trifluoromethyl ketones used: Eleven TFs (*1-9*, *11* and *12*) were synthetized by reaction of the corresponding 2-methylbenzazoles with trifluoroacetic or chlorodifluoroacetic anhydride (107). Compound *10* was prepared by treatment of 2-lithiomethylbenzoxazole with ethyl acetate (108, 109). *N*-Hexanoyl-DL-homoserine lactone was purchased from Sigma (Budapest, Hungary). TFs were dissolved in, DMSO.The structures of the TFs is presented in **Table 1.**

Table 1.

Trifluoromethyl ketones investigated in the study

R^1—[ring]—N—CH$_2$COR^3, R^2, X

	R^1	R^2	R^3	X
1	H	H	CF$_3$	O
2	Cl	H	CF$_3$	O
3	F	H	CF$_3$	O
4	CH$_3$	H	CF$_3$	O
5	CH$_3$O	H	CF$_3$	O
6	Ph	H	CF$_3$	O
7	COOC$_2$H$_5$	H	CF$_3$	O
8	H	COOC$_2$H$_5$	CF$_3$	O
9	H	H	CClF$_2$	O
10	H	H	CH$_3$	O
11	H	H	CF$_3$	NH
12	H	H	CF$_3$	S

2.4. Essential oils used: Rose (*Rosa damascena* L., *Rosaceae*), lavender (*Lavandula angustifolia* L., *Labiatae*), chamomile (*Matricaria recutica* L., *Asteraceae*), orange (*Citrus sinensis* L., *Myrtaceae*), eucalyptus (*Eucalyptus globulus* L.,*Myrtaceae*), geranium (*Geranium robertianum* L., *Geraniaceae*), juniper (*Juniperus communis* L., *Cupressaceae*), citrus (Citrus lemon) and rosemary oils (*Rosmarinus officinalis* L., *Lamiaceae*) were purchased from Phoenix Pharma Ltd. (Hungary). The oils were either used directly (concentrated) or dissolved in DMSO to yield a dilution of 10% (v/v). The stock solution was either used directly or further diluted with 10% DMSO. Both AO and 5-FU were dissolved in distilled water.

2.5. Bacterial strains:

C. violaceum 026 (CV026) served as QS sensor. *C. violaceum* is a common bacterium which lives in soil and water. When *C. violaceum* reaches a high cell density, it produces a purple pigment, violacein (110). The CV026 sensor strain is a Tn*5* mutant which alone cannot synthesize AHLs; it produces the purple pigment only in the presence of externally added inducers (111). This strain has been used to detect a wide range of short-chain AHLs or QS inhibitors (112-114).

EZF 10-17 was isolated from a grapevine crown gall tumour. This strain induced pigment production by CV026 and proved to be efficient for the study of QS interactions (115).

Enterobacter cloaceae (*E.* cloaceae) 31298; a clinical wound isolate, was used as AHL producer.

E. coli wild-type AG100 [argE3 thi-1 rpsL xyl mtl (gal-uvrB) supE44] was employed for determination of the effects of TFs on the activity of the intrinsic efflux pump of this organism (116).

P. aureginosa 49010 was clinical strains isolated from the human trachea.

Strains used in the interference of QS amongst microbial species experients:

Fungi: *Candida krusei, Candida tropicalis and C. albicans*

Bacteria*: Achromobacter xylosoxidans 40502, Acinetobacter baumannii 32703, A. baumanni 42701, Bacillus cereus, Bacillus subtilis, Bacillus clausii, Bacillus megaterium PV 361, Bacillus megaterium MS 941, Bacillus megaterium 216, Staphylococcus epidermidis, S. aureus and E. coli* strains were isolated from extraintestinal infections numbered *5536, 10902, 10904, 11925, 14525, 14584, 18596, 19579, 19672, 24310, 24409, 24442, 33444, 36446* and *40312*.

2.6. Media

LB (Luria-Bertani) medium: containing yeast extract 5 g/l, trypton 10 g/l and NaCl 10 g/l

Modified LB agar (LB*) containing yeast extract 5 g, trypton 10 g, NaCl 10 g, K_2HPO_4 1 g, $MgSO_4$**.**$7H_2O$ 0.3 g and FeNaEDTA 36 mg in 1.0 l of distilled water, Potato dextrose agar (PDA), Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) purchased in powder form from Sigma (Madrid, Spain), Blood agar, complemented with sheep blood

Methods

2.7. QS tests: LB* was used for the experiments. The sensor strain CV026 and the AHL producer strains EZF 10-17, *E. cloaceae* 31298 or *P. aureginosa* 49010 were inoculated as parallel lines and incubated at room temperature (20 ^{o}C) for 24-48 h. QS inhibition was monitored by the agar diffusion method. Filter paper discs (7.0 mm in diameter, Whatmann 3MM) were impregnated with 10 µl of stock solutions of the compounds in distilled water or DMSO. The discs were placed between the parallel lines of sensor and AHL producer strains on the surface of nutrient agar. The plates were incubated at room temperature for a further 24-48 h and the interactions between the strains and compounds were evaluated as concerns

the reduction in size of the zone of pigment production and the zone of growth inhibition of the affected strains, in mm. 5-FU and AO was applied as positive controls (117).

2.8. QS interference tests

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 ºC) 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 a further 24-48 h. LB* medum was 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 ºC, and further incubation being continued at room temperature. QS inhibition was revealed as a decreased level of violacein production by CV026.

2.9. Evaluation of complex formation between N-hexanoyl-DL-homoserine lactone and QS inhibitors:

Biological method: 1 µl of a solution of 5, 10 or 25 ng of AHL was mixed with 10 µl of stock solution (25 mg/ml) of the potential QS inhibitor. Filter paper discs (7.0 mm in diameter, Whatmann 3MM) were impregnated with 11 µl of the mixture of QS inhibitor and AHL solution. The discs were placed upon the inoculation line of the CV026 sensor strain on the surface of nutrient agar medium. The plates were incubated at room temperature (18-22 °C) for a further 24-48 h, and the interactions between the AHL and the tricyclic compounds were evaluated in terms of the reduction in diameter of pigment production, and the reduced colour intensity.

2.10. Taxonomic identification of EZF 10-17 and analysis of its AHL production

The V3 region of 16S rDNA from EZF 10-17 was amplified by using the forward primer (5'-ACTCCTACGGGAGGCAGCAG-3') and reverse primer (5'-ATTACCGCGGCTGCTGG-3') and sequenced. Sequence data were compared and analysed by BLAST to the published 16S V3 sequences available in the database. The AHLs from the liquid culture of EZF 10-17 were extracted and concentrated by using acidified ethyl acetate liquid-liquid extraction. The

purified AHLs were analysed by using thin-layer chromatography (TLC) overlaid with the *C. violaceum* CV026 biosensor strain.

2.11. Minimum inhibitory concentration (MIC) of each TF on CV026 and *E. coli* AG100. The MICs of TFs were determined by the broth dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (118).

2.12. Assessment of the effects of each TF on the activities of the efflux pump systems of CV026 and *E. coli* AG100. The activities of the TFs on the real-time accumulation of EB were assessed by the automated EB method previously described in detail (119), using the Rotor-Gene 3000^{TM} thermocycler with real-time analysis software (Corbett Research, Sydney, NSW, Australia). Briefly, *E. coli* AG100 was cultured in MHB medium until the culture reached an optical density (OD) of 0.6 at 600 nm. The culture was then centrifuged at 13,000 rpm for 3 min, the pellets were resuspended in phosphate-buffered saline (PBS; pH 7.4), with a final concentration of 0.4% glucose, and the OD was adjusted to 0.6 at 600 nm. Aliquots of 45 μl of the cell suspension were distributed to 0.2 ml tubes. The TFs were individually added at concentrations equal to half their MIC against the strain in 5 μl volumes of their stock solutions, and finally 45 μl of EB to yield a final concentration of 1 mg/l (Sigma-Aldrich Química SA, Madrid, Spain) in PBS, with or without glucose, was added. The selection of the concentration of each TF at half its MIC was due to the empirical fact that at this concentration there is no significant effect on the viability of the organism (119, 120). It is also important to note that prior to the experiments described, the maximum concentration of EB which was within the capacity of the bacterium to extrude was determined at least three times. For the wild-type *E. coli* AG100 reference and the CV026 strains employed in the study, these concentrations of EB were determined to be 1 and 0.5 mg/l, respectively (116, 119, 120). The tubes were placed into a Rotor-Gene 3000^{TM} thermocycler and the fluorescence was monitored on a real-time basis. From the real-time data, the activity of the TF, i.e. the relative final fluorescence (**RFF**) of the last time point (at 30 min) of the EB accumulation assay was calculated according to the formula:

$$\textbf{RFF} = \frac{\textbf{RF}_{treated} - \textbf{RF}_{untreated}}{\textbf{RF}_{untreated}}$$

where $\mathbf{RF}_{treated}$ is the relative fluorescence at the last time point of the EB retention curve in the presence of an inhibitor, and $\mathbf{RF}_{untreated}$ is the relative fluorescence at the last time point of the EB retention curve of the untreated control. The greater the difference between $\mathbf{RF}_{treated}$ and $\mathbf{RF}_{untreated}$, the greater the degree of EB accumulated, and therefore the greater the degree of inhibition of the efflux pump system of the bacterium promoted by the agent at that concentration.

The $\mathbf{RFF}$ was then divided by the concentration of the TF that corresponded to half its MIC. This yielded a measure of the effect of each TF at a milligram level (specific activity) and therefore afforded comparison of each TF for activity against the efflux pump systems of the CV026 and *E. coli* AG100 strains. The experiments were repeated three times and the specific activity values presented are the averages of three independent assays. This method of analysis has been described previously (121). TZ an efflux pump inhibitor (122), served as a positive control.

3. Results

3.1. QS inhibition of essential oils

The agar diffusion method provides a convenient and semiquantitative method with which to assess antibacterial activities and QS signal production in the presence of essential oils. Using this method, we tested 9 essential oils compared to the positive controls of AO and 5-FU. Most of these oils are known as inhibitors of bacterial growth (123). Our data demonstrate that several plant-derived essential oils not only inhibit bacterial growth, but also block QS regulation processes.

As evident from Fig. 13, DMSO did not affect bacterial growth or AHL-induced violacein production. As demonstrated by Fig. 14, the positive controls 5-FU and AO produced the anticipated inhibition of QS. However, the inhibitory activity of 5-FU was considerably greater than that of AO.

The use of discs impregnated with DMSO containing various essential oils afforded an understanding of which oils inhibited the QS response of CV026 to AHL released by the bacteria employed in this study. As examples, although geranium oil was most effective as an inhibitor of the QS response of CV026 (Fig. 15a) following induction by the AHL-producing strains *E. cloaceae* 31298 and EZF 10-17, rose oil also displayed significant inhibitory activity on QS responses (Fig. 15b). Rose, lavender and rosemary oils also inhibited the

colour development to varying degrees (Table 2). Eucalyptus oil inhibited violacein production only after induction with *E. cloaceae* 31298, while citrus oil reduced the colour formation only in the CV026 + EZF 10-17 combination. As expected, the oils also inhibited bacterial growth to variable degrees. The other tested essential oils too exhibited weak antibacterial activity on CV026 but the growth of the AHL-producing bacteria was not affected. QS was moderately inhibited by lavender, eucalyptus and citrus oils, while chamomile, orange and juniper oils were ineffective (Table 2).

Figure 13.

Negative control: DMSO did not affect colour production by the QS sensor *C. violaceum* CV026 in response to an AHL-producing bacterium.

A **B** **C**
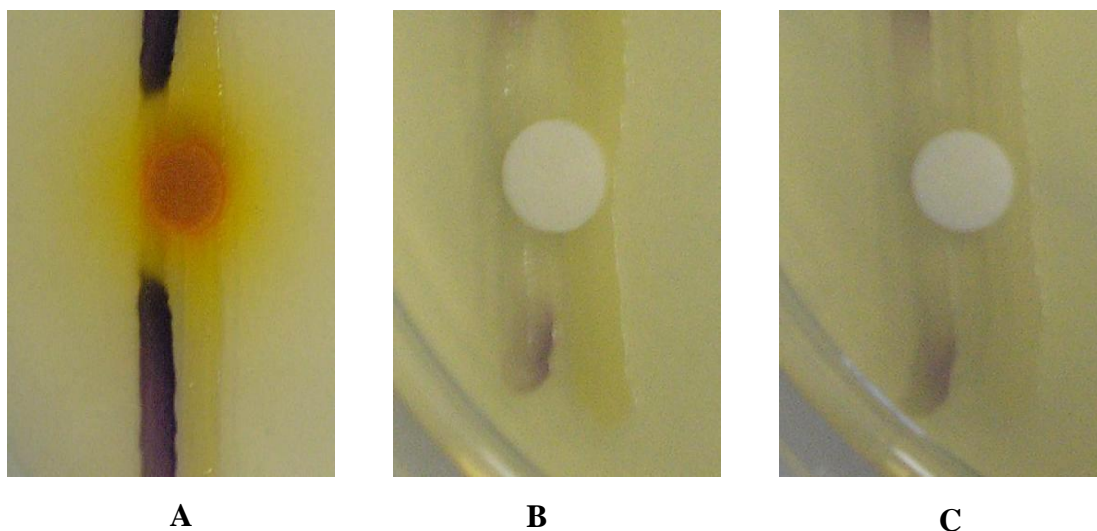
Figure 14.

Positive controls: Disc impregnated with AO (left) or 5-FU (middle, right) inhibited the production of colour by *C. violaceum* CV026 (left lines) in response to the AHL-producing bacterium EZF 10-17 (Fig. 2a,b) and *E. cloaceae* 31298 (Fig. 2c).

Figure 15a.
Effect of geranium oil on QS. The disc contained 10 µl geranium oil.

Figure 15b.
Effect of rose oil on QS. The disc contained 10 µl rose oil.

Table 2. Effects of tested essential oils on QS signal production between *C. violaceum* CV026 as sensor, and *E. cloaceae* 31298 and EZF 10-17 as producer strains after a 48 h incubation.

Essential oils	10% oil		100% oil	
	CV026 + *E. cloaceae* 31298	CV026 + EZF 10-17	CV026 + *E. cloaceae* 31298	CV026 + EZF 10-17
Rose	15-20	10	18	20
Lavender	0	5	10	15
Chamomile	0	0	0	0
Geranium	0	3	13	15
Eucalyptus	0	3	6	0
Juniper	0	0	0	0
Citrus	0	2.5	0	16
Orange	0	0	0	0
Rosemary	0	3	18	18

The numbers in the Table indicate the sizes of the inhibition zones in mm

3.2. QS inhibition of tricyclic compounds

The agar diffusion method provided an opportunity to investigate the potential inhibition of
tricyclic compounds on QS between the QS sensor *C. violaceum* and 3 AHL-producing
bacteria. For control experiments, EZF 10-17 adjacent to the QS sensor CV026 cultured in the
presence of a water-containing disc resulted in the purple colouration of the swabbed sensor,
an indication of a positive response to the presence of AHLs produced by EZF 10-17. To
demonstrate that the colouration of the sensor strain is prevented by a known inhibitor of the
QS response, 5-FU and AO were used as positive controls. Since the phenothiazines exert
antimicrobial activity against *E. coli* (124), the inhibition of violacein production as QS signal
on the growth of inoculated bacteria was measurable only at subinhibitory concentrations.
Consequently, the production of AHL as a QS mediator was evaluated below the MICs of the
phenothiazines against the AHL-producing bacteria.

 The amount of each phenothiazine placed onto the disc was chosen so that it did not produce
any antimicrobial effect against the given bacterium (less than one-half of the MIC). In
summary, amitriptyline, desipramine, imipramine and promethazine inhibited the production
of the purple colour by the sensor CV026 strain. Although the concentration of each
phenothiazine was identical, the degree of inhibition varied as the distance between the disc
and the margins of the diameter of the produced colouration. **Table 3** shows the antibacterial
effects of the phenothiazines: the diameters of the growth inhibitory zones are indicated in
mm.

Table 3.

Investigation of antibacterial effects of tricyclic compounds with the agar diffusion method.

Tricyclic compounds	Growth inhibition zones in mm					
	EZF 10-17	*E. cloaceae 31298*	*P. aeruginosa 49010*	*CV026 (+EZF 10-17)*	*CV026 (+ E. cloaceae 31298)*	*CV026 (+ P. aeruginosa 49010)*
AO	-	-	-	-	-	-
Amitriptyline	8	-	-	9	10	12
Desipramine	9	7	-	11	16	9
Imipramine	-	-	-	9	9	7
Diethazine	7	-	-	9	7	8
Promazine	8	-	-	10	12	15
Promethazine	9	7	-	11	15	10
Chlorprothixene	-	-	-	7	7	7

10 µl of a 25 mg/ml solution was added to the filter paper discs

To study the effects of phenothiazines and structurally related compounds on QS, the tricyclic compounds were examined in a concentration below the growth inhibitory doses. The inhibition of QS signal transmission was measured as described in the Methods section. The production of colourless zones by the phenothiazine compounds impregnated into the filter paper discs was measured around CV026, in response to the inhibited AHLs produced by EZF 10-17, *E. cloaceae* and *P.aeruginosa.* As shown in **Table 4**, the degree of inhibition of purple colour formation by CV026 in response to the AHLs produced by the various bacteria differed, indicating that the effectivities of the tricyclics differed as regards the QS response to the sensor CV026.

Table 4.

Inhibitory effects of tricyclic compounds on QS signal transmission.

Tricyclic compounds	QS inhibition zone in mm		
	EZF10-17	*E. c. 31298*	*P. a. 49010*
AO	14	12	14
Amitriptyline	64	30	60
Desipramine	26	26	-
Imipramine	15	12	-
Diethazine	18	12	-
Promazine	13	13	20
Promethazine	26	20	15
Chlorprothixene	-	-	-

10 µl of 25 mg/ml stock solution was impregnated into the filter paper discs (25 µg/disc) and the colourless zone around the *C. violaceum* was measured after incubation for 24-36 h at room temperature.

The mechanism of QS inhibition was studied by measuring the effects of the direct interactions between the AHL and QS inhibitors on violacein production in the bioassay. The most effective inhibitors were imipramine, promethazine, desipramine and amitriptyline, which in high concentration inactivated the AHL and probably formed micellar complexes. The activities of the complexes between AHL and its inhibitors were measured in AHL-specific chromogenic tests. The biological activities of the AHL complexes were apparently reduced by complex formation, compared with the uncomplexed control AHL (Table 5).

Table 5.

The effects of QS inhihibitors on N-hexanoyl-DL-homoserine lactone (C6 HSL)-mediated violacein production.

Quantity of C6 HSL	0 ng	5 ng	10 ng	25 ng
Tested compounds	Diameter of colouration in mm (18 h)			
IDU 10 µg	0	15	25	30
Desertomycin 200 µg	0	22	25	30
5-FU 30 µg	0	29	35	35
Promethazine 250 µg	0	0	30	33
Desipramine 250 µg	0	0	28	32
Promazine 250 µg	0	0	30	32
Imipramine 250 µg	0	0	20	26
Amitriptyline 250 µg	0	0	25	30
C6 HSL control	0	25	34	35

3.3. Identification of EZF 10-17 and its AHLs

In our previous studies, the unidentified grapevine tumour isolate EZF 10-17 proved to be an inducer of violacein production by *C. violaceum* CV026. Thus, this pair of inducer/sensor strains was successfully used to study potential QS inhibitors (115, 125). To identify EZF 10-17, we sequenced the V3 region of the 16S rDNA gene from its genome. On comparison of the sequence data with those found in the databases, this strain proved to be a member of the *Sphingomonadaceae* family.

The signal production of EZF 10-17 was analysed by TLC overlaid with *C. violaceum* CV026. As compared with the standard AHLs, EZF 10-17 produces a strong signal that co-migrated with 3-oxo-C6 AHL. Additionally, weaker signals which seemed to be identical to C6 AHL, 3-oxo-C8 AHL and C8 AHL were also observed (**Figure 16**). These data support our earlier observations on the suitability of EZF 10-17 in QS assays with *C. violaceum* CV026 (125).

Figure 16. Detection of AHLs from EZF 10-17.
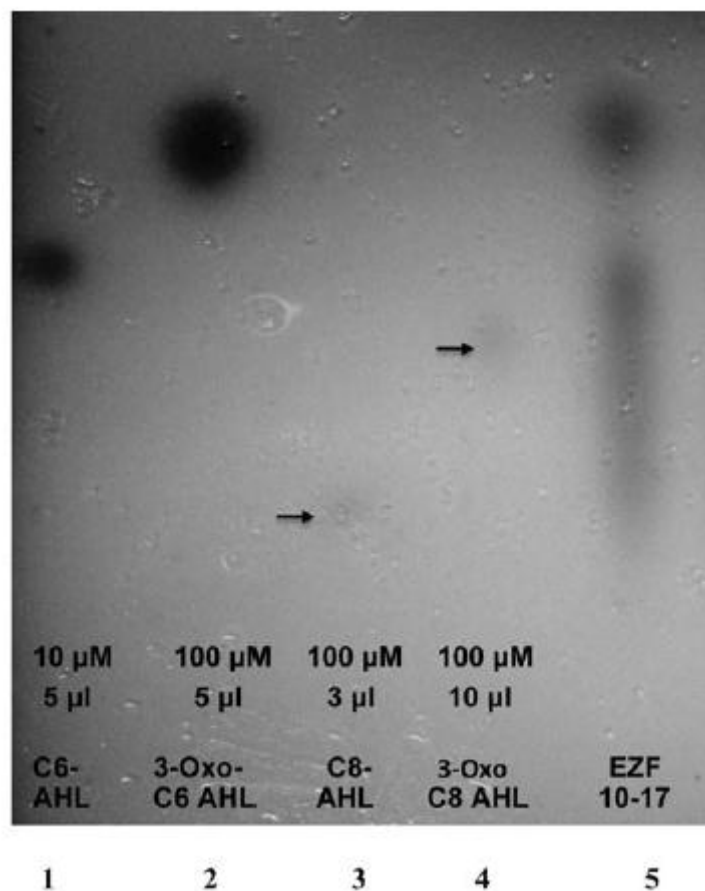
Legend. Pure AHLs (lanes 1-4) and purified AHL produced by EZF 10-17 (lane 5) isolated by TLC. The plate was overlaid with the responder CV026. The application of varying concentrations of pure AHLs provided standards of the degree of response (intensity of colour)

3.4. QS and efflux pump inhibitor activity of TF compounds

In order for an agent to be correctly evaluated for effects on a QS system whose intensity of colour is dependent upon the growth of the producer of the QS signal and the growth of the responding bacterium, the concentration of the agent that is to provide a meaningful interpretation must be one that does not affect the viability of either bacterium. As summarized in **Table 6,** various TFs displayed antimicrobial activities against the producer and responding bacteria.

Table 6. Inhibitory effects of the TFs on QS and the growth of producing and QS sensor bacterial strains, after 24 h of incubation.

TF	MIC (µg/ml)		Extent of QS inhibition (zone of discolouration) at 20 µg/disc (mm)
	CV026	EZF 10-17	
1	50	25	26
2	12,5	25	28
3	12,5	12,5	30
4	50	50	20
5	50	50	16
6	>200	>200	0
7	>200	>200	0
8	>200	>200	0
9	50	25	29
10	>200	>200	0
11	>200	>200	0
12	>200	>200	0

The extent of the colourless zone indicates the inhibitory effect of the given compound on QS signal transmission. (CV026: *C. violaceum* 026)

Consequently, the amounts of TFs selected for the evaluation of effects on the QS system were at or below those that had no effect on the growth of either species. Because of the limitations of space, the range of effects of the TFs on the QS system cannot be presented pictorially. Rather, the effects are illustrated in **Figure 17** and **Table 7.** Briefly, TF *5* had the least inhibitory effect (a deep colour associated with the responder CV026) and TF *3* had the greatest inhibitory effects on the response of the CV026. The effect of the TFs on the QS system were clearly inhibitory. Whether the effect is due to the TF inhibiting the release of the QS signal or due to the inhibition of the response of the responding species cannot be distinguished from the above evaluation.

The direct effects of each TF on the QS response by CV026 were determined with the use of discs impregnated with combinations of a constant amount of the AHL and differing amounts of TF. The discs with TF alone did not produce the purple colour associated with CV026. The presence of pure AHL in the disc led to the production of the deep purple colour associated with CV026. The presence of the TF that inhibited the production of colour in the QS assay described, when in combination with the AHL, inhibited the production of the purple colour by the responding CV026.
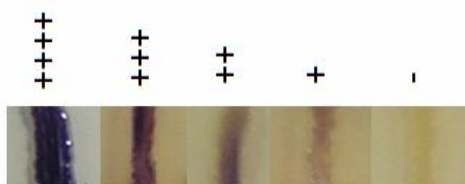
Figure 17.

The length and intensity of the purple colouration induced by 10 ng AHL/disc on *C. violaceum* 026, after a 24 h incubation in the presence of TFs applied at 20 µg/disc. The effective inhibitors reduced the length and intensity of the purple colouration. The scale indicates an increasing intensity of colouration. A lower colour intensity means a greater inhibition of QS.

-, No colouration; +, white-purple; ++, pale-purple; +++, purple; ++++, dark-purple;

Table 7.

Effects of TFs on AHL-mediated QS.

TF 20 µg/disc	Intensity of colouration (mm)	Length of colouration (mm)	Growth inhibition (mm)
1	+++	34	0
2	+	30	17
3	+	34	22
4	+++	35	0
5	+++	37	0
9	+	36	22
AHL control	++++	40	0

Intensity scored as described in Figure 17

These results clearly show that the TF has a powerful inhibitory effect on the QS responding strain. However, the question of whether this same TF can inhibit the secretion of the QS signal by the producer species remains unanswered. The activity of each TF at half its MIC on the efflux pump system of the CV026 is exemplified by **Figure 18** (126).

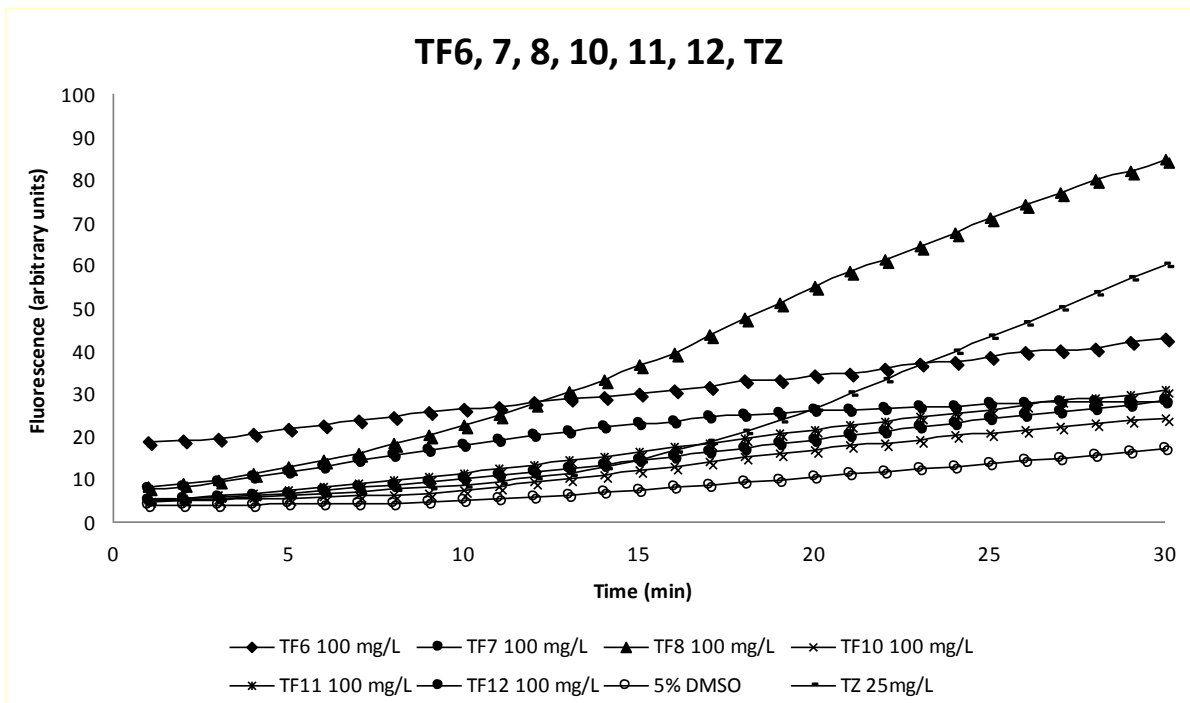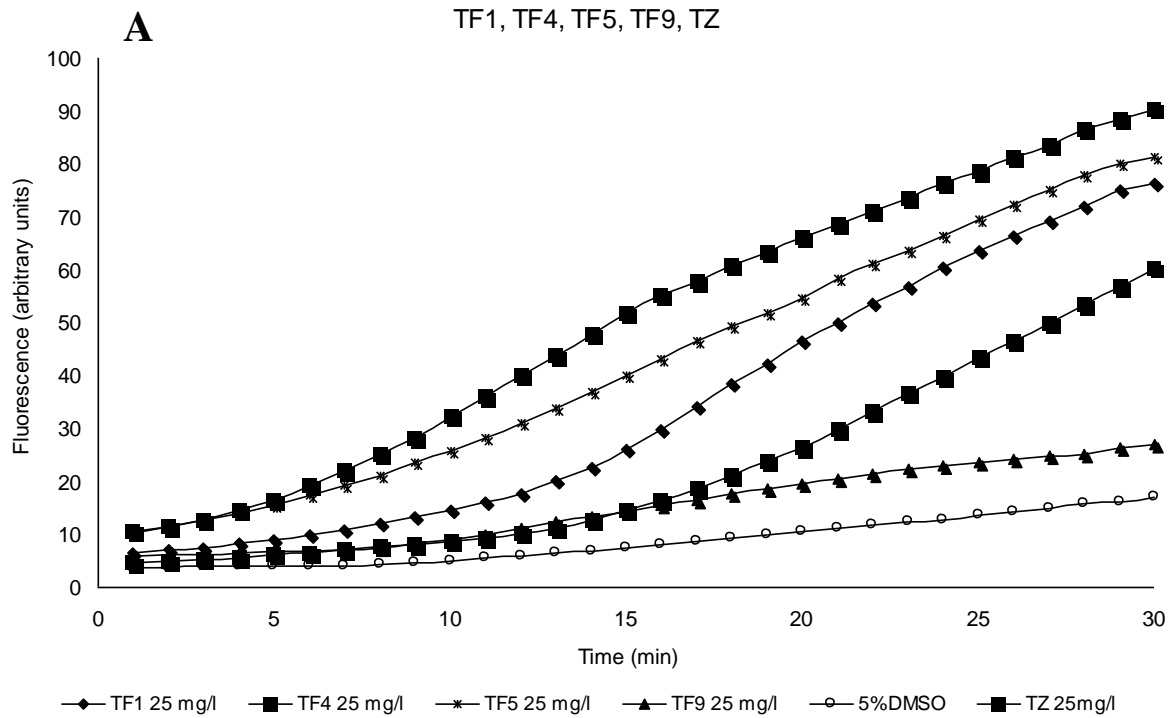**Figure 18.** The effects of half the MIC of TFs 1, 4, 5, 9 (A), and 6, 7, 8, 10, 11, 12 (B) as compared with the positive phenothiazine control TZ.

The data presented by these Figures suggest that all of the TFs have activity against the efflux pump of CV026. However, as the concentrations of TFs used in the assay were half of their MICs, and the MICs of the TFs against the strain differed significantly, the activities shown in **Figure 18** do not permit a comparison of the activities between the TFs. However, calculation of the specific activity of each TF by the formula for RFF presented in the Materials and Methods section affords a comparison, and these data are presented in **Table 8**.

Table 8. The related final fluorescence (RFF) and specific activity (SA) of each (TF) on the efflux pump system of CV026.

TF		RFF	SA
1 (25 mg/l)		4.20	**1.68**
2 (6.25 mg/l)		2.99	**4.78**
3 (6.25 mg/l)		2.21	**3.53**
4 (25 mg/l)		4.42	**1.77**
5 (25 mg/l)		5.10	**2.04**
6 (100 mg/l)		4.31	**0.43**
7 (100 mg/l)		0.92	0.09
8 (100 mg/l)		1.24	0.01
9 (25 mg/l)		3.97	**1.59**
10 (100 mg/l)		0.44	0.04
11 (100 mg/l)		0.73	0.07
12 (100 mg/l)		1.18	0.12

Samples consisted of saline plus 1 mg/l of EB, 0.4% glucose, without or with half the MIC of the positive control and TFs. The fluorescence was assessed at 37 °C for 30 min. Data in bold indicate TFs that express very high inhibitory activity against the efflux pump system of CV026. SA=RFF/(0.5 MIC).

Briefly, the activity of the positive control TZ was 1.02. On comparison of the activity of each TF relative to the positive control, TFs-*2* and *3* prove to exhibit the greatest activities against the efflux pump system of CV026; TFs-*1, 4, 5* and *9* displayed significant activity; and TFs-*7, 8, 10, 11* and *12* had no activity. The facts that the TFs inhibited the response of an environmental strain to a QS signal and the same TFs inhibited the efflux pump of the environmental responding strain themselves did not support clinical interest in the TFs for possible use in the therapy of a bacterial infection. Therefore, in order to establish the necesarry support for the claim that the TFs do indeed have clinical value, the TFs were examined for activity against the efflux pump system of *E. coli*, a pathogenic bacterium. Since a large number of graphs would be needed to depict each effect, an example of the data obtained is presented in **Figure 19** for TF-*4*. As evident from the Figure, the presence of TF-*4*

promoted an increase of the fluorescence due to the accumulation of EB, whereas in the absence of the compound there was no significant increase of fluorescence (the curve is rather flat).

▲ *E. coli* AG100 control; ■ *E. coli* AG100 and TF-*4*
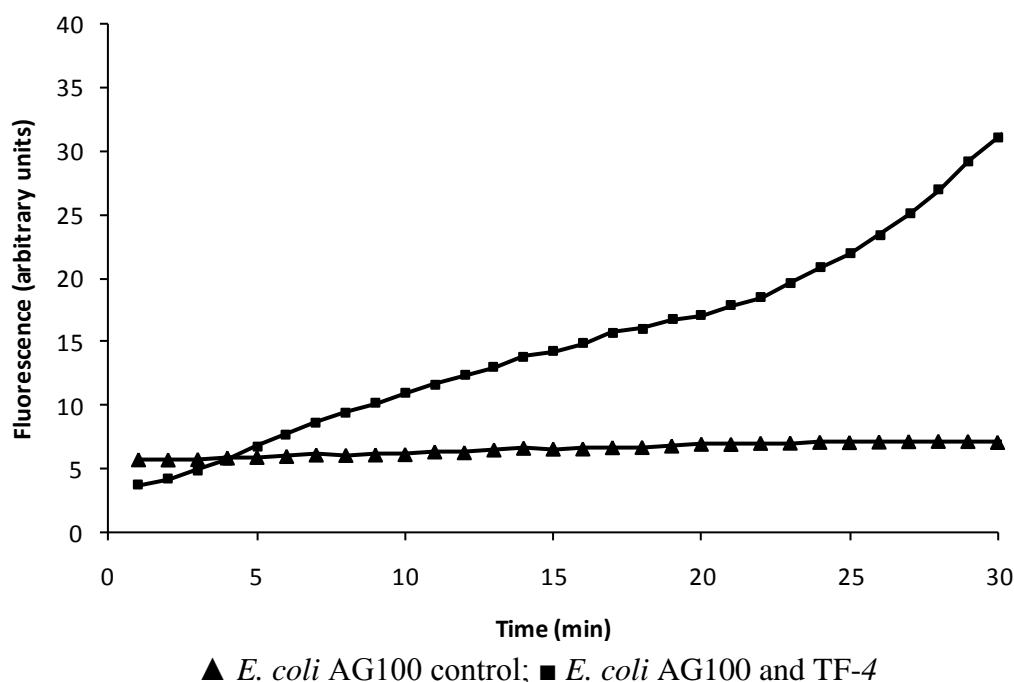
Figure 19. The effect of TF-4 on the activity of the efflux pump system of *E. coli* AG100. The concentration of TF-*4* that corresponded to half the MIC was 30 mg/l. Note that the control did not accumulate EB during the 30 min of the assay.

The effects of the TFs on the activity of the efflux pump are summarized in **Table 9.** This Table provides the concentrations of the positive control TZ and each of the TFs that corresponded to half their MIC. As noted in **Table 9**, TFs-*2* and *3* exerted the highest activities against the efflux pump system of *E. coli*. TFs-*1, 4, 5* and *9* were also very active since their inhibitory activities exceeded that of TZ, the efflux pump inhibitor that served as positive control.

Table 9. The effects of (TFs) on the efflux pump system of *E. coli* AG 100.

Sample	RFF	SA
AG100 control	0.5397	0.000
+ TZ positive control. 30 mg/l	11.8703	0.6998
+ TF-1 7.5 mg/l	5.0638	**1.1176**
+ TF-2 3.75 mg/l	12.9179	**6.1160**
+ TF-3 3.75 mg/l	10.4916	**4.9172**
+ TF-4 30 mg/l	31.1324	**1.8894**
+ TF-5 7.5 mg/l	7.8839	**1.8143**
+ TF-6 30 mg/l	5.1950	0.2875
+ TF-7 240 mg/l	7.2390	0.0517
+ TF-8 60 mg/l	6.9595	0.1982
+ TF-9 7.5 mg/l	6.4451	**1.4589**
+ TF-10 240 mg/l	19.3553	0.1452
+ TF-11 120 mg/l	6.6206	0.0938
+ TF-12 60 mg/l	7.0865	0.2021

RFF: relative final fluorescence; SA: specific activity = RFF/(0.5 MIC).

Samples consisted of saline plus 1 mg/l of EB, 0.4% glucose, without or with half the MIC of the positive control and TFs. Fluorescence was assassed at 37 °C for 30 min. Data in bold idicate TFs that expressed very high inhibitory activities against the efflux pump system of *E. coli.*

3.5. Bacterial-bacterial interactions in QQ

Our results reflect *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 10** and **11**), of which 2 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 (**Fig. 20**), while *B. subtilis* and *B. clausii* inhibited QS moderately, and the 3 *B. megaterium* strains (PV361, MS941 and 216) did not exhibit any QS inhibitory activity (Table 10). Surprisingly, 14 of the 15 investigated *Escherichia* clinical isolates were effective inhibitors, and only 1 had no inhibitory effect. We investigated the QS inhibitory activity between *E. cloaceae* 31298 and CV026 and also that 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 11**). 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.
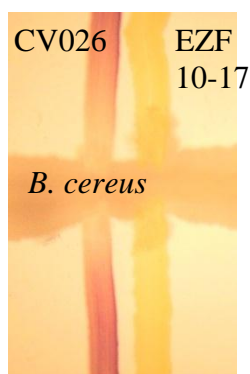
Figure 20.
QS inhibitory activity of *B. cereus* in the system containing the CV026 sensor and the EZF 10-17 producer strain. The inhibition of QS is revealed in the decreased level of violacein production.

Table 10.
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	-

All of the 15 tested E. coli isolates except strain 19579 modified the QS (Table 11). The most exciting strains were 5539, 24310, 33444 and 40312, which strongly inhibited the established

QS system, but the tested isolates did not affect the growth of the indicator and 2 producer strains (Fig. 21).

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

Table 11.

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

Figure 21.
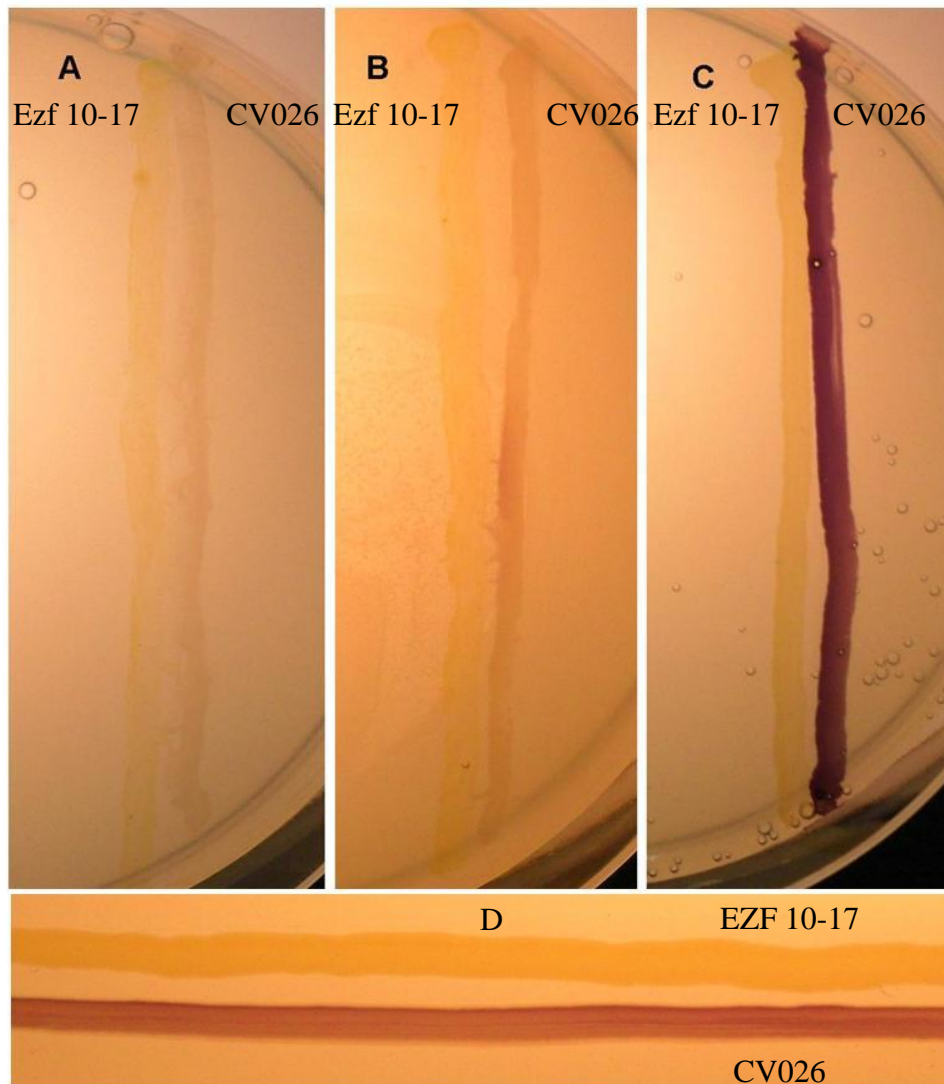The interference of different *E. coli* strains with the QS signal between the CV026 sensor and the EZF 10-17 producer strain.
The sensor and producer strains are situated on the top of the medium containing different *E. coli* strains.
A: Strain 5536 lacks QS between the sensor CV026 and producer EZF 10-17 strains.
B: Strain 11925 has high QS-inhibitory activity.
C: Strain 19579 has no QS-inhibitory activity.
D: Control.

4. Discussion

The discovery of QS has opened up new perspectives in modern microbiology. QS is necessary in many crucial bacterial features e.g. biofilm formation (127), virulence factor production (128), competence (1), sporulation (129), antibiotic production (130) and resistance (131). Without communication, the microbes are lose the ability to fight as a unit against the immune system, produce virulence factors or organize biofilms. On the whole, it is impossible for most the bacteria to cause infections without QS. On the other hand, the increasing level of antibiotic resistance is currenly the greatest problem in microbiology. Most bacteria tolerate at least a few antibiotics, and, unfortunately, numerous multiresistant strains have appeared, which have also adapted to the latest antibiotics. The investigation of QS is therefore of increasing significance. In the future, QS inhibitors may offer a new perspective in the fight against multiresistant infections and take over the function of decaying antibiotics. Numerous compounds have been investigated in this search of QS inhibitors. Three main categories, essential oils, tricyclic compounds, and TFs proved to be the best inhibitors. These compounds and bacterial-bacterial co-existence are discussed below.

Essential oils: Land plants have co-evolved with microorganisms during 700 million years ago (132). Plants therefore produce various molecules which have effects on bacteria. Some attract microbes with beneficial effects, some are antibacterials, and some influence the bacterial QS (133). Because of the long co-evolution, probably the best source of different QSI compounds is to be found in plants. We investigated numerous essential oils and pure compounds for their QS inhibition. Many of them exert a QSI effect and it was found that most ethnomedical plants used against bacterial infections, also have a QSI ability. Purified plant QSI compounds, e.g. ajoene from garlic or thymol from thyme, in combination with antibiotics, will probably prove a good alternative to fight against complex bacterial infections.

Tricyclic compounds: The effects of tricyclic compounds on various Gram-positive and Gram-negative bacteria and R-plasmid elimination were examined earlier (124, 134), and the inhibition of horizontal plasmid transfer due to the blocked conjugation (135), antibacterial and plasmid curing in co-existing bacterial populations is known. The importance of tricyclic compounds as a class of "non-antibiotics" which may provide alternatives to the ineffective therapy of serious bacterial infections has already been reviewed (136). Despite this considerable body of evidence, the effects of tricyclic compounds on QS have not been

studied before. Obviously, if the specific QS system is blocked during the bacterial infectious process, the therapy of serious infections may be improved. In view of the demonstration that certain tricyclic compounds can block the QS signal response of diverse bacterial species, it is conceivable that in the future these compounds may be used to modify biological signal transmission at a population level, e.g. by the modification of antibiotic resistance in biofilms, the production of virulence factors and the antibiotic production of some microbes in combination with conventional therapy to reduce the virulence of certain types of bacterial infections.

TFs: The results presented in this study show that some TFs have inhibitory activities against the response of CV026 to a QS signal such as AHL and the efflux pump systems of the CV026 and *E. coli* strains. Comparison of the efflux pump inhibitory activities of the TFs towards both strains suggests that the inhibition is practically identical in each case. This suggests that the TFs are of clinical value. Comparison of the individual inhibition induced by each TF on the QS response by CV026 to AHLs indicates that it is TF-*3* that exerts the greatest inhibition on the QS response. From comparisons of the MIC of each effective TF, it is clear that the most effective inhibitors of the efflux pump system of *E. coli* also have the most potent antibacterial activities. TFs such as TF-*7, 10* and *11* are devoid of any significant antibacterial activity (the MIC for TF-*7* and 10 is 480 mg/l, and for TF-*11*, it is 240 mg/l) and have little activity against the efflux pump of *E. coli*. The demonstration of a QS response by the method used in this study requires the growth of the responding organism. If an agent inhibits growth, it pre-empts any response since there are no bacteria present to respond. The application of 20 μg of each TF to discs promoted strong antibacterial effects by TFs with very low MICs. Applying amounts of a TF that has significant antibacterial properties to a disc below the inhibitory concentration exceeds the sensitivity of the system, since the distance between evident growth and the absence of a response (no colour) is masked by the deep purple colour associated with the growing population that is less than a millimetre from the disc. This is why TFs with very high MICs were able to produce evidence of an inhibition of the QS response by CV026, whereas for TFs with low MICs, with the exception of TF-*3* (MIC 7.5 mg/l), the antibacterial effect of the TF pre-empted growth. Our previous study demonstrated that phenothiazines were able to inhibit the QS system that involved EZF 10-17, the producer of the signal and CV026, the responder to the signal (125). The phenothiazine TZ, an inhibitor of efflux pumps of Gram-negative bacteria (119-122, 137), also inhibits the response of CV026 to pure AHL. Since phenothiazines and TFs that inhibit the efflux pumps of the CV026 and *E. coli*, as shown in the current study, also inhibit access to energy supplied

by the proton motif force (PMF) (138,139), we believe that the response to a QS signal depends upon a functional efflux pump system that deflects the noxious QS signal before it reaches its intended target. Moreover, as all the secretory activity of a bacterium, such as the secretion of a QS signal, is controlled (140-142), and the main efflux pump systems of Gram-negative bacteria are the secretory paths of internally produced noxious agents (143-146), inhibition of the efflux pump system of a QS signal producer will result in obviating the secretion of the QS signal. Therefore, it is our contention that inhibitors of an efflux pump, such as phenothiazines and now also TFs, will inhibit the QS. The results of the current study clearly show that various TFs have the ability to inhibit the response of *C. violaceum* 026 to the QS signal AHL and to inhibit the efflux pump of the QS-responding CV026 and that of *E. coli*. This ability is a direct one since none of the TFs formed a complex with AHL. In view of the fact that some TFs inhibit the QS response, inhibit an efflux pump system and also have significant antibacterial activity, TFs have a promising future for the therapy of problematic infections that rely on an efflux-mediated multidrug-resistant phenotype and which, due to QS, make their therapy problematic.

Bacterial interactions: 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 co-exist with their neighbours and to maintain their own optimum population level. They may gain benefit from the QQ 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 (139 Molnár). Nature provides numerous examples of bacterial-bacterial and eukaryotic-bacterial interactions (63,64 148-153). As an example, a furanosyl borate diester, AI-2, 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, *P. 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 (149). 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 (147). 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 (150), whereas that of Streptomyces sp. strain M664 exerts a high AHL-degrading effect on AHLs with a short acyl side-chain (64). 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 molecules which compete with the AHL signals of CV026, or metabolize the signal molecules, or use systems such as the AI-2 importers. *E. coli* possesses a special strategy to compete with the AI-2 signals of other bacteria. *E. coli* strains have AI-2 specific importers, which are activated at a high level of the inducer molecules. The import of AI-2 eliminates these signals from the extracellular environment (148). These importers probably play a role in the AHL-dependent QS-inhibitory effects too in our system. Another, and 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 (154, 155).

In spite of the fact that most QSI investigations, including my own studies, have been *in vitro* experiments, numerous *in vivo* studies underpin the effectiveness of anti-QS treatments. Two excellent examples are found in the serous pathogens: *V. cholerae* and *S. aureus*. The QS system of *V. cholerae* is really interesting. It promotes virulence factor production in low cell density, and switches to the dispersion state at high cell density. The application of externally added AIs can theoretically abolish cholera toxin production. Douan and March created an *E. coli* Nissle strain wich expresses the CAI-1 gene (156). This probiotic strain has proved very effective in *in vivo* mouse models. This experiment not only reveals a new treatment of cholera, but also highlights the potential power of probiotic bacteria to serve as AI vectors. Another example is found in *S. aureus*. These dangerous, highly antibiotic-resistant bacteria can be categorized in several groups as concerns their signal molecules. At least 4 types of *S. aureus* exist as concerns their AIPs. The AgrC receptor responds to its own AIP, but binds the

signal molecules from the other groups without modifying the gene expression. This competitive binding ensures the seperiority of the first colonizing strain (89 Ji G 1997). The Lyons group synthetized an antagonist molecule traAIP-II, which inhibit the QS in all the 4 groups. It also inhibits the toxin production of the pathogen in mouse models (90 lyon 2000). To summarize, in the past nearly 40 years we have learned much about bacterial communication, but in my opinion what we see is nearly the tip of the iceberg. QS is still one of the most rapidly developing fields in microbiology. Many QS molecules, receptors or complete systems and QSI compounds are already known, but the great breakthrough is still to come. I fully expect that, in the near future, QSI based treatments against complicated bacterial infections will become available in human medicine.

The following of our results are considered novel:

The QSI activity of the investigated essential oils

The QSI activity of phenothiazines

The QSI activity of TFs, and the connection between efflux pump inhibitor activity and QSI activity of TFs

The AHL profile of and identification of EZF 10-17

The interaction of *E. coli* with AHL-based QS systems

5. Summary

QS is population size-dependent gene regulation, mediated by small signal molecules called AIs. The natures of these molecules are very different, but most belong in 3 groups: AHLs are mainly used by Gram-negative bacteria, autoinducer II (AI-2) is used by both Gram-negatives and Gram-positives, and AIPs are used by Gram-positives. Some bacteria use AIs which do not fit these groups, e.g. the PQS, DSF or AI-3. QS is a widely prevalent feature of both Gram-negative and Gram-positive bacteria. There are many different types of QS systems, but each system relies on the same basics. Each bacterium synthesizes signal molecules in low amounts. When the population density is low, the number of signal molecules is also low, when the number of bacteria increases, the concentration of QS molecules increases. When the concentration of signal molecules exceeds a threshold concentration, a positive feedback starts, and the bacteria increase the production of signal molecules. At this very high concentration of QS molecules, they bind their receptors. The nature of the receptors can differ considerably in each case, but in the end of the process is the same: the initiation of target gene transcription. QS is necessary in many crucial bacterial features e.g. biofilm formation (127), virulence factor production (128), competence (1), sporulation (129), antibiotic production (130) and resistance (131). Without communication, the microbes are lose the ability to fight as a unit against the immune system, produce virulence factors or organize biofilms. In the present thesis, my attention focused on in vitro models of QS to investigate QSI alternatives. Numerous compounds have been investigated in this search of QS inhibitors. Three main categories, essential oils, tricyclic compounds, and triofluoroketone proton pump inhibitors (TFs) proved to be the best inhibitors. The bacterial-bacterial co-existence also investigated. 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. Antibiotic resistance is the most important problem in modern microbiology. Bactericidal or bacteriostatic compounds exert strong selective pressure on microbes. Because of the irresponsible use of antibiotics, pathogenic bacteria nowadays often display resistance to at least a few medicines. The appearance of multidrug-resistant strains which tolerate the latest antibiotics too enhances this problem. There is an urgent need to discover new antimicrobial, antipathogenic and antivirulence drugs. QS inhibitors may be a good alternative to solve this situation.

6. Összefoglaló

A „quorum" egy görög eredetű szó, igazán jó magyar megfelelője nincsen. Az ókori görög és római fórumokkal lehet összefüggésbe hozni. A döntéshozás ezen színterein, egy törvény elfogadásához vagy elvetéséhez létre kellett jönnie a quorumnak, azaz egy minimális többségnek ami a szavazás eredményességéhez szükséges. A quorum sensing, lényegében a mikrobák között sem zajlik másként, rengeteg olyan bakteriális gént ismerünk, amely csak bizonyos sejtkoncentráció felett aktiválódik. Számos különféle QS rendszert és jelmolekulát ismerünk, de a működési elvük minden esetben azonos alapokon nyugszik. A baktériumok egy alap szinten mindig termelnek jelmolekulákat. Alacsony mikrobaszámnál a szignálmolekulák száma is alacsony marad, és nem indukálnak változást a génkifejeződésben. A baktériumszám növelésével a jelmolekulák száma is növekszik, amint elérik a küszöbkoncentrációt kötődnek a receptoraikhoz. A receptor típusok és szignál transzdukciós utak szintén nagyon változatosak, de végső soron minden esetben egyrészt a jelmolekulák termeléséért felelős gének, másrészt a struktúrgének transzkripcióját indukálják. A pozitív visszacsatolás hatására egyre több jelmolekula kerül a környezetbe, egyre több struktúrgén is íródik át, és bekövetkezik a baktériumpopuláció fenotipusbeli változása. Rendszerint a jelmolekulák szintézisének növelése együtt jár a receptorok szintézisének csökkenésével, így téve szabályozottá a reakciót. A szignál molekulák többsége 3 nagy csoportba sorolható: a Gram-pozitívokra jellemző autoinducer peptidekre, a Gram-negatívokra jellemző acyl homoszerin laktonokra és az interbakteriális kommukációban szerepet játszó autoinducer 2-re. Egyre több szignál molekulát ismerünk meg, amely nem sorolható be egyik csoportba sem, ilyenek például az AI-3, DSF, PQS és HHQ szignálok. Quorum sensing rendszerükkel az egysejtű élőlények is képesek bizonyos mértékben többsejtűként viselkedni. Az adott mikrobaközösségek egy egységként képesek organizálni biológiai folyamataikat. Napjaink egyik legnagyobb, minél sürgetőbb megoldást igénylő mikrobiológiai problémáját az egyre szélesebb körben terjedő antibiotikum rezisztencia jelenti. Ennek leküzdésére a különféle quorum sensing gátló szerek jelenthetnek alternatívát. A QS rendszerek a baktériumok legkülönfélébb tulajdonságait befolyásolják. Szerepük lehet az antibiotikum rezisztenciában, biofilm képződésben, a kompetencia kialakításában, virulencia faktorok termelésében, az antibiotikum termelésben, a biolumineszcenciában, a konjugációban, sporulációban, motilitásban és még számos egyéb tulajdonság kialakításában. Így egy hatásos QS blokkolóval meggátolhatjuk az antibiotikum rezisztencia terjedését, a biofilmek létrejöttét, vagy a virulencia faktorok termelését is. Ezáltal a kórokozók zöme elveszítené, vagy

csökkentené az antibiotikum rezisztenciáját, virulenciáját és más QS mediált tulajdonságait. Munkám során célul tűztem ki, a quorum sensing gátlási lehetőségeinek vizsgálatát, különféle gátló anyagok keresését és tanulmányozását, a mikrobák egymás kommunikációs rendszereire való hatásának megfigyelését. Kísérleteim alapját a CV026 (*Chromobacterium violaeum* 026) szenzortörzs jelentette, mely rövid szénláncú AHL-ek jelenlétében lila pigmentet, violaceint termel. Segítségével több batériumtörzsnél is detektáltunk AHL termelést, melyek közül a *P.aeriginosa* 49010-et és az *E. cloaceae* 31298-at és az akkor még identifikálatlan EZF 10-17-et használtuk a további munkákhoz. Kísérleteimben elsőként különféle illóolajok QS-re kifejtett hatását vizsgáltam. Az irodalmakban már említett (fokhagyma, kakukkfű) kivonatokon kívül is számos esetben mutattam ki QS gátlást, így például a rózsa, citrom, levendula, gólyaorr és rozmaringolaj esetében is. Két vegyületcsoport az ABC transzporter gátló háromgyűrűs vegyületek és a proton pumpa gátló trifluorometil ketonok többsége esetében szintén erőteljes QS gátlás volt tapasztalható. A háromgyűrűs vegyületek közül az amitriptilin fejtette ki a legerősebb hatást az AHL termelő törzsek segítségével végzett kísérletek esetében. A C6 HSL jelmolekula és a háromgyűrűs vegyületek direkt interakcióján alapuló vizsgálatoknál pedig az imipramin gátolta legeredményesebben a QS-et. A vizsgált 12 protonpumpa gátló trifluorometil keton közül 6 bizonyult erős QS gátlónak. A trifluoro keton proton pumpa gátló TF vegyületek efflux pumpákra kifejtett hatását is vizsgáltuk. A legeredményesebb efflux pumpa gátlóknak ugyanazok a vegyületek bizonyultak, mint a legeredményesebb QS inhibitorok (TF-1, 2, 3, 4, 5, 9,). A különféle baktériumok egymás QS rendszereire való hatásának tanulmányozása során pedig a vizsgált 15 *E. coli* törzs közül 14 bizonyult hatásosnak. A jövőben a különféle QS gátló szerek óriási jelentőségre tehetnek szert a komplikált bakteriális fertőzések kezelésénél.

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I.

SHORT COMMUNICATION
Inhibition of Quorum-sensing Signals by Essential Oils

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The role of quorum sensing (QS) is well known in microbial pathogenicity and antibiotic resistance. QS is responsible for motility, swarming, and biofilm production based on the signal molecules, e.g., acylated homoserine lactones (AHLs) produced by micro-organisms above certain population density. The inhibition of QS may reduce pathogenicity, antibiotic resistance and biofilm formation in systemic and local infections. The homoserine lactones and other transmitters contribute to antibiotic resistance and pathogenicity of several bacteria; consequently the inhibition of QS signals reduces the problem of resistance and virulence. Due to the increasing number of persistent non-treatable infections, there is an urgent need to develop new strategies to combat infections that destabilize bacterial communities in the host.

The effect of essential oils on bacterial growth and QS were evaluated using the sensor strain *Chromobacterium violaceum* CV026 and *N*-acyl homoserine lactone (AHL) producing *Escherichia coli* ATTC 31298 and the grapevine colonizing Ezf 10-17 strains. Of the tested oils, rose, geranium, lavender and rosemary oils were the most potent QS inhibitors. Eucalyptus and citrus oils moderately reduced pigment production by CV026, whereas the chamomile, orange and juniper oils were ineffective. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: antibiotic resistance; *Chromobacterium violaceum* CV026; rose oil; geranium oil; natural compounds; rosemary oil.

INTRODUCTION

Intercellular communication among bacterial cells resulting in coordinated gene expression that leads to a 'community-behaviour' of bacteria is a rather common phenomenon in nature. The process itself depends on cell density, thus it has been called 'quorum sensing' (QS). Among others, *N*-acyl homoserine lactones (AHLs) are the most common mediators and are auto-inducers of QS-regulated gene expression in bacteria. The specificity of AHLs is determined by the length of the acyl chain and the substitution (–H, –OH or =O) on the 3 carbon position. This regulatory system consists of two genes: one produces the inducer homoserine lactones (usually designated *luxI* type genes) and the other codes for the *luxR* type receptor protein (Williams, 2007; Braeken *et al.*, 2008; Case *et al.*, 2008).

Cell-density-dependent regulation of bacterial genes has significant importance in several ecological relationships in plant and human pathology, as well as in the communication between prokaryotes and eukaryotes (von Bodman *et al.*, 2003; 2008; Braeken *et al.*, 2008: Hughes and Sperandino, 2008). Manipulation of QS may also be an efficient tool for modification of microbial communities, for improved plant protection strategies and for curing human bacterial diseases (Hentzer and Givskov, 2003; Cui and Harling, 2005; Waters and

Bassler, 2005; González and Keshavan, 2006; Scott *et al.*, 2006; Williams, 2007).

In the case of plant pathogens, the QS in agrobacteria is one of the best studied auto-inductive signal transfer systems. *Agrobacterium tumefaciens* produces only one single AHL, *N*-3-oxo-octanoyl-L-homoserine lactone that contributes to conjugative plasmid transfer and increases copy number of the tumor inducing plasmid pTi (Li and Farrand, 2000; Pappas and Winans, 2003). The grapevine pathogen *Agrobacterium vitis* produces several types of AHLs coded by chromosomal or plasmid genes but their functions have not been completely determined. The chromosomal AHLs contribute to tissue necrosis and hypersensitive reactions of grapevines and tobacco, respectively (Li *et al.*, 2005; Hao and Burr, 2006). Besides these, the role of QS regulation is well documented in several additional plant-bacterial interactions (Lithgow *et al.*, 2001; Molina *et al.*, 2005; Quinones *et al.*, 2005; Braeken *et al.*, 2006; 2008).

Besides plant pathology, QS plays a key impotant role in several human diseases. *Pseudomonas aeruginosa*-related diseases are the most studied (Hentzer and Givskov, 2003; Rasmussen *et al.*, 2005; Ishida *et al.*, 2007). Manipulations of the QS system may open new possibilities and improve therapies for chronic bacterial and other diseases (Hentzer and Givskov, 2003; Rasmussen *et al.*, 2005; Ishida *et al.*, 2007) inluding cancer (Li *et al.*, 2004; Shiner *et al.*, 2006; Hickson *et al.*, 2009). Our main aim was to construct a model for investigation of QS signal transmission regulation by using natural compounds such as essential oils.

MATERIAL AND METHODS

Essential oils. Rose (*Rosa damascena* L., *Rosaceae*), lavender (*Lavandula angustifolia* L., *Labiatae*), chamomile (*Matricaria recutica* L., *Asteraceae*), orange (*Rutaceae*), eucalyptus (*Eucalyptus globulus* L., *Myrtaceae*), geranium (*Geranium robertianum* L., *Geraniaceae*), juniper (*Juniperus communis* L., *Cupressaceae*), citrus (*Citrus sinensis* L., Rutaceae) and rosemary oils (*Rosmarinus officinalis* L., *Lamiaceae*) were purchased from Phoenix Pharma Ltd. (Hungary, Budapest) in quality according to the requirements of Hungarian pharmacopoeia (Ph. Hg. VII.). The essential oils used in this study were either used directly (concentrated) or dissolved in dimethyl sulfoxyde (DMSO) to yield a dilution of 10% (v/v). This stock solution was either used as it is or further diluted with 10% DMSO. Acridine orange was purchased from Reanal (Budapest, Hungary) and 5-Fluorouracil purchased from Sigma-Aldrich (Budapest, Hungary). Both compounds were dissolved in distilled water.

Bacterial strains. To detect auto-inducer production of tested bacteria, the biosensor strain *Chromobacterium violaceum* CV026 (obtained from Prof. Thomas J. Burr, Cornell University, Geneva, NY) was used. This Tn*5* mutant strain produces the purple pigment violacein upon induction with externally added short-chain auto-inducers (McClean *et al.*, 1997) and it has been widely used for the detection of *N*-acyl homoserine lactones or quorum sensing inhibitors (Lithgow *et al.*, 2001; Molina *et al.*, 2003; McClean *et al.*, 2004). *Escherichia coli* ATTC 31298 and the partially characterized Ezf 10/17 isolated from a grapevine crown gall tumor (Szegedi, unpublished) were used as AHL producers. Both strains induce pigmentation of CV026 and therefore they can be used to monitor AHL-induced pigment production by *C. violaceum* CV026.

Culture medium. An MIC equivalent inoculum from each bacterial overnight culture were streaked on agar modified Luria Bertani (LB) agar plates containing yeast extract (1 g/l), tryptone (10 g/l), NaCl (10 g/l), K2HPO4 (1 g/l), MgSO4 × 7H2O (0,3 g/l), FeNaEDTA (36 mg/l) and agar (20 g/l). pH was adjusted to 7.15. Cultures were grown at 25°C for 48 h.

Agar diffusion. Pair combinations of the sensor strain CV026 and the producer strains ATTC 31298 or Ezf 10-17 each at an MIC equivalent inoculum were swabbed on LB plates as approx. 5 cm line cultures at a 5 mm distance from each other. Whatman 3MM filter paper discs, 7 mm in diameter, impregnated with 10 µl of concentrated or DMSO-diluted oils and placed on the inoculated line cultures. Discs containing acridine orange and 5-fluoro-uracil served as positive controls and that containing DMSO (10 µl) served as the negative control. Signal transmission was assessed by the effect of the agent present on the disc that did not affect the development of purple colour defined as 'No QS inhibitory activity' and by the absence of colour and the size of the colourless zone defined as 'QS activity' after 24- and 48-h incubation periods.

The diameter of the reduced bacterial growth around the filter paper discs in mm yields the degree of the antibacterial effect of the tested compounds. Drug-free DMSO blanks served as controls for any effect produced by the DMSO diluent.

RESULTS AND DISCUSSION

The agar diffusion method provides a convenient and semi-quantitative method to assess the antibacterial activities and QS signal production in the presence of essential oils. Using this method we tested 9 essential oils compared to the positive controls of acridine orange and 5-fluoro-uracil. Most of these oils are known as inhibitors of bacterial growth (Schelz *et al.*, 2006). Our present data demonstrate that several plant-derived essential oils not only inhibit bacterial growth but also block QS regulation processes.

As evident from Fig. 1, DMSO did not effect bacterial growth or AHL-induced violacein production. As demostrated by Fig. 2, the positive controls 5-fluoro-uracil and acridine orange produced the anticipated inhibition of QS. However, the inhibitory activity of 5FU is considerably greater than that produced by acridine orange.

The use of impregnated disks with DMSO containing various essential oils afforded an understanding of which oils inhibited the sensor response of *Chromobacterium violaceum* CV026 to AHL released by the bacteria employed in this study. As examples, although the geranium oil was most effective as an inhibitor of the QS response of *Chromobacterium violaceum* CV026 (Fig. 3A) following induction by the strains ATTC 31298 and Ezf 10-17, rose oil also has significant inhibitory activity on QS responses (Fig. 3B). Rose, lavender
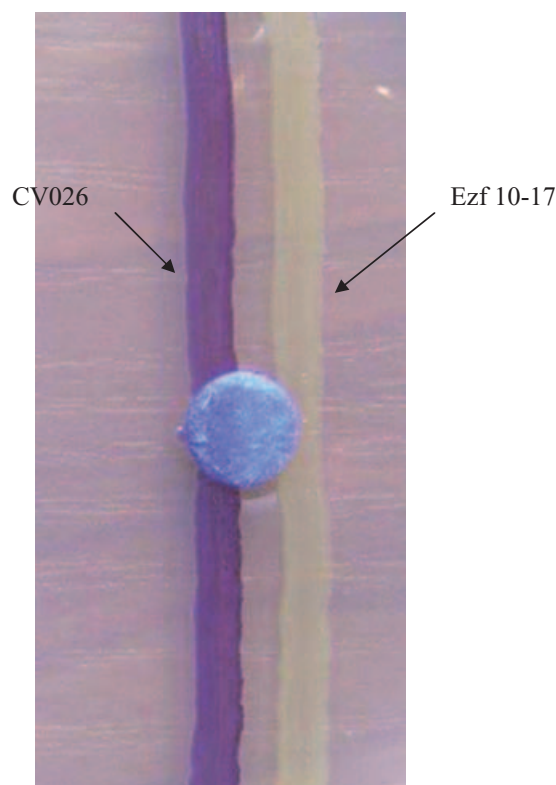

Figure 1. Negative control: DMSO does not affect colour production by the QS sensor *Chromobacterium violaceum* CV026 in response to an AHL producing bacterium. Control demonstrating color production by the CV responding strain and that 10 µl DMSO does not affect this response.
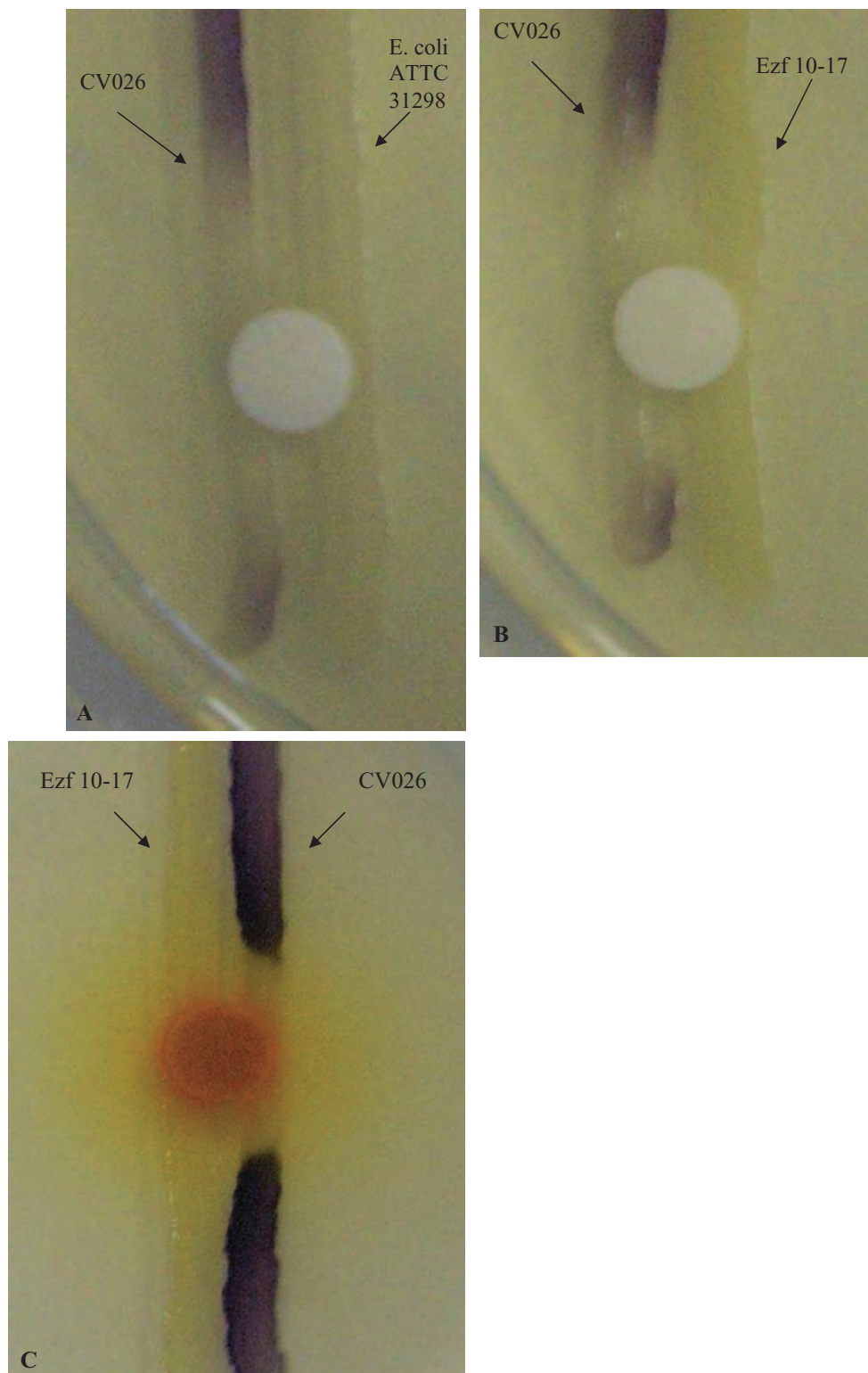
Figure 2. Positive controls: Impregnated disks with acridine orange and 5-FU inhibit the production of color by *Chromobacterium violaceum* CV026 in response to the AHL producing bacterium. (A) 5-FU inhibits colour response by CV 026 to the AHL produced by E. coli ATTC 31298. (B) 5-FU inhibits colour production by CV 026 to the AHL produced by Ezf 10-17. (C) Acridine orange inhibits response of CV026 to AHL produced by Ezf 10-17. Comparison of the degree of response to that affected by 5FU indicates that acridine orange has a lower inhibitory effect than 5FU.

and rosemary oils also inhibited colour development to varying degrees (Table 1). Eucalyptus oil inhibited violacein production only after induction with ATTC 31298 while citrus oil reduced colour formation only in the CV026 + Ezf 10-17 combination. As expected they also inhibited bacterial growth to variable degrees (data not shown). The other tested essential oils also had a weak antibacterial activity on *C. violaceum* CV026 but the growth of the AHL producing bacteria were not affected (data not shown). QS was moderately inhibited by lavender, eucalyptus and citrus oils, while the chamomile, orange and juniper oils were ineffective (Table 1).

The role of QS is well known in plant pathology; however it also plays an important role in several human
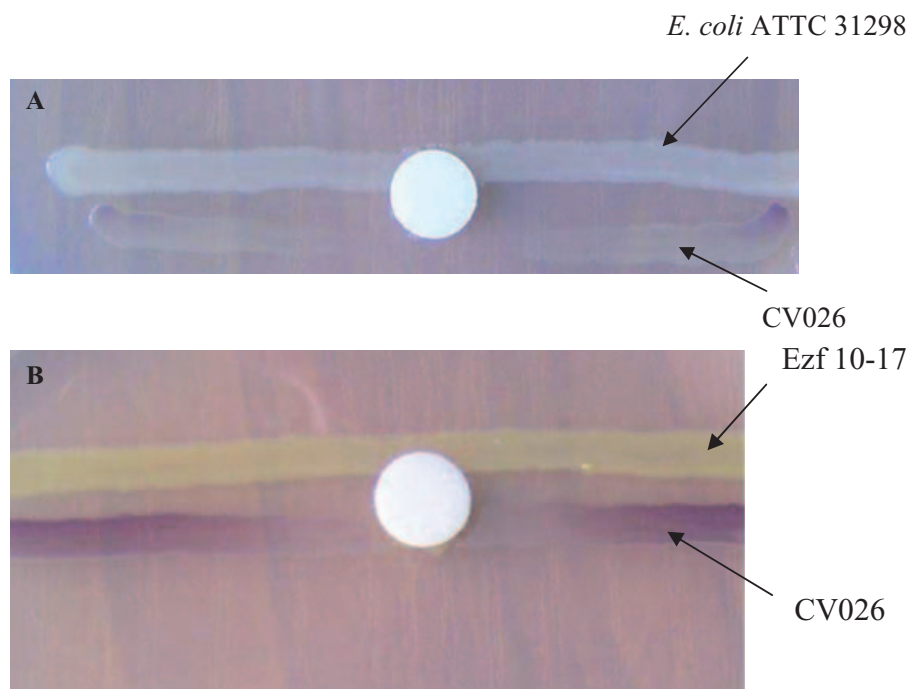
Figure 3. Demonstration of the QS inhibitor activity of essential oils. (A) Effect of Geranium oil on QS. The disc contains 10 μl Geranium oil. (B) Effect of Rose oil on QS. The disc contains 10 μl Rose oil.

Table 1. Effect of tested essential oils on quorum sensing signal production between *Chromobacterium violaceum* CV026 as sensor, and *Escherichia coli* ATTC 31298 and Ezf 10-17 as producer strains after 48 hr incubation

Essential oils	10% oil		100% oil	
	CV026 + E. coli ATTC 31298	CV026 + Ezf 10-17	CV026 + E. coli ATTC 31298	CV026 + Ezf 10-17
Rose	15–20	10	18	20
Lavender	0	5	10	15
Chamomile	0	0	0	0
Aeth. Geranium	0	3	13	15
Eucalyptus	0	3	6	0
Juniper	0	0	0	0
Citrus	0	2.5	0	16
Orange	0	0	0	0
Rosemary	0	3	18	18

diseases. To cure bacterial diseases through the manipulation of QS-regulated processes, several natural and synthetic compounds have already been tested (Rasmussen *et al.*, 2005; Ishida *et al.*, 2007). The results obtained in our study show that some essential oils can also inhibit QS. Although at this time we are not suggesting that these compounds be used for the modification of QS in bacterial populations of a host, we do suggest that because these compounds modify QS at least in the laboratory environment, that the approaches employed in our study may eventually lead to a new form of therapy for some mixed human infections which, at this time, are problematic for therapy. (Hentzer and Givskov, 2003; Rasmussen *et al.*, 2005; Ishida *et al.*, 2007) including cancer (Shiner *et al.*, 2006).

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II.

Quorum Sensing Inhibition by Phenothiazines and Related Compounds

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Abstract: A quorum is the smallest number of people able to organize the decisions concerning functional activity. Similarly microbes use chemical signal molecules to make population size-dependent "decisions" by changing their gene regulations.

The inhibition of quorum sensing (QS) by phenothiazines and structurally related molecules, e.g. amitriptyline, promethazine, acridine orange, imipramine, promazine, diethazine, desipramine, desertomycin and 5-fluorouracil as positive control was studied with *Chromobacterium violaceum* 026 as a sensor strain, which detects short carbon chain AHLs by the development of a purple pigment. The AHL was produced by *Novospingobium* Ezf 10-17, and the antibiotic-resistant clinical isolates, *E. coli* 31298.

The QS was demonstrated as a signal transmission between the two bacterial strains. The most effective inhibitors of QS were amitriptyline, promethazine, acridine orange and desertomycin. Imipramine and diethazine were moderately active, while chlorprothixene was ineffective relative to 5-fluorouracil as positive control. The direct complex formation between AHL and QS inhibitors markedly reduced the QS in a chromogenic test. The AHL-neutralizing effect of the related compounds was shown by chromogenic method.

The inhibition of QS signal transmission appears to be related to the quasi-planar structure and electron donor capacity of the conjugated π-electron system of the tricyclic framework. The results can be exploited in rational drug design as a new way to reduce the QS mediated processes eg. virulence of pathogens, to vary the formation of biofilms and to modify antibiotic resistance.

Keywords: 5-Fluorouracil, Amitriptyline, Homoserine lactone complexes, Imipramine, Promethazine, Quorum quenching.

INTRODUCTION

Phenothiazines and structurally related tricyclic compounds exert various effects on bacterial cells including the inhibition of growth and the reversal of antibiotic resistance [1-3].

Quorum sensing (QS) is a communication system mediated by chemical signals which depends on the population density of bacterial species within a common environment and contributes to their ability to compete and survive [4, 5]. It is common to both Gram-positive and Gram-negative bacteria and plays a role in intra- and interspecies communications, additionally allowing communications between microbes and their hosts [6]. QS is of great significance in the formation of biofilms [7], the production of virulence factors [8], the development of intrinsic antibiotic resistance [9], the facilitation of growth in a competitive environment [10] and the enhancement of motility [11]. It may also provide genetic advantages due to the promotion of conjugational gene transfer [12] and control of the competent state [13].

The most common mediators in Gram-negative bacteria are the *N*-acyl homoserine lactones (AHLs). The specificity of the AHLs is determined by the length of the acyl chain and the substitution at C-3. The QS regulation system consists of two types of genes. The *luxI*-type genes produce autoinducer molecules such as AHLs, and the *luxR*-type genes produce their receptor proteins [14-16]. In Gram-positive bacteria, various types of peptides have the same role in QS.

The bacterial interactions focused on QS between various species have not yet been studied in the presence of tricyclic compounds. Since these compounds have various effects on bacterial functions, QS signal transmission seems to be a good model for the characterization of molecular interactions between AHLs and commonly used tricyclic drugs.

The aim of our study was to investigate the effects of some phenothiazines and structurally related compounds on the QS signal transmission between the sensor strain *Chromobacterium violaceum* 026 and three different AHL-producing bacterial isolates.

MATERIALS AND METHODS

Chemicals

The following tricyclic compounds were used: promethazine (Pipolphen, EGIS, Hungary), amitriptyline

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Table 1.   **Investigation of Antibacterial Effects of Tricyclic Compounds with the Agar Diffusion Method**

Tricyclic Compounds	Growth Inhibition Zones in mm					
	Ezf10-17	*E. coli 31298*	*P. aeruginosa 49010*	*CV026 (+Ezf 10-17)*	*CV026 (+ E. coli. 31298)*	*CV026 (+ P. aeruginosa 49010)*
Acridine orange	-	-	-	-	-	-
Amitriptyline	8	-	-	9	10	12
Desipramine	9	7	-	11	16	9
Imipramine	-	-	-	9	9	7
Diethazine	7	-	-	9	7	8
Promazine	8	-	-	10	12	15
Promethazine	9	7	-	11	15	10
Chlorprothixene	-	-	-	7	7	7

10 µl of a 25 mg/ml solution was added into the filter paper discs.

(Teperin, EGIS, Hungary), acridine orange (Reanal, Hungary), imipramine (Melipramin, EGIS, Hungary), desipramine, chlorprothixene (Truxal, Lundbeck, Denmark), promazine, diethazine (Parkazin, Rhone-Poulenc, France) and desertomycin. Stock solutions of these tricyclic compounds were prepared in distilled water at 25.0 mg/ml before use. For control experiments 5-fluorouracil (5-FU), and acridine orange were used as known inhibitors [2, 17]. Iodine deoxy uridine (IDU). N-hexanoyl-DL-homoserine lactone (SIGMA, Budapest).

Bacterial Strains

Chromobacterium violaceum 026 (CV026) served as QS sensor. *C. violaceum* is a common bacterium which lives in soil and water. When *C. violaceum* reaches a high cell density, it produces a purple pigment called violacein [18]. The CV026 sensor strain is a Tn*5* mutant which alone cannot synthesize AHLs; it produces the purple pigment only in the presence of externally added inducers [19]. This strain has been used to detect a wide range of short-chain AHLs or QS inhibitors [20-22]. As AHL signal producers inducing CV026, the following strains were used: Ezf 10-17 is an as yet unidentified grapevine crown gall tumour isolate which resembles *Novospingobium* sp.; *Esherichia coli* 31298 and *Pseudomonas aureginosa 49010* were clinical strains isolated from a wound lesion and the human trachea, respectively.

Quorum Sensing Tests

A modified LB medium containing yeast extract 5 g/l; Trypton 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) 20g /l) (pH 7.2) was used for the experiments. The sensor strain CV026 and the AHL producer strains Ezf10-17, *E. coli* 31298 or *P. aureginosa* 49010 were inoculated as parallel lines and incubated at room temperature (20 ^{o}C) for 24-48 h.

QS inhibition was monitored by the agar diffusion method. Filter paper discs (7.0 mm in diameter, Whatmann 3MM) were impregnated with 10 µl of stock solutions of the tricyclic compounds in distilled water. The discs were placed on the surface of nutrient agar between the parallel lines of sensor and AHL producer strains. The plates were incubated

at room temperature for further 24-48 h and the interactions between the strains and tricyclic compounds were evaluated as concerns the reduction in the size of the zone of pigment production and the size of the zone of growth inhibition of the affected strains, in mm. 5-FU was applied as positive control [17].

Evaluation of Complex Formation Between the Comercially AHL and QS Inhibitors

Biological method: 1 µl of a solution of 5, 10 or 25 ng of AHL was mixed with 10 µl of stock solution (25 mg/ml) of the potential QS inhibitor. Filter paper discs (7.0 mm in diameter, Whatmann 3MM) were impregnated with 11 µl of the mixture of QS inhibitor and AHL solution. The discs were placed on the surface of nutrient agar media, upon the inoculation line of the CV026 sensor strain. The plates were incubated at room temperature (18-22 °C) for a further 24-48 h and the interactions between the AHL and the tricyclic compounds were evaluated in terms of the reduction in the diameter of pigment production (due to complex formation when the AHL was inactivated), and the reduced colour intensity.

RESULTS

The agar diffusion method provides an opportunity to investigate the potential inhibition of tricyclic compounds on QS between the QS sensor *C. violaceum* and three AHL producing bacteria. For control experiments, Ezf 10-17 adjacent to the QS sensor CV026 cultured in the presence of a water-containing disc resulted in the purple colouration of the swabbed sensor, an indication of a positive response to the presence of AHLs produced by Ezf 10-17. To demonstrate that the colouration of the sensor strain is prevented by a known inhibitor of the QS response, 5-FU and acridine orange were used as positive controls. Since the phenothiazines have antimicrobial activity against *E. coli* [1], the inhibition of violacein production as quorum signal was measurable only at subinhibitory concentrations on the growth of inoculated bacteria. Consequently, the production of AHL as a QS mediator was evaluated below the MICs of the phenothiazines against the AHL-producing bacteria.

Table 2. **Inhibitory Effects of Tricyclic Compounds on QS Signal Transmission**

Tricyclic Compounds	QS Inhibition Zone in mm		
	EZF10-17	*E. c. 31298*	*P. a. 49010*
Acridine orange	14	12	14
Amitriptyline	64	30	60
Desipramine	26	26	-
Imipramine	15	12	-
Diethazine	18	12	-
Promazine	13	13	20
Promethazine	26	20	15
Chlorprothixene	-	-	-

10 µl of 25 mg/ml stock solution was impregnated into the filter paper discs (25 µg/disc) and the colourless zone around the disc measured on *C. violalaceum* was measured after incubation for 24-36 h at room temperature.

Table 3. **The Effects of QS Inhihibitors on N-hexanoyl-DL-homoserine Lactone (C6 HSL) Mediated Violacein Production**

Quantity of C6 HSL	0 ng (K)	5 ng	10 ng	25 ng
Tested Compounds	Diameter of Coloration in mm (18h)			
IDU 10 µg	0	15	25	30
Desertomycin 200 µg	0	22	25	30
5-FU* 30 µg	0	29	35	35
Promethazine 250 µg	0	0	30	33
Desipramine 250 µg	0	0	28	32
Promazine 250 µg	0	0	30	32
Imipramine 250 µg	0	0	20	26
Amitriptyline 250 µg	0	0	25	30
C6 HSL control	0	25	34	35

The amount of each phenothiazine placed onto the disk was chosen so that it did not produce any antimicrobial effect against the given bacterium (less than one-half of the MIC). In summary, amitriptyline, desipramine, imipramine and promethazine inhibited the production of the purple colour by the sensor CV026 strain. Although the concentration of each phenothiazine was identical, the degree of inhibition varied as the distance between the disk and the margins of the diameter of the produced colouration. Table **1** shows the antibacterial effects of the phenothiazines: the diameters of the growth inhibitory zones are indicated in mm. To study for the effects of phenothiazines and structurally related compounds on QS, the tricyclic compounds were examined in a concentration below the growth inhibitory doses. The inhibition of QS signal tranmission was measured as described in the Methods section. The production of colourless zones by the phenothiazine compounds impregnated into the filter paper discs was measured around CV026, in response to the inhibited AHLs produced by Ezf10-17, *E.coli* and *P.aeruginosa*. As shown in Table **2**, the degree of inhibition of purple colour formation by CV026 in response to AHLs produced by the various bacteria differed, indicating that the effectivities of the tricyclics differed as regards the QS response on the senzor CV026.

The mechanism of QS inhibition was studied by measuring the effects of the direct interactions between the AHL and QS inhibitors on violacein production in the bioassay. The most effective inhibitors were imipramine, promethazine, desipramine and amitriptyline, which in high concentration inactivated the AHL and probably formed micellar complexes.

The activities of the complexes between AHL and its inhibitors were measured in AHL-specific chromogenic tests. The biological activities of the AHL complexes were apparently reduced by complex formation, compared with the uncomplexed control AHL (Table **3**).

DISCUSSION

The effects of tricyclic compounds on various Gram-positive and Gram-negative bacteria and R-plasmid elimination were examined earlier [1, 24], and the inhibition of horizontal plasmid transfer due to the blocked conjugation [25], antibacterial and plasmid curing in coexisting bacterial populations is known. The importance of tricyclic compounds as a class of "non-antibiotics" which may

provide alternatives to the ineffective therapy of serious bacterial infections has already been reviewed [3]. Despite this considerable body of evidence, however the effects of tricyclic compounds on QS have not been studied before.

QS signal transmission plays a key role in several microbial processes since the inhibition of QS may modify pathogenicity, virulence and antibiotic resistance.

Obviously, if the specific QS system is blocked during the bacterial infectious process, the therapy of serious infections may be improved. In view of the demonstration that certain tricyclic compounds can block the QS signal response of diverse bacterial species, it is conceivable, that in the future these compounds may be used to modify biological signal transmission at a population level, e.g. by the modification of antibiotic resistance in biofilms, the production of virulence factors and the antibiotic production of some microbes in combination with conventional therapy to reduce the virulence of certain types of bacterial infections.

We presume that in a mixed bacterial population the partners may outcompete competitors in vivo too, consequently gaining benefit within the population. There are other practically important opportunities when QS can also be a signal, mediating messages in microorganisms and host relationships.

The effects of known QS inhibitors can be exploited to prevent or lower virulence and antibiotic resistance and the formation of biofilms on catheters. The results obtained may lead to a rational drug design with perspectives of improved chemotherapy. We assume that the inhibition of proton motive forces plays a key role in the modification of QS signal transmission.

CONCLUSION

The inhibition of quorum sensing signal transmission by the studied tricyclic compounds may open a new perspective to modify various quorum sensing mediated processes: like biofilm formation, bacterial pathogenicity and antibiotic resistance.

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III.

Inhibition of Quorum Sensing and Efflux Pump System by Trifluoromethyl Ketone Proton Pump Inhibitors

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Abstract. *Background: One major microbiological problem is the widespread antibiotic resistance. There is an urgent need for new antibiotics and ways to treat multi-drug-resistant infections. Inhibition of bacterial quorum sensing (QS) systems could be an effective alternative in a smuch as they regulate a broad spectrum of cell functions, including, virulence factor production, biofilm organisation and motility. Influx and efflux bacterial systems involved in quorum sensing (QS) are known to depend on the proton motive force (PMF). Thus, a new series of 12 trifluoromethyl ketones (TFs) known to inhibit the PMF, was investigated for effects on the efflux pump of a QS responding bacterium, for its subsequent effect on the response to a QS signal and its direct inhibition of the response to a QS signal. Materials and Methods: Chromobacterium violaceum 026 (CV026) was used as the indicator strain to evaluate the QS inhibitory effect of TFs. This strain responds to the presence of short carbon chain acyl-homoserine lactones (AHLs) by the development of a purple pigment. Effect on the QS response of CV026 to externally added AHLs was evaluated. In addition, the specific activity of the TFs on the efflux pump system of the CV026 strain and a wild-type Escherichia coli strain was assessed with the aid of the automated real-time ethidium bromide method. Results: From the 12 compounds, 6 proved to be effective inhibitors of the QS response by CV026, as well as inhibit the efflux pumps of CV026 and Escherichia coli. Conclusion: Our results show that TFs have QS inhibitory properties that are mediated through their inhibition of efflux pumps that extrude the noxious QS signal before it reaches its intended target. Because the TFs also inhibit the efflux pump of a pathogenic bacterium, the method used for the evaluation of the TFs in the current study has clinical relevance and may be exploited for the prevention of QS responses of infecting bacteria.*

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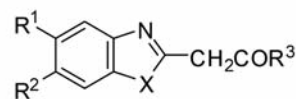
The antimicrobial and antimotility effects of 30 trifluoromethyl ketones (TFs) on various bacterial species have already been studied (1-5). Some of these TFs only inhibit the growth of various Gram-positive bacteria, while others exhibit antimicrobial activity against Gram-negative bacteria and yeasts. The combination of certain derivatives of TFs with promethazine results in a synergistic antibacterial effect (1).

TFs at subinhibitory concentrations inhibit the motility of *Proteus vulgaris*. Detailed analysis demonstrated that the proportion of mobile bacterial cells is reduced (2), suggesting that the action of TFs at concentrations that have no effect on the viability of the studied bacteria involves the inhibition of an energy source upon which flagellae depend for their action, namely, the proton motive force (PMF) (2).

Quorum sensing (QS) signal systems regulate a broad spectrum of cell functions in bacterial populations (6-8). Among the functions that depend or interact with QS is the secretion of biofilms. Bacteria whose main efflux pump has been deactivated or deleted are deficient in the secretion of QS signals (9) as well as production of biofilm (10). Efflux pumps of the resistance nodulation division (RND) family of transporters, depend upon the energy provided by the PMF

Table I. *Inhibitory effects of the (TFs) on (QS) and the growth of producing and QS sensor bacterial strains, after 24 hours of incubation. The extent of the colourless zone indicates the inhibitory effect of the given compound on QS signal transmission.*

TF	MIC (µg/ml)		Extent of QS inhibition [zone of discolouration) at 20 µg/disc (mm)]
	CV026	EZF 10-17	
1	50	25	26
2	12.5	25	28
3	12.5	12.5	30
4	50	50	20
5	50	50	16
6	>200	>200	0
7	>200	>200	0
8	>200	>200	0
9	50	25	29
10	>200	>200	0
11	>200	>200	0
12	>200	>200	0

CV026: Chromobacterium violaceum 026.

TF	R^1	R^2	R^3	X
1	H	H	CF_3	O
2	Cl	H	CF_3	O
3	F	H	CF_3	O
4	CH_3	H	CF_3	O
5	CH_3O	H	CF_3	O
6	Ph	H	CF_3	O
7	$COOC_2H_5$	H	CF_3	O
8	H	$COOC_2H_5$	CF_3	O
9	H	H	$CClF_2$	O
10	H	H	CH_3	O
11	H	H	CF_3	NH
12	H	H	CF_3	S

Figure 1. *Structures of trifluoromethyl ketones investigated in this study.*

for effective function (11, 12). These efflux pumps extrude noxious agents that penetrate the outer cell wall of the organism prior to reaching their intended targets (13). QS signals that are produced by one species for the inhibition of growth of a competing bacterial species can be considered noxious agents; consequently one would expect that compounds shown to inhibit access to energy provided by the PMF would have a negative effect on the main efflux pump of the responding competitor and hence the response to a QS signal such as *N*-acyl homoserine lactone (AHL) would be negatively affected. Since TFs have been shown to affect access to energy provided by the PMF (5) we have been studying the effects of a series of 12 systematically synthesized TFs (**1-12**) (Table I) on the QS signal system. This study evaluated the effect of 12 TFs on the expression of the AHL-mediated signal in a system containing the environmental member *Sphingomonas* sp. Ezf 10-17 strain, as producer of AHL and the *Chromobacterium violaceum* CV026 as the responding sensor to AHL. Since the effects of the TFs on the QS system by the former system do not distinguish an effect on the producer *versus* the responder, the effects of the TFs on the responder were directly assessed by combinations of pure commercial *N*-Hexanoyl-DL-homoserine lactone AHLs and each TF. In addition, we have studied the effects of the TFs on the activity of the efflux pumps of the QS-responding CV026 strain, as well as on the efflux pump system of *Escherichia coli*, in order to determine the role that an efflux pump system might play in the response to a QS signal.

Materials and Methods

Chemicals. Eleven TFs (**1-9, 11** and **12**) were synthesized by reaction of the corresponding 2-methylbenzazoles with trifluoroacetic or chlorodifluoroacetic anhydride (14). Compound **10** was prepared by treatment of 2-lithiomethylbenzoxazole with ethyl acetate (15, 16). *N*-Hexanoyl-DL-homoserine lactone was purchased from Sigma (Budapest, Hungary). TFs were dissolved in, dimethyl sulfoxide (DMSO).

The structures of the TFs are presented in Figure 1. Ethidium bromide and thioridazine were purchased from Sigma (Madrid, Spain).

Bacterial strains. *Chromobacterium violaceum* is a common bacterium which lives in soil and water. When it attains a high cell density, it produces a purple pigment called violacein (17). The *C. violaceum* CV026 used as a sensor strain to study the effect of TFs on QS is a Tn5 mutant that cannot synthesize AHLs; it produces the purple pigment only in the presence of externally added inducers (18). This strain has been used to detect a wide range of short-chain AHL molecules and QS inhibitors (19-21). Ezf 10-17 was isolated from a grapevine crown gall tumor. This strain induced pigment production by CV026 and proved to be efficient to study QS interactions (22). *Escherichia coli* wild-type AG100 [argE3 thi-1 rpsL xyl mtl (gal-uvrB) supE44] was employed for the determination of effects of TFs on the activity of the intrinsic efflux pump of this organism (13).

Medium. A modified LB agar containing yeast extract 5 g, trypton 10 g, NaCl 10 g, K_2HPO_4 1 g, $MgSO_4\cdot 7H_2O$ 0.3 g and FeNaEDTA 36 mg in 1.0 liter of distilled water was used to study the effect of TFs of QS. Ezf 10-17 was grown on potato dextrose agar (PDA) to prepare signal compounds. *E. coli* was cultured on Mueller-Hinton broth (MHB) and colonies isolated on Mueller-Hinton agar (MHA) purchased in powder form from Sigma (Madrid, Spain).

Taxonomic identification of Ezf10-17 and analysis of its AHL production. The V3 region of 16S rDNA from Ezf 10-17 was amplified using the forward primer (5'-ACTCCTACGGGAGGCA GCAG-3') and reverse primer (5'-ATTACCGCGGCTGCTGG-3') and sequenced. Sequence data were compared and analysed by BLAST against the published 16S V3 sequences available in the database.

The AHLs from the liquid culture of Ezf 10-17 were extracted and concentrated by using acidified ethyl acetate liquid-liquid extraction. The purified AHLs were analysed using thin layer chromatography (TLC) overlaid with *C. violaceum* CV026 biosensor strain.

Evaluation of the effect of TFs on the response by CV026 to AHL. Isolated colonies of the AHL-responding CV026 were plated directly as a 6 to 7 cm line on the surface of the modified LB agar. One microlitre of solution containing 5 or 10 ng of *N*-Hexanoyl-DL-homoserine lactone AHLs was mixed with 10 µl of the stock (2 mg/ml or 0.4 mg/ml) solution of the potential QS inhibitor TF compound. Filter paper discs (7.0 mm in diameter) were impregnated with 11 µl of the mixture of different concentrations of each TF and AHL, and the disks were placed on the inoculated line of the CV026 sensor strain (18). For sets of assays that aimed to evaluate the interaction between AHL and each TF, blank filter disks were separately impregnated with 11 µl of AHL alone, with 11 µl each of the TFs alone, and with 11 µl of DMSO, the latter serving as an absolute control. The plates were incubated at room temperature (*ca*. 20˚C) for 24-48 hs.

Minimum inhibitory concentration (MIC) of each TF on CV026 and E. coli AG100. The MIC of TFs was determined by the broth dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (23).

Assessment of the effects of each TF on the activity of the efflux pump systems of CV026 and Escherichia coli AG100. The activity of the TFs on the real-time accumulation of ethidium bromide (EB) was assessed by the automated EB method, previously described in detail (24), using the Rotor-Gene 3000™ thermocycler with real-time analysis software (Corbett Research, Sydney, NSW, Australia). Briefly, *E. coli* AG100 was cultured in MHB medium until the culture reached an optical density (OD) of 0.6 at 600 nm, the culture was then centrifuged at 13,000 rpm for 3 min, the pellets were re-suspended in phosphate-buffered saline (PBS; pH 7.4) with a final concentration of glucose of 0.4% and the OD adjusted to 0.6 at 600 nm. Aliquots of 45 µl of the cell suspension were distributed to 0.2 ml tubes. The TFs were individually added at concentrations equal to half their MIC against the strain in 5 µl volumes of their stock solutions, and finally 45 µl of EB to yield a final concentration of 1 mg/l (Sigma-Aldrich) in PBS, with and without glucose, were added. Note that the selection of a concentration of each TF at half its MIC is due to the empirical fact that at this concentration there is no significant effect on the viability of the organism (24, 25). It is also important to note that prior to the experiments described, the maximum concentration of EB which was within the capacity of the bacterium to extrude, was determined at least three times. For the wild-type *E. coli* AG100 reference and the CV026 strains employed in the study, these concentrations of EB were determined to be 1 and 0.5 mg/l, respectively (13, 24, 25). The tubes were placed into a Rotor-Gene 3000TM thermocycler and the fluorescence monitored on a real-time basis. From the real-time data, the activity of the TF, namely the relative final fluorescence (RFF) of the last time point

(minute 30) of the EB accumulation assay was calculated according to the formula:

$$RFF = \frac{RF_{treated} - RF_{untreated}}{RF_{untreated}}$$

Where $RF_{treated}$ is the relative fluorescence at the last time point of the EB retention curve in the presence of an inhibitor, and $RF_{untreated}$ is the relative fluorescence at the last time point of the EB retention curve of the untreated control. The greater the difference between $RF_{treated}$ and $RF_{untreated}$, the greater the degree of EB accumulated and, therefore, the greater the degree of inhibition of the efflux pump system of the bacterium promoted by the agent at that concentration.

The RFF was then divided by the concentration of the TF that corresponded to half its MIC. This yielded a measure of the effect of each TF at a milligram level (specific activity) and therefore afforded comparison of each TF for activity against the efflux pump systems of the CV026 and *E. coli* AG100 strains. The experiments were repeated three times and the specific activity values presented are the average of three independent assays. This method of analysis has been previously presented (26). Thioridazine (TZ) an efflux pump inhibitor (12), was used as as a positive control.

Results

In our previous studies, the unidentified grapevine tumor isolate Ezf 10-17 proved to be an inducer of violacein production by *C. violaceum* CV026. Thus, this pair of inducer/sensor strains was successfully used to study potential QS inhibitors (21) (Figure 2). To identify Ezf 10-17, we sequenced the V3 region of 16S rDNA gene from its genome. Comparing the sequence data to those found in databases, this strain proved to be a member of the *Sphingomonadaceae* family.

Signal production of Ezf 10-17 was analysed by TLC overlaid with *C. violaceum* CV026. As compared to the standard AHLs, Ezf 10-17 produces a strong signal that co-migrates with 3-oxo-C6 AHL. Additionally, weaker signals which seem to be identical with C6 AHL, 3-oxo-C8 AHL and C8 AHL were also observed (Figure 3). These data support our earlier observations on the suitability of Ezf 10-17 in QS assays with *C. violaceum* CV026 (21).

In order for an agent to be correctly evaluated for effects on a QS system whose intensity of colour is dependent upon the growth of the producer of the QS signal and the growth of the responding bacterium, the concentration of the agent that is to provide meaningful interpretation, must be one that does not affect the viability of either bacterium. As summarized in Table I, various TFs have antimicrobial activities against the producer and responding bacteria. Consequently, the amounts of TFs selected for the evaluation of effects on the QS system were at or below those that had no effect on the growth of either species. Because of space limitation, the range of effects of the TFs on the QS system

Figure 2. *The quorum sensing response of CV026 to the presence of the (AHL) producer Ezf 10-17. A: CV026 responder strain alone. B: CV026 responder strain (right) with Ezf 10-17 AHL producer strain (left). The deep colour associated with the responder strain CV026 was found only when CV026 was cultured in parallel with the AHL producer Ezf 10-17.*

Figure 3. *Detection of N-acyl-homoserine lactones (AHL) from Ezf 10-17. Pure, commercial AHLs (lanes 1-4) and purified AHL produced by Ezf 10-17 (lane 5) isolated by thin layer chromatography. The plate overlaid with the responder CV026. The application of varying concentrations of pure commerical AHLs provide standards of the degree of response (intensity of colour). EZF 10-17 AHL extracts were prepared with acidified ethyl acetate of re-suspended 4-day old cultures of EzF 10-17 grown on potato dextrose agar.*

cannot be pictorially presented. Rather, the effects are presented in Figure 4 and Table II. Briefly, TF *5* had the least inhibitory effect (deep colour associated with the responder CV026) and TF *3* had the greatest inhibitory effect on the response of the CV026. The effect of the TFs on the QS system is clearly inhibitory. Whether the effect is due to the TF inhibiting the release of the QS signal or to the inhibition of the response of the responding species cannot be distinguished from the above evaluation.

The direct effects of each TF on the QS response by CV026 was determined with the use of disks impregnated with combinations of a constant amount of the AHL and differing amounts of TF. As evident from the example provided in Figure 5, whereas the disks with TF alone (Figure 5A) do not produce the purple colour associated with CV026, the presence of pure AHL in the disk led to the production of the deep purple colour associated with CV026 (Figure 5B). The presence of the TF that inhibited the production of colour in the QS assay described, when in combination with the AHL, inhibits the production of the purple colour by the responding CV026 (Figure 5C). These results clearly show that the TF has a powerful inhibitory effect on the QS responding strain. However, the question of whether the same TF can inhibit the secretion of the QS signal by the producer species remains
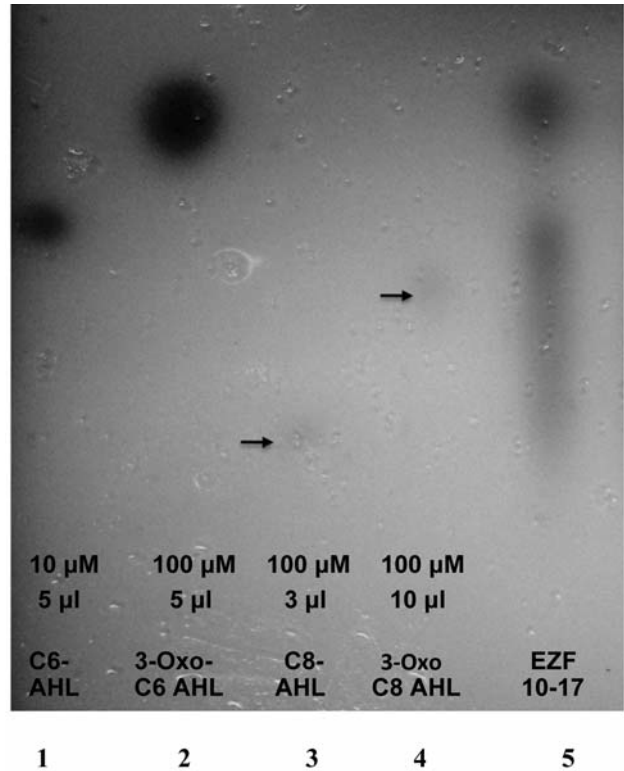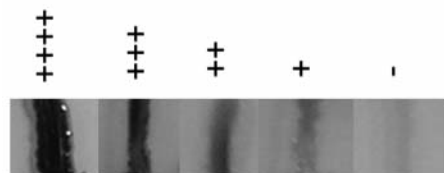

Figure 4. *The length and intensity of the purple colouration induced by 10 ng AHL/disc on Chromobacterium violaceum 026, after 24-hour incubation in the presence of TFs applied at 20 μg/disc. The effective inhibitors reduce the length and intensity of the purple colouration. The scale indicates an increasing intensity of colouration. A lower colour intensity means higher inhibition of QS. −, No coloration; +, white-purple; ++, palepurple; +++, purple; ++++, darkpurple.*

unanswered. The activity of each TF at half its MIC on the efflux pump system of the CV026 is exemplified by Figure 6. The data presented on those figures suggest that all of the TFs have activity against the efflux pump of CV026. However,
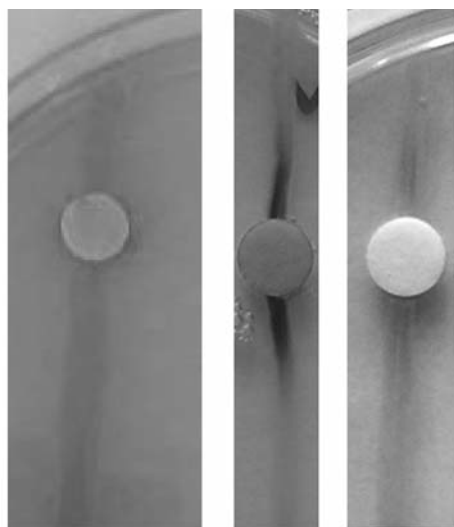
Figure 5. *The effect of (TFs) on the response of CV026 (AHL). A: Control disc with **TF4** (20 μg) alone (no colour).B: Control disc with AHL (10 ng) alone (deep purple colouration). C: Disc with **TF4** (20 μg/disc) and AHL (10 ng) (very light purple colouration).*

Table III. *The related final fluorescence (RFF) and specific activity (SA) of each (TF) on the efflux pump system of CV026. Samples consisted of saline plus 1 mg/l of ethidium bromide, 0.4% glucose, and without and with half (MIC) of positive control and TFs. Assessment of fluorescence took place at 37°C for 30 minutes. Data in bold identifies TFs that express very high inhibitory activity against the efflux pump system of CV026.*

TF	RFF	SA
1 (25 mg/l)	4.20	**1.68**
2 (6,25 mg/l)	2.99	**4.78**
3 (6,25 mg/l)	2.21	**3.53**
4 (25 mg/l)	4.42	**1.77**
5 (25 mg/l)	5.10	**2.04**
6 (100 mg/l)	4.31	**0.43**
7 (100 mg/l)	0.92	0.09
8 (100 mg/l)	1.24	0.01
9 (25 mg/l)	3.97	**1.59**
10 (100 mg/l)	0.44	0.04
11 (100 mg/l)	0.73	0.07
12 (100 mg/l)	1.18	0.12

SA=RFF/(0.5 MIC).

Table II. *Effects of (TFs) on (AHL)-mediated quorum sensing.*

TF 20 μg/disc	Intensity of colouration (mm)	Length of colouration (mm)	Growth inhibition (mm)
1	+++	34	0
2	+	30	17
3	+	34	22
4	+++	35	0
5	+++	37	0
9	+	36	22
AHL control	++++	40	0

Intensity scored as described in Figure 4.

Table IV. *The effects of (TFs) on the efflux pump system of Escherichia coli AG 100.*

Sample	RFF	SA
AG100 control	0.5397	0.000
+ TZ positive control. 30 mg/l	11.8703	0.6998
+ TF **1** 7.5 mg/l	5.0638	**1.1176**
+ TF **2** 3.75 mg/l	12.9179	**6.1160**
+ TF **3** 3.75 mg/l	10.4916	**4.9172**
+ TF **4** 30 mg/l	31.1324	**1.8894**
+ TF **5** 7.5 mg/l	7.8839	**1.8143**
+ TF **6** 30 mg/l	5.1950	0.2875
+ TF **7** 240 mg/l	7.2390	0.0517
+ TF **8** 60 mg/l	6.9595	0.1982
+ TF **9** 7.5 mg/l	6.4451	**1.4589**
+ TF **10** 240 mg/l	19.3553	0.1452
+ TF **11** 120 mg/l	6.6206	0.0938
+ TF **12** 60 mg/l	7.0865	0.2021

RFF: Relative final fluorescence; SA: specific activity=RFF/(0.5 MIC). Samples consisted of saline plus 1 mg/l of ethidium bromide, 0.4% glucose, and without and with half (MIC) of positive control and TFs. Assessment of fluorescence took place at 37°C for 30 min. Data in bold identifies TFs that express very high inhibitory activity against the efflux pump system of *E. coli.*

because the concentrations of TFs used in the assay were half of their MICs, and the MICs of the TFs against the strain differed significantly, the activities presented in Figure 6 do not permit a comparison of the activities between TFs. However, calculation of the specific activity of each TF by the formula for RFF presented in the Materials and Methods section affords a comparison, and these data are presented in Table III. Briefly, the activity of the positive control thioridazine was 1.02. Comparison of the activity of each relative to the positive control, TFs **2** and **3** have the greatest activity against the efflux pump system of CV026; TFs *1, 4, 5* and *9* have significant activity; and, TFs *7, 8, 10, 11* and *12* have no activity. The demonstrations that the TFs inhibit the response of an environmental strain to a QS signal and the same TFs inhibit the efflux pump of the environmental

responding strain, by themselves, do not support clinical interest in the TFs for possible use in the therapy of a bacterial infection. Therefore, in order to establish the needed support for the claim that indeed, the TFs have clinical value, the TFs were examined for activity against the efflux pump system of *E. coli*, a pathogenic bacterium. Since the number of graphs needed to depict each effect is large, an example of the data
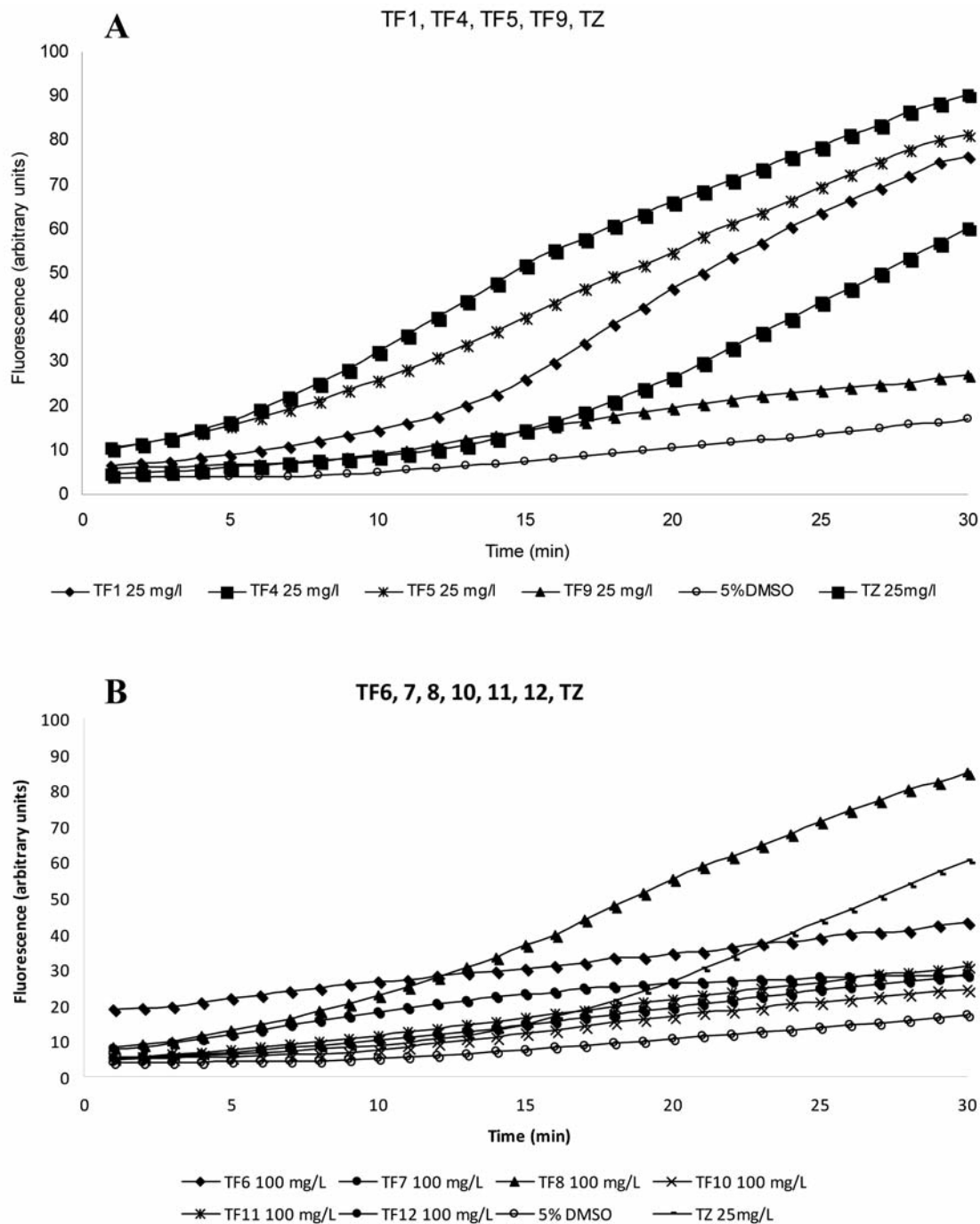
Figure 6. *The effect of half the minimum inhibitory concentration of (TFs)* ***1, 4, 5, 9*** *(A) , and* ***6, 7, 8, 10, 11, 12*** *(B) as compared to the positive control thioridazine (TZ).*

obtained is presented in Figure 7 for TF **4**. As evident from the figure, the presence of TF **4** promotes an increase of fluorescence due to the accumulation of EB whereas in the absence of the compound, no significant increase of fluorescence takes place (the curve is rather flat). The effects

of the TFs on the activity of the efflux pump is summarised in Table IV. This Table provides the concentration of the positive control thioridazine and each of the TFs that corresponds to half their MIC. As noted in Table IV, TFs **2** and **3** have the highest activity against the efflux pump system of
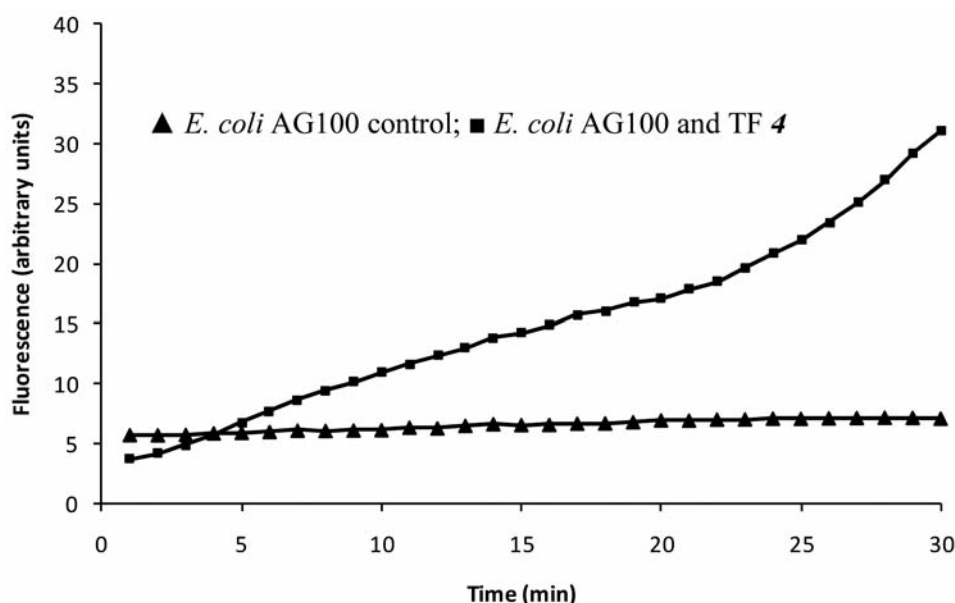
Figure 7. *The effect of TF 4 on the activity of the efflux pump system of Escherichia. coli AG100. The concentration of the TF 4 that correspond to half minimum inhibitory concentration was 30 mg/l. Note that the control does not accumulate ethidium bromide during the 30 minutes of the assay.*

E. coli. TFs *1, 4, 5* and *9* are also very active since their inhibitory activity exceeds that of thioridazine, the efflux pump inhibitor that serves as positive control.

Discussion

The results presented in this study show that some TFs have inhibitory activities against the response of CV026 to a QS signal such as AHL and the efflux pump systems of the CV026 and *E. coli* strains. Comparison of the efflux pump inhibitory activities of the TFs towards both strains suggests that the inhibition is practically identical in both cases. This suggests that the TFs have clinical value. Comparison of the individual inhibition induced by each TF on the QS response by CV026 to AHLs indicates that TF *3* exerts the greatest inhibition on the QS response. By comparison of the MIC of each effective TF, it is clear that the most effective inhibitors of the efflux pump system of *E. coli* also have the most potent antibacterial activity. TFs such as TF *7, 10* and *11* are devoid of any significant antibacterial activity (MIC for TF *7* and *10* is 480 mg/l, and for TF *11*, it is 240 mg/l) and have little activity against the efflux pump of *E. coli*. The demonstration of a QS response by the method used in this study requires the growth of the responding organism. If an agent inhibits growth, it pre-empts any response since there are no bacteria present to respond. The application of 20 μg of each TF to discs promoted strong antibacterial effects by TFs with very low MICs. Applying amounts of a TF that have significant antibacterial properties to a disc below the inhibitory concentration exceeds the sensitivity of the system,

since the distance between evident growth and the absence of a response (no colour) is masked by the deep purple colour associated with the growing population that is less than a millimetre from the disc. This is why TFs with very high MICs were able to produce evidence of an inhibition of the QS response by CV026, whereas for TFs with low MICs, with the exception of TF *3* (MIC 7.5 mg/l), the antibacterial effect of the TF pre-empted growth. QS is essential for various pathogens. In nature there are many types of organisms which can quench QS signals of other species. For example, *Bacillus* species produce lactonase enzyme to degrade the AHL signals of Gram-negative species. Eukaryotic cells also deploy QS inhibitors to prevent bacterial infection. QS has great importance in the organisation of biofilms (6), the production of virulence factors (8), and also in the spread of antibiotic resistance. The antibiotic resistance of bacteria in biofilms is several hundred or even thousand-fold higher than the one of free living bacteria. Without the ability to make biofilms or virulence factors production, pathogens lose the ability to cause infections. Therefore, if we reduce or completely block bacterial QS, we can reduce these QS-dependent/related activities as well. Our previous study demonstrated that phenothiazines were able to inhibit the QS system that involved Ezf 10-17, the producer of the signal and CV026, the responder to the signal (27). The phenothiazine thioridazine, an inhibitor of efflux pumps of Gram-negative bacteria (11, 12, 24, 25) also inhibits the response of CV026 to pure AHL (data not shown). Because phenothiazines and TFs that inhibit the efflux pumps of the CV026 and *E. coli*, as shown in the current study, also inhibit

access to energy supplied by the PMF (4, 5), we believe that the response to a QS signal depends upon a functional efflux pump system which extrudes the noxious QS signal before it reaches its intended target. Moreover, because all secretory activity of a bacterium such as the secretion of a QS signal, is controlled (28-30), and the main efflux pump systems of Gram-negative bacteria are the secretory paths of internally produced noxious agents (31-34), the inhibition of the efflux pump system of a QS signal producer will result in obviating the secretion of the QS signal. Therefore, it is our contention that inhibitors of an efflux pump, such as phenothiazines and now also TFs, will inhibit the secretion of the QS signal and the response to the QS signal. The results of the current study clearly show that various TFs have the ability to inhibit the response of *C. violaceum* 026 to the QS signal AHL and to inhibit the efflux pump of the QS-responding CV026 as well as the one of *E. coli*. This ability is a direct one since none of the TFs formed a complex with AHL (spectrophotometric data not shown). Since some TFs inhibit the QS response, inhibit an efflux pump system and also have significant antibacterial activity, TFs have a promising future for therapy of problematic infections that rely on an efflux-mediated multidrug-resistant phenotypes and which, due to QS, make their therapy problematic.

Acknowledgements

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IV.

INTERFERENCE IN QUORUM SENSING SIGNAL TRANSMISSION AMONGST MICROBIAL SPECIES

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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.

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

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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 be 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 through 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 through 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, $MgSO_4 \times 7H_2O$ 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 (20ºC) 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 37ºC and further incubation being continued at room temperature. QS inhibition was revealed as a decreased level of violacein production by CV026.

Results

Our results reflect *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 I and II), 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 (Fig. 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 I).

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 II). 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.

Table I

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	–

All of the tested isolates except strain 19579 modified the QS (Table II). 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 (Fig. 2).
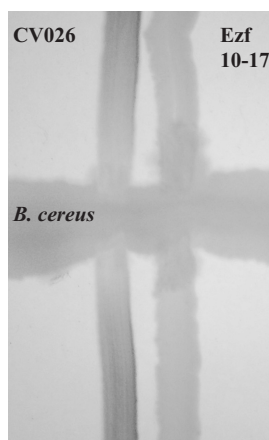

Figure 1. QS inhibitory activity of *B. cereus* in the system containing the CV026 sensor and Ezf 10-17 producer strain.
The inhibition of QS is revealed in the decreased level of violacein production

Table II

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

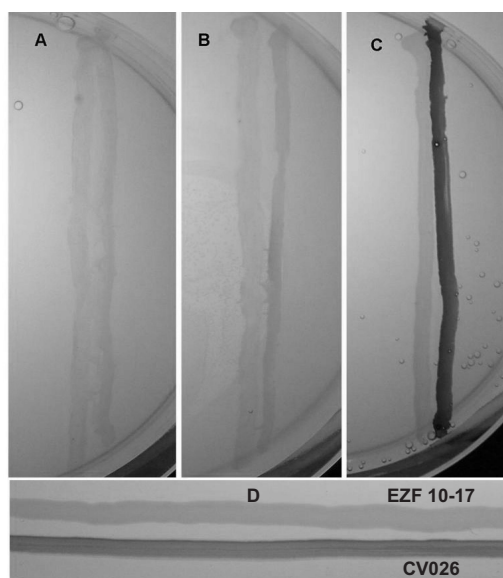| *E. coli* strain number	Growth inhibition of CV026	Growth inhibition of Ezf 10-17	Growth inhibition of *E. cloaceae* 31298	QS inhibition	Origin of isolate (specimen)
5536	–	–	–	+	abscess
10902	+	+	+	+	blood culture
10904	+	+	+	+	blood culture
11925	–	–	+	+ (very low)	blood culture
14525	–	–	+	+	abdominal 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

Figure 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

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.

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].

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.

Acknowledgements

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