Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 4 (9): 345-354, 2009
ISSN 1816-4935

Swimming Motility in *Agrobacterium tumefaciens* is Controlled by Quorum Sensing and Inhibited by Garlic Bulb Extract

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Abstract: Bacteria can produce and sense signal molecules, allowing the whole population to initiate a concerted action once a critical concentration (corresponding to a particular population density) of the signal has been reached; a phenomenon known as Quorum Sensing (QS). The current study was conducted to examine the possible role of QS in the regulation of swimming motility of *Agrobacterium tumefaciens*. In addition, we investigated the anti-QS or Quorum-Quenching (QQ) activity of garlic bulb and *Salvadora persica* extracts. We found that treatment of *A. tumefaciens* culture with different exogenous QS compounds induced swimming motility. C4 AHL, C6 AHL, C7 AHL, C8AHL, C10 AHL and C14 AHL induced bacterial swimming motility by about 3.5, 4, 4.5, 4.5, 3.5 and 4 fold, respectively, providing strong evidence that quorum sensing in *A. tumefaciens* controls cell motility, or at least plays a major role in its regulation. We also found that different QS compounds affect the bacterial phenotype, including the colony pattern and morphology. In addition, garlic bulb and *Salvadora persica* extracts were investigated for their QQ activity. While *S. persica* extract did not show any significant QQ activity, garlic bulb extract showed QQ activity against C4 AHL, C8 AHL, C10 AHL and C14 AHL, repressing the *A. tumefaciens* swimming motility induced by these QS compounds. To the best of our knowledge, this is the first report of a possible role for QS in the regulation of swimming motility in *A. tumefaciens*.

Key words: *Agrobacterium tumefaciens*, quorum sensing, quorum quenching, swimming motility, garlic bulb, *Salvadora persica*

INTRODUCTION

Bacterial populations co-ordinately regulate gene expression by producing diffusible signal molecules. These signals, known as auto-inducers or, more recently, quormones, accumulate extracellularly and interact specifically with a receptor protein to effect changes not related to their own metabolism (Gonza´lez and Marketon, 2003; Daniels *et al*., 2004). These diffusible signals frequently act to induce gene expression in response to bacterial cell density, a process often referred to as quorum sensing or cell-to-cell communication (Gonza´lez and Marketon, 2003; Bassler, 2002; Fuqua *et al*., 2001; Daniels *et al*., 2004).

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An important class of quormones is the family of N-acylhomoserine lactones (AHLs) used by Gram-negative bacteria. Variation in the N-acyl chain length and the oxidation state of AHLs allows for bacterial strain specificity in the signalling process and subsequent synchronisation of gene expression (Kaufmann *et al*., 2005). Quorum sensing within bacterial populations can promote pathogenesis, symbiosis, cellular dissemination or dispersal, DNA transfer and microbial biofilm development (Atkinson *et al*., 2006). One factor important for colonisation and pathogenesis is bacterial surface translocation, which can be achieved by swimming, swarming or sliding motility. For many pathogens, mutants unable to swim or swarm have been found to be unable to establish infection (Swift *et al*., 2001; Atkinson *et al*., 2006). It has been reported that in several different Gram-negative bacteria, different kinds of motility, including swarming, sliding and swimming motility, are regulated at least in part by AHL-dependent quorum sensing (Huber *et al*., 2001; Horng *et al*., 2002; Lupp and Ruby, 2005; Atkinson *et al*., 2006).

The critical role of QS in bacterial virulence and survival makes it a prime target for attacking bacterial pathogens (Adonizio *et al*., 2006). There are a number of ways to interrupt the QS system, including inhibition of a QS component or depletion of the signal itself. These disruptions result in an attenuation of the response in a process called quorum quenching (QQ). Thus, anti-quorum sensing (anti-QS) compounds are of great interest for the treatment of bacterial infections (Fast, 2003; Rice *et al*., 2005; Adonizio *et al*., 2006).

The main goal of the current study was to investigate the possible role of quorum sensing in the regulation of swimming motility in *Agrobacterium tumefaciens* (best known as the causative agent of crown gall, a neoplastic disease of plants). In addition, we investigated the activity of garlic bulb and *Salvadora persica* (a commonly used medicinal plant in Saudi Arabia) extracts as quorum quenchers.

MATERIALS AND METHODS

The following homoserine compounds were purchased from Sigma-Aldrich chemicals Co., (MO., USA): N-Butyryl-DL-homoserine lactone BHL (C4 AHL), N-Butyryl-DL-homocysteine thiolactone (AHTL), N-Hexanoyl-DL-homoserine lactone HHL (C6 AHL), N-(β-Ketocaproyl)-DL-homoserine lactone OHHL (3oxo-C6 AHL), N-Heptanoyl-DL-homoserine lactone (C7 AHL), N-Octanoyl-DL-homoserine lactone OHL (C8 AHL), N-Decanoyl-DL-homoserine lactone DHL (C10 AHL), N-Dodecanoyl-DL-homoserine lactone dDHL"(C12 AHL) and N-Tetradecanoyl-DL-homoserine lactone tDHL (C14 AHL).

Bacterial Strain and Media

Agrobacterium tumefaciens was a gift from the culture collection of the department of plant pathology, King Saud University. Luria-Bertani (LB) media was used for bacterial growth and preservation.

Bacterial Activation with QS Compounds

The effects of different QS compounds on *Agrobacterium tumefaciens* were investigated using a modification of previously reported methods (Qin *et al*., 2007; Rampioni *et al*., 2007). Stock solutions (1 mM) of QS compounds were prepared in acetic acid-acidified ethyl acetate (0.01% vol/vol) (BDH) (HPLC grade) except C14AHL, which was prepared in dichloromethane (BDH) (HPLC grade). The stock QS solution was sterilised by filtration using a 0.22 µm-pore-size filter membrane (Millipore). One hundred microlitres of this

solution were added to 30 mL sterile glass tubes and the solvent was evaporated in a 35°C water bath before the addition of 10 mL of LB broth to give a final concentration of 10 μM for the QS compounds. *Agrobacterium tumefaciens* was grown in LB broth and incubated at 28°C with shaking at 250 rpm for 24 h. This culture was used to inoculate the QS compound-containing media and the resulting culture was incubated at 28°C with shaking at 250 rpm for 24 h. These cultures were then used for the swimming motility assays.

Swimming Motility Assay of *A. tumefaciens*

The swimming migration assay was performed as described previously (Atkinson *et al*., 2006). Briefly, 5 μL of *A. tumefaciens* overnight cultures (grown in LB media or LB media containing different QS compounds, as described above), were inoculated onto the centre of a 0.4% soft LB agar plate and then incubated at 28°C. The swimming migration distance was assayed by following the colony fronts of the bacterial cells. Progress was recorded at 60 min intervals for 48 h.

Garlic Bulb and *Salvadora persica* Extract Preparation

Garlic bulb and *Salvadora persica* extracts were prepared as previously reported (Adonizio *et al*., 2006). Briefly, garlic bulbs and *Salvadora persica* were cut into small pieces and dried in a plant drier for approximately 24 h at room temperature. Dried plant materials was ground and added to 95% ethanol (100 g dry wt. L^{-1}) and allowed to stand for 24 h before vacuum filtration with filter paper (No. 1 Whatman Filter Paper, Whatman Ltd., England) to remove particulate materials. The solution was evaporated to dryness using a rotary evaporator (Evapotec™). The dry materials were stored at -20°C and reconstituted as needed in 95% ethanol and filtered through a 0.22 μm-pore-size filter membrane (Millipore).

Bacterial Cell Viability Test

The effects of garlic bulb and *Salvadora persica* extracts on *A. tumefaciens* viability were determined as previously reported (Rudi *et al*., 2005), using a bacterial viability kit (LIVE/DEAD® *Bac*Light™, Molecular Probes, Germany) according to the manufacturer's instructions to differentiate between viable and non-viable cells.

Anti-Quorum Sensing Activity

For anti-QS activity testing, garlic bulb or *S. persica* extract was mixed with different QS compounds in LB broth (5 mL) at a final concentration of 1 mg mL^{-1} and 10 μM, respectively and incubated for 10 min at room temperature with periodic shaking. LB media with only QS compounds, LB media with only garlic extract and LB media with only *S. persica* were used as controls. All media were inoculated with overnight cultures of *A. tumefaciens* and incubated at 28°C with shaking at 250 rpm for 24 h.

Extraction of QS Molecules from Bacterial Cultures

AHLs were extracted from spent cell-free *A. tumefaciens* culture supernatants (three replicates for each) using a modification of a method described previously (Rasmussen *et al*., 2005; Bazire *et al*., 2005; Gould *et al*., 2006; Catharine and Finan, 2009). The cell-free supernatants were extracted twice with an equal volume of dichloromethane (BDH) (HPLC grade). The extract was then passed through anhydrous Na_2SO_4 (BDH, UK) to remove excess water. The solution was evaporated at 35°C to dryness using a rotary evaporator (Evapotec™), then reconstituted in 50 μL of acetonitrile and filtered through

0.22 µm-pore-size filter membrane (Millipore). The samples were analyzed using an HPLC system (Shimadzu, Japan) under conditions described previously using a C18 reverse-phase column (100 mm×4.6 mm, 5 µm) at 40°C, fluorescence detector at 210 nm, flow rate of 1 mL min^{-1}. The column was re-equilibrated for a total of 3.5 min. Samples were re-dissolved in 50 µL acetonitrile prior to use and a 10 µL volume was injected onto the column. The mobile phase was acetonitrile:water. The gradient profile was as follows: 10% acetonitrile in water over 0 to 2 min, followed by a linear gradient from 10 to 70% acetonitrile over 12 min, 100% acetonitrile over 4 min, 100% down to 10% acetonitrile for 2 min and finally 10% acetonitrile for 8 min.

RESULTS AND DISCUSSION

Agrobacterium tumefaciens is a member of the Alphaproteobacteria, which forms complex biofilms on abiotic surfaces and plant tissues (Danhorn *et al*., 2004; Ramey *et al*., 2004; Peter *et al*., 2007). *Agrobacterium tumefaciens* is best known as the causative agent of crown gall, a neoplastic disease of plants. Pathogenesis involves the horizontal transmission of a segment of *A. tumefaciens* DNA, carried on the Tumour-inducing (Ti) plasmid, into the host plant genome, a process that is known to be completely controlled by N-acylhomoserine lactone (AHL)-mediated quorum sensing (Gelvin *et al*., 2003; Peter *et al*., 2007). However, our understanding of the activities that lead to plant association and productive attachment is far more limited. One factor important for colonisation and pathogenesis is bacterial surface translocation. Motility and chemotaxis have been implicated in plant association and the early steps of disease (Peter *et al*., 2007). Swimming motility in *A. tumefaciens* is mediated by flagella and there is no evidence of swarming or twitching motility. Multiple flagella are typically localised as a small tuft positioned at or around a single pole of the cell (Chesnokova *et al*., 1997; Peter *et al*., 2007). However, there have been no previous reports about the regulation of cell motility in *A. tumefaciens*.

Effect of Exogenous Quorum Signalling Molecules on *A. tumefaciens* Swimming Behavior

Agrobacterium tumefaciens culture was treated with different QS compounds and then the bacterial swimming behavior was observed. While the control showed limited cell swimming only after 4-5 days of incubation, treatment with different QS compounds resulted in an induction of bacterial swimming motility after only 24 h of incubation (Fig. 1). The results presented in Table 1 shows that C4 AHL, C6 AHL, C7 AHL, C8AHL, C10 AHL and C14 AHL induced bacterial swimming by about 3.5, 4, 4.5, 4.5, 3.5 and 4 fold, respectively.

Table 1: Swimming test of *Agrobacterium tumefaciens* in the presence of different QS molecules

	Colony and halo diameter (cm)	
Quorum sensing compounds	24 h	48 h
Control	1.5	2*
C4 AHL	5	7
AHTL	1	1
C6 AHL	5	8
3oxo-C6 AHL	1	1.5
C7 AHL	6	9
C8 AHL	7	9
C10 AHL	5	7
C12 AHL	1	1
C14 AHL	5	8

Induction of *A. tumefaciens* swimming motility by the addition of exogenous quorum signalling molecules provides strong evidence that quorum sensing in *A. tumefaciens* controls cell motility, or at least plays a major role in its regulation. Interestingly, treatment of the cells with C8 AHL also induced a new type of swimming behavior called vortex swimming (in a counter-clockwise pattern), which has not been reported in this bacterium before (Fig. 2). To the best of our knowledge, this is the first report suggesting a role for QS in the regulation of motility in *A. tumefaciens*.

It has been previously reported that QS, mediated by AHL, controls swimming and swarming motility in *Yersinia pseudotuberculosis.* However, researchers have been unable to identify precisely which homoserine lactone compound (s) is involved (Atkinson *et al*., 1999; Catharine and Finan, 2009). Interestingly, C8-AHL, which induces motility in *A. tumefaciens*, is one of the QS signalling molecules shown to be involved

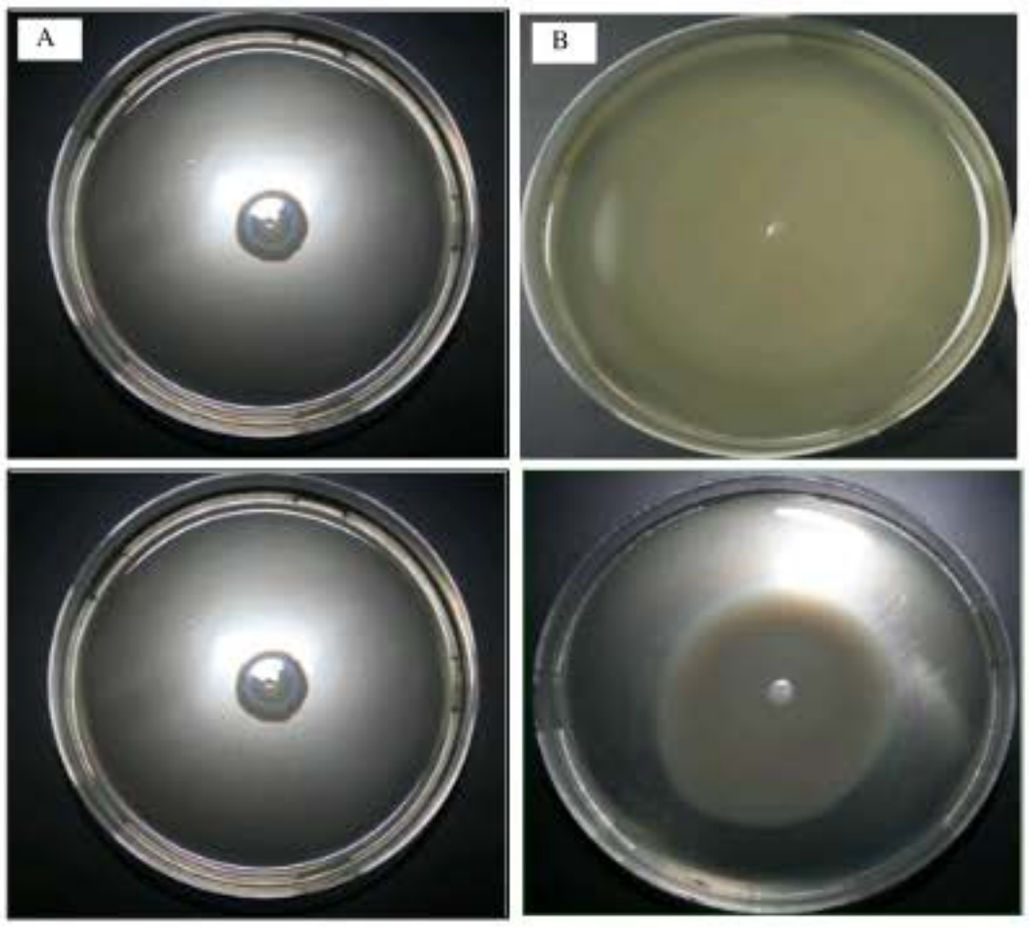

Fig. 1: Swimming motility test of *Agrobacterium tumefaciens* in the presence of different QS compounds. A: Control (with no QS compound treatment), B: *A. tumefaciens* cells treated with QS compounds followed by swimming motility testing

Fig. 2: Vortex movement (anti-clockwise pattern) of *A. tumefaciens* induced by C8 AHL. A: Control (with no QS compound treatment), B: *A. tumefaciens* cells treated with C8 AHL followed by a test of swimming behavior

in motility regulation in *Yersinia pseudotuberculosis* (Atkinson *et al*., 1999; Catharine and Finan, 2009). *Serratia liquefaciensis* and *S. marcescens* are generally motile bacteria, by means of peritrichous flagella. The formation of swarming colonies in these bacteria was also found to be controlled by an AHL-mediated QS system (Givskov *et al*., 1998; Fuqua *et al*., 2001).

Extraction and Detection of Quorum Sensing Molecules in *A. tumefaciens* Culture

As mentioned above, the addition of different exogenous QS molecules, including C4 AHL, C6 AHL, C7 AHL, C8AHL, C10 AHL and C14 h, induces cell swimming motility in *A. tumefaciens*. Trials were carried out to detect and extract endogenous AHLs in *A. tumefaciens* culture media using solvent extraction and HPLC. However, none of the QS compounds used in the study were detected in the culture media, even using large volumes (up to 2 L) of media. This failure to detect QS molecules in the culture may be due to the presence of the compounds at concentrations below our limits of detection. Another possibility is that these QS molecules are not produced naturally by *A. tumefaciens*, but instead are produced by other micro-organisms in the natural habitat of *A. tumefaciens* to interfere with their cell motility, a process known as bacterial cross-talk or interspecies quorum signalling (Daniels *et al*., 2004; Catharine and Finan, 2009). Signalling molecules produced either by unrelated bacteria (such as other AHLs and diketopiperazines) or excreted by plants (such as furanones) might influence the quorum sensing-regulated swarming behaviour of bacteria (Daniels *et al*., 2004).

Quorum Sensing and Colony phenotype of *A. tumefaciens*

In addition to the induction of swimming motility in *A. tumefaciens* by exogenous QS molecules, we also found that different QS molecules affect the bacterial phenotype, including the colony pattern and morphology. Although, AHTL had no effect on cell motility, treatment of the *A. tumefaciens* with AHTL resulted in the formation of smaller, thinner and more opaque colonies, indicating a reduction in slime production (Fig. 3). C6 AHL, in contrast, induced excess slime production and brown pigmentation of the *A. tumefaciens* colony (Fig. 4). It has been previously reported that violacein pigment production in *Chromobacterium violaceum* and exopolysaccharide production in *Pantoea stewartii* are controlled by QS systems (Salmond *et al*., 1995; Swift *et al*., 1996; Gonza´lez and Marketon, 2003).

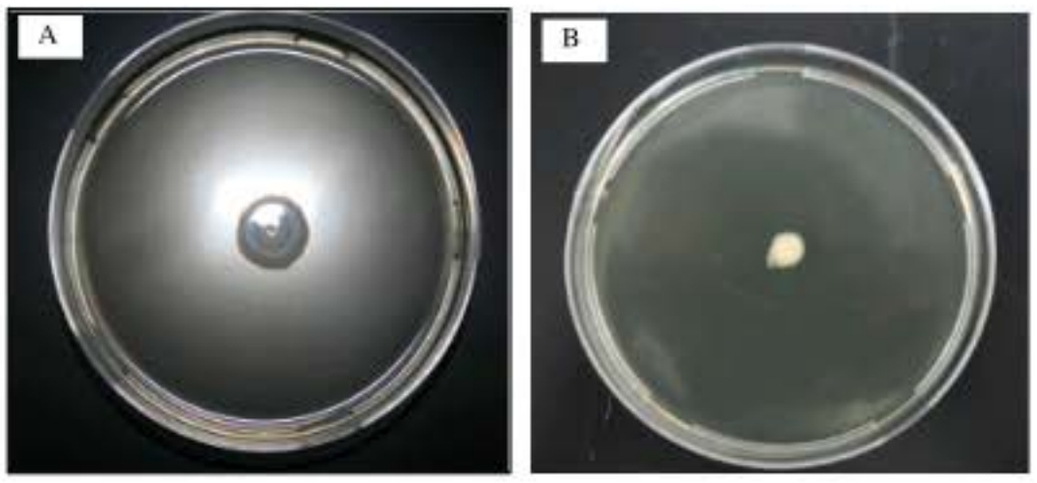

Fig. 3: Phenotype and colony morphology *A. tumefaciens*. A: Control, B: *A. tumefaciens* treated with AHTL

Anti-Quorum Sensing Activity

A relatively new and exciting area in the field of quorum sensing that has received much recent attention is Quorum Quenching (QQ), or the inhibition of QS signalling (Catharine and Finan, 2009). In this study, two plant extracts, garlic bulb and *Salvadora persica* (a commonly used medicinal plant in Saudi Arabia), were investigated for their anti-QS activities. First, the effects of garlic bulb and *S. persica* extracts (final concentration 1 mg mL^{-1} in LB media) on *A. tumefaciens* viability were tested using a bacterial viability kit according to the manufacturer's instructions. We found that neither the garlic bulb nor the *S. persica* extract showed any significant toxicity to *A. tumefaciens*. Therefore, this concentration was used to test these extracts for anti-QS activity. While *S. persica* extract did not show any significant QQ activity, we found that garlic extract had QQ activity against C4 AHL, C8 AHL C10 AHL and C14 AHL, as indicated by its repression of the swimming motility induced by these QS compounds (Fig. 5, Table 2).

It has been previously reported that a crude extract of garlic bulb specifically inhibits QS-regulated gene expression in *P. aeruginosa* (Rasmussen *et al*., 2005; Bjarnsholt *et al*., 2005). Ninety-two expressed *P. aeruginosa* genes (out of 167 genes) are regulated by QS and repressed by garlic bulb extract (Rasmussen *et al*., 2005). The mechanism by which garlic compounds block QS is presently unknown (Rasmussen *et al*., 2005; Bjarnsholt *et al*., 2005). Anti-quorum sensing agents were first characterized in the red marine alga *Delisea pulchra*, (Manefield *et al*., 1999;

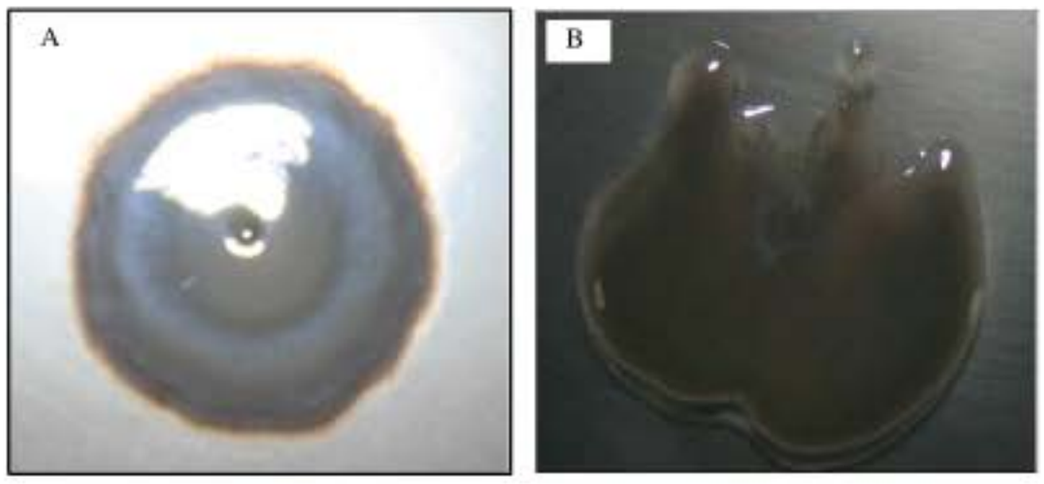

Fig. 4: Induction of polysaccharide production by C6AHL. A: Control, B: *A. tumefaciens* treated with C6AH

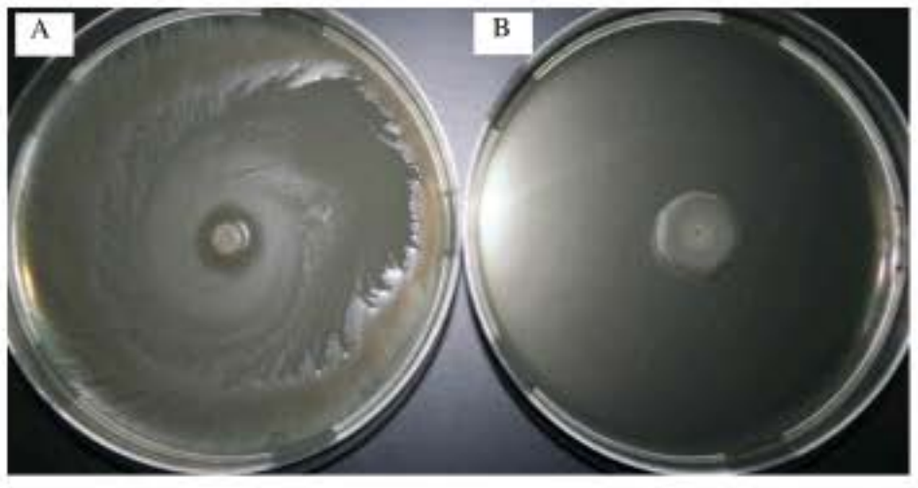

Fig. 5: Inhibition of *A. tumefaciens* vortex swimming by garlic extract. A: Cells treated with C8 AHL. B: Cells treated with mixture of C8 AHL and garlic bulb extract

Table 2: Inhibition of *A. tumefaciens* swimming by garlic extract

	Colony and halo diameter*			
	With garlic extract		Without garlic extract	
QS	24 h	48 h	24 h	48 h
Control	1.5	2.0	1.5	2.0
C4 AHL	5.0	7.0	1.5	3.0
AHTL	1.0	1.0	1.0	1.0
C6 AHL	5.0	8.0	5.0	8.0
3oxo-C6 AHL	1.0	1.5	1.0	1.5
C7 AHL	6.0	9.0	6.0	9.0
C8 AHL	7.0	9.0	1.0	2.0
C10 AHL	5.0	7.0	1.0	1.0
C12 AHL	1.0	1.0	1.0	1.0
C14 AHL	5.0	8.0	1.0	1.0

*All results are the means of triplicates

Cumberbatch, 2002) and more recently, in a south Florida alga (Gao *et al*., 2003; Adonizio *et al*., 2006) and a few higher medicinal plants (Teplitski *et al*., 2000; Bjarnsholt *et al*., 2005; Adonizio *et al*., 2008). A number of QQ enzymes that hydrolyse AHLs have been also identified in bacteria (Dong and Zhang, 2005).

CONCLUSION

The current study was conducted to investigate the possible role of quorum sensing in the regulation of swimming motility of *Agrobacterium tumefaciens,* as well as to investigate the QQ activity of garlic bulb and *Salvadora persica* extracts. We found that swimming motility of *A. tumefaciens* was induced by the addition of different exogenous QS compounds, providing strong evidence that quorum sensing in *A. tumefaciens* controls swimming motility, or at least plays a major role in its regulation. In addition to motility regulation, we also found that different QS compounds affect the bacterial phenotype, including the colony pattern and morphology. As motility and chemotaxis have been implicated in plant association and the early steps of disease, targeting the QS system that regulates swimming motility in *A. tumefaciens* could be a novel way to attack the pathogen and attenuate bacterial pathogenicity in an effort to control plant crown gall disease. However, more research is needed to further investigate this QS system at the molecular level. We also tested two plants, garlic bulb and *Salvadora persica*, for their QQ activity. While, *S. persica* extract showed no significant QQ activity, garlic bulb extract displayed QQ activity against several QS compounds. Given the promise of anti-QS compounds, efficient screening for these agents is imperative. To best of our knowledge, this is the first report suggesting a possible role for quorum sensing in the regulation of swimming motility in *A. tumefaciens*

ACKNOWLEDGMENTS

The authors are grateful to the Deanship of higher education, Deanship of Scientific Research, King Saud University and King Abdulaziz City for Science and Technology (KACST) for funding this project.

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