Influence of Polyphenols on Bacterial Biofilm Formation and Quorum-sensing

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Many bacteria utilize sophisticated regulatory systems to ensure that some functions are only expressed when a particular population density has been reached. The term 'quorum-sensing' has been coined to describe this form of density-dependent gene regulation which relies on the production and perception of small signal molecules by bacterial cells. As in many pathogenic bacteria the production of virulence factors is quorum-sensing regulated, it has been suggested that this form of gene regulation allows the bacteria to remain invisible to the defence systems of the host until the population is sufficiently large to successfully establish the infection. Here we present first evidence that polyphenolic compounds can interfere with bacterial quorum-sensing. Since polyphenols are widely distributed in the plant kingdom, they may be important for promoting plant fitness.

Key words: Quorum-sensing Inhibition, Biofilm Formation, Polyphenols

Introduction

Evidence has accumulated over the past few years that numerous bacteria employ cell-cell communication systems that rely on small signal molecules to express certain phenotypic traits in a density-dependent manner. This regulatory principle is now generally referred to as quorum-sensing (Fuqua *et al.*, 1994). Among Gram-negative bacteria the most intensively investigated and probably the most wide-spread signaling molecules are *N*-acyl-homoserine lactones (AHLs). AHL-based regulatory systems typically rely on two proteins, an AHL synthase, usually a member of the LuxI family of proteins, and an AHL receptor protein belonging to the LuxR family of transcriptional regulators. At low population densities cells produce a basal level of AHL *via* the activity of the AHL synthase. As the cell density increases, AHLs accumulate in the growth medium. On reaching a critical threshold concentration, the AHL molecule binds to its cognate receptor, which in turn activates or inhibits expression of target genes (for reviews see Fuqua *et al.*, 1996; Eberl, 1999).

Given that AHL-mediated quorum-sensing is utilized by many pathogens, it may be expected that higher organisms have evolved strategies to disrupt these signaling pathways. The best-investigated example of an eukaryotic organism which produces metabolites that specifically interfere with bacterial communication is the Australian macroalga *Delisea pulchra*, which produces a range of metabolites known as halogenated furanones (de Nys *et al.*, 1993). These compounds exhibit a broad range of biological activities, including antifouling and antimicrobial properties (de Nys *et al.*, 1995). More recent results have shown that some of the furanones specifically interfere with AHL-regulated processes (Givskov *et al.*, 1996) by accelerating the degradation of the AHL receptor protein (Manefield *et al.*, 2002).

Many resistant interactions with fungi and bacteria involve the accumulation of toxic concentrations of phenolic compounds. Catechin and proanthocyanidins were found to complex with spores and hyphae of pathogenic fungi of fruit crops (Feucht *et al.*, 2000), and phenolic polymer deposition was correlated with a decrease in bacterial multiplication rates (Leach *et al.*, 2001). In this study we show that some polyphenolic compounds having a gallic acid moiety (as for example, epigallocatechin gallate, EGCG, and ellagic acid as well

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as tannic acid) that are commonly produced by various plant species specifically block AHL-mediated communication between bacteria.

Materials and Methods

(−)-Epigallocatechin gallate (EGCG) was purchased from Sigma (E-4143, Sigma-Aldrich Chemie, Steinheim, Germany), (+)-catechin and tannic acid from Carl Roth (Karlsruhe, Germany).

Bacterial growth and analysis of toxic effects

Escherichia coli and *Pseudomonas putida* were grown in Luria-Bertani (LB) broth (Bertani, 1951). *Burkholderia cepacia* was grown in AB minimal medium (Clark and Maaløe, 1967) supplemented with 10 mM citrate (ABC medium). To investigate the toxicity of the investigated compounds growth experiments were performed in the presence of various concentrations (ranging from 20 to 80 μg/ml) of EGCG, ellagic acid and tannic acid. Tannic acid was dissolved in $H_2O_{dest.}$, ellagic acid and EGCG in DMSO (40 mg/ml w/v). As controls, growth experiments were also performed in respective media supplemented with the solvent DMSO. Cultures were grown at 30 °C under vigorous shaking. Growth of the cultures was monitored spectrophotometrically by a Ultraspec Plus spectrophotometer (Pharmacia, Upsala, Sweden) by measurement of optical density at 600 nm (OD_{600}) at various time intervals. In all subsequent experiments the three substances were used in concentrations that did not interfere with bacterial growth.

Inhibition of AHL sensor strains

Quorum-sensing inhibition by EGCG, ellagic acid and tannic acid was investigated using the following sensor strains: (1) *P. putida* harbouring the sensor plasmid pKR-C12 (Steidle *et al.*, 2001) and (2) *E. coli* MT102 harbouring the sensor plasmid pSB403 (Winson *et al.*, 1998). The sensor strains were grown over night in Luria-Bertani (LB) medium at 30 °C, diluted 4-fold in fresh medium and grown for another hour. After addition of the respective signal molecule [50 nM of *N*-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL) for pKR-C12 and 100 nM of *N*-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HSL) for pSB403], 100 μl aliquots of culture were pipetted into the wells of a microtitre plate (FluoroNunc Polysorp, Nunc, Wiesbaden, Germany). The compounds were added to the wells in final concentrations ranging from 1.25 to 60 μg/ml. Thereafter, microtitre plates were incubated at 30 °C for 4 h. Fluorescent or bioluminescent signals were measured with a Lambda Fluoro 320 Plus Reader (Bio-Tek Instruments, Winooski, Vermont, USA). Inhibitor-mediated reduction of the reporter strain signal was correlated to the value obtained without addition of the compounds.

Inhibition of biofilm formation

Biofilm formation in polystyrene microtitre dishes was assayed essentially as described previously (O'Toole and Kolter, 1998) with a few modifications. An overnight culture of *B. cepacia* in ABC-medium was diluted 1:100 in fresh medium and grown for another hour. After the addition of different concentrations of the compounds, 100 μl aliquots of culture were pipetted into the wells of the microtitre dishes and incubated for 48 h at 30 °C. Thereafter, the medium was removed, and 100 μl of a 1% (w/v) aqueous solution of crystal violet (CV) was added. Following staining at room temperature for 20 min, the dye was removed and the wells were washed thoroughly. For quantification of attached cells the CV was solubilized in DMSO and the absorbance was determined at 570 nm. Inhibitor-mediated reduction of biofilm formation was correlated to the value obtained without addition of the compounds.

Swarming motility

For the examination of swarming motility ABC medium supplemented with 0.1% Casamino acids was solidified with 0.4% (w/v) agar (Huber *et al.*, 2001). The compounds were added to the medium and agar plates were allowed to solidify for one hour. Thereafter, they were point-inoculated with *B. cepacia* H111 and incubated at 30 °C for 24 h.

Results

EGCG, ellagic acid and tannic acid are all derived from gallic acid. Epigallocatechin is esterified with gallic acid to form EGCG. In ellagic acid, two gallic acid molecules are linked by ester

bonds. Tannic acid is a mixture containing galloyl-D-glucose as monomeric and oligomeric structures.

Growth inhibition

Tannic acid inhibited growth of *E. coli* at concentrations higher than 60 μg/ml, while *B. cepacia* and *P. putida* were already inhibited at concentrations above 30 μg/ml. *E. coli* and *B. cepacia* were not inhibited by ellagic acid in concentrations up to 40 μg/ml, while for *P. putida* the maximum non-inhibitory concentration was 30 μg/ml. EGCG could be added to the medium at concentrations up to 40 μg/ml without affecting growth of the three strains.

Quorum-sensing inhibition

The potential of EGCG, ellagic acid and tannic acid to interfere with bacterial cell-cell communication systems was investigated using two different AHL biosensors that, depending on the components used for their construction, respond to different types of AHL molecules. pKR-C12 contains a translational fusion of the *lasB* elastase gene of *Pseudomonas aeruginosa* to *gfp* (ASV) encoding an unstable version of the green fluorescent protein (GFP) (Steidle *et al.*, 2001). Furthermore, the sensor contains the *lasR* gene, which encodes the cognate 3-oxo-C12-HSL receptor protein under control of a lac-type promoter. Since expression of *lasB* is controlled by the *las* quorum-sensing system, this sensor is most sensitive for 3-oxo-C12 HSL and related long-chain AHLs. pSB403 contains the *Photobacterium fischeri luxR* gene together with a transcriptional fusion of the *luxI* promoter region to the bioluminescence operon *luxCDABE*. Although this sensor exhibits the highest sensitivity for the *P. fischeri* signal 3-oxo-C6-HSL, a relatively wide range of other AHL molecules can be detected by the sensor (Winson *et al.*, 1998; Geisenberger *et al.*, 2000).

To analyse whether EGCG, ellagic acid and tannic acid were capable of antagonizing AHL-dependent quorum-sensing systems, decreasing concentrations of the substances were added to cultures of the different biosensors in the presence of the respective inducing AHL. In the case of AHL-inhibitory activity a reduction in the expression of the reporter gene(s) (*luxAB* or *gfp*) is anticipated.

In these assays all three substances exhibited significant antagonistic effects (Fig. 1). Using the biosensor *E. coli* MT102 (pSB403) EGCG caused a more than 50% reduction of the signal intensity at a concentration of 40 μg/ml. In the case of the sensor *P. putida* (pKR-C12) the fluorescence signal was reduced by 40%. Ellagic acid caused a reduction in signal intensity of about 40% at a concentration of 40 μg/ml with *E. coli* MT102 (pSB403) and at a concentration of 30 μg/ml with *P. putida* (pKR-C12), respectively. Less pro-

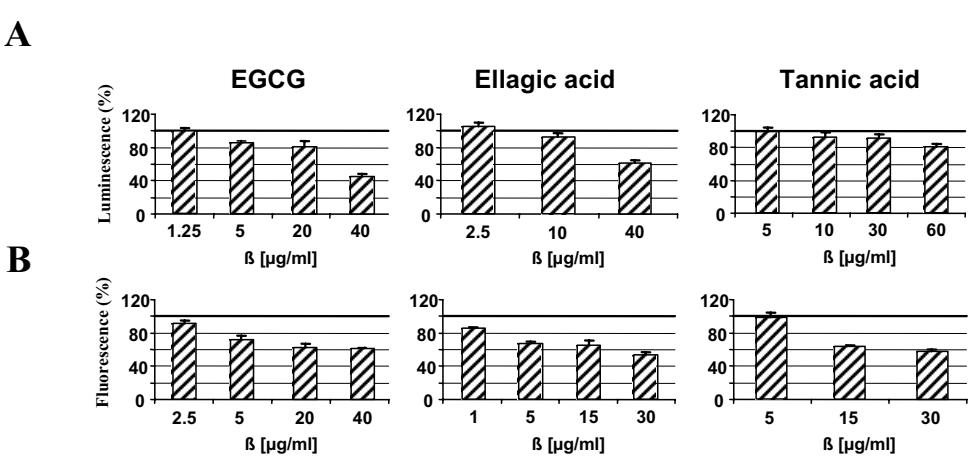

Fig. 1. Response of different quorum-sensing reporter strains to the respective signal molecules and (–)-epigallocatechin gallate (EGCG), ellagic acid, and tannic acid. A: *Escherichia coli* harbouring pSB403; B: *Pseudomonas putida* harbouring pKR-C12. The reporter strain signals (bioluminescence and fluorescence) in absence of the compounds were set to 100%.

nounced effects were observed with tannic acid: signal intensities were reduced by 20% at a concentration of 60 μg/ml with *E. coli* MT102 (pSB403) and by 40% at a concentration of 30 μg/ml with *P. putida* (pKR-C12).

Inhibition of biofilm formation and swarming motility

To further analyse the AHL-antagonistic acitivites of EGCG, ellagic acid, and tannic acid, their effects on two quorum-sensing controlled phenotypes, biofilm formation and swarming motility, were investigated. In a number of strains, including *B. cepacia*, the formation of biofilms is quorum-sensing regulated (Davies *et al.*, 1998; Huber *et al.*, 2001). AHL-negative mutants produce significantly thinner biofilms than the wild type and thus inhibition of quorum-sensing would be expected to prevent colonization of surfaces. The addition of tannic acid did not result in a significant reduction of the thickness of biofilms formed by *B. cepacia* (data not shown). In contrast, formation of biofilms was reduced by 30% by the addition of EGCG and by 50% by the addition of ellagic acid (both at a concentration of 40 μg/ml) (Fig. 2).

Besides biofilm formation, swarming motility on a surface is a second form of multicellular behaviour controlled by quorum-sensing in a number of bacteria (Eberl *et al.*, 1996). In *B. cepacia*, the quorum-sensing negative mutant H111-I is no longer able to swarm (Huber *et al.*, 2001; Fig. 3) Like biofilm formation, swarming motility did not seem to be affected by the addition of tannic acid to the medium (data not shown). However, ellagic acid at a concentration of 20 μg/ml completely inhibited swarming and EGCG at 40 μg/ml significantly reduced the ability of *B. cepacia* to swarm (Fig. 3).

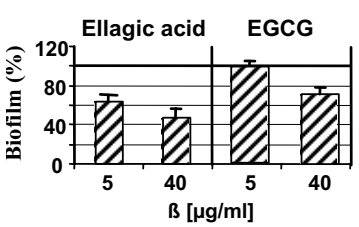

Fig. 2. Inhibition of biofilm formation of *Burkholderia cepacia* in% of control by ellagic acid and (–)-epigallocatechin gallate (EGCG) in different concentrations. Cells were grown in AB minimal medium supplemented with 10 mM citrate in the wells of polypropylene microtitre dishes. After incubation for 48 h at 30 °C planktonic cells were removed and attached cells were then stained with crystal violet. Biofilm thickness in the absence of compounds is set to 100%. Error bars represent the standard deviation of the mean for six independent wells.

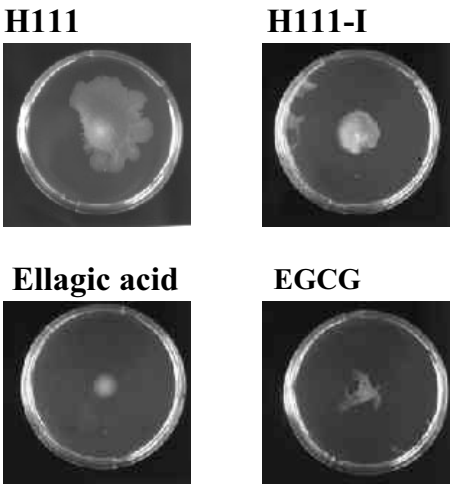

Fig. 3. Ellagic acid and EGCG inhibit swarming motility on ABC medium with 0.4% agar. Shown are swarming of *B. cepacia* H111, the QS mutant H111-I, and of *B. cepacia* on agar plates with ellagic acid (20 μg/ml) and (–)-epigallocatechin gallate (EGCG) (40 μg/ml), respectively.

Discussion

Many plants have co-evolved and established carefully regulated symbiotic or syntrophic associations with bacteria and it therefore may not be too surprising that higher organisms are capable of perceiving and responding to these molecules. In a recent study Teplitski *et al.* (2000) showed that several plants secrete substances that mimic bacterial AHLs and affect quorum-sensing regulated behaviours in respective plant-associated bacteria. Exudates from pea (*Pisum sativum*) were demonstrated to contain several separable activities that mimicked bacterial AHL signals. While some of these activities stimulated AHL-dependent phenotypes, others inhibited expression of AHL-regulated traits. Our results show that tannic acid, ellagic acid and EGCG, compounds that are commonly produced by many plants, specifically interfere with AHL-mediated signaling. Similar to the furanones of *D. pulchra* the specific AHL-antagonistic effect of these compounds is limited to

a certain concentration range, as at higher concentrations growth inhibition, *i.e.* bacteriocidal effects, is observed. Hence these substances exhibit at least two biological activities: inhibition of growth at high concentrations and blockade of bacterial signaling and thus prevention of plant surface colonization at lower concentrations. The inhibitory efficiencies of plant compounds investigated here are considerably lower than those of the furanones. However, it has become clear that the furanone inhibitors have limitations because they are reactive molecules and thus can only serve as model compounds in research. In contrast, some plants produce EGCG, ellagic acid and tannic acid in relatively high amounts, for example in fruit and leaves. Furthermore, these compounds were shown to be beneficial for human health (Bravo, 1998). EGCG is the main catechin component of green tea often reaching more than 10% of the dry weight. In accordance with our results, it was shown previously that tea extracts (and purified components thereof) inhibit biofilm formation (Shimomura, 1992). Likewise, fruit trees utilize catechin-derived flavanols to protect the fruit surface against pathogenic attack (Feucht *et al.*, 1994; Feucht and Schwalb, 2000).

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