Dietary plant components ellagic acid and tannic acid inhibit *Escherichia coli* biofilm formation

Bacteria usually live as surface-associated communities, rather than as planktonic cells. These compact microbial consortia, referred to as biofilms, are commonly associated with many health problems (Costerton *et al.*, 1999; Donlan, 2002). A few common examples of where biofilms form are in dental plaque, in lung infections and in infections related to the use of medical devices, such as catheters. Many persistent and chronic bacterial infections are now thought to be linked to biofilm formation; more than 60 % of all bacterial infections have been estimated to involve biofilm formation (Lewis, 2001). Virtually all medical implants are prone to colonization and biofilm formation by pathogenic bacteria, and such biofilms can serve as a source for recurrent infections (Costerton *et al.*, 1999). Biofilm-linked infections are particularly problematic, because biofilm-associated bacteria can tolerate immune defences, antibiotics, biocides and hydrodynamic shear forces far better than the corresponding planktonic bacteria. The intrinsic tolerance of biofilm-associated bacteria towards antimicrobial agents makes biofilm-associated infections particularly recalcitrant toward treatment. New types of antibiotics are therefore needed; development of candidate drugs that target biofilm formation will be of great importance. Most conventional antibiotics have targeted biochemical and physiological functions that are present both in pathogenic as well as saprophytic bacteria. Specific targeting of disease-associated bacterial lifestyles such as biofilm formation but not free-living pelagic bacteria is a highly attractive approach that has not yet been exploited. Drugs specifically aimed at bacterial biofilm formation are unlikely to be cross-resistant to existing therapies.

Plants have been known for many years to contain health-improving substances. One example is green tea, which has been consumed for over 4000 years by a large

number of people. Traditionally green tea has been recommended against infectious diseases and was frequently taken by patients suffering from infections (Hamilton-Miller, 1995). Dietary plant material contains numerous metabolites with antibacterial effects; the antibacterial properties have been attributed to the high level of polyphenols (30 % of dry weight in green tea) such as ellagic acid (EA) and tannic acid (TA) (Akiyama *et al.*, 2001). On this background, we have investigated the anti-biofilm properties of these plant compounds.

TA and EA (Fig. 1a) were tested for their ability to inhibit biofilm formation by *Escherichia coli*. TA and EA were purchased from Sigma. Stock solutions of TA and EA (each 10 mg ml⁻¹) were prepared in water and DMSO, respectively, and filter sterilized before use. MIC determinations were performed in FBA minimal medium (with 0.02 % glucose, 0.02 % Casamino acids, 1 µg thiamine ml⁻¹ and, in the case of VR50, 10 µg panthotenic acid ml⁻¹) according to the broth microdilution method and the concentrations used in the biofilm experiments were set to <1/20 of the MICs to ensure non-toxic conditions. Bacterial susceptibility was also assessed in urine, where the compounds showed no significant effect on planktonic growth under the conditions chosen for the biofilm experiments. The final concentration of TA and EA was 30 µg ml⁻¹. After addition of each of the tested compounds to the growth medium, the pH was checked and no change in pH due to the addition of compounds was observed.

Two *E. coli* strains with excellent biofilm-forming abilities were chosen to monitor the biofilm-reducing potential of TA and EA, viz. VR50, a urinary tract infectious strain (Roos *et al.*, 2006), and F18, a commensal isolate (Myhal *et al.*, 1982). Both strains grew well in the presence of the two plant compounds and no

reduction of growth was observed in the presence of the compounds at the concentrations used in the biofilm experiments (MIC values of >600 µg ml⁻¹).

Biofilm formation in FBA minimal medium in 24-well flat-bottom microplates (Iwaki) at 37 °C of *E. coli* strains VR50 and F18 alone (control) or in the presence of EA and TA was monitored. TA and EA were added to static cultures at the time of inoculation. After incubation overnight, biofilm formation was determined by crystal violet staining as described previously (Ferrières *et al.*, 2007) and the amount of biofilm was related to that of controls grown in medium without any additions. Each strain and compound was tested in three wells per plate and all experiments were repeated three to seven times. Both compounds reduced biofilm formation by VR50 and F18 significantly. TA and EA reduced biofilm formation by 44–80 % and 22–26 %, respectively (Fig. 1b). In the presence of both TA and EA, no synergistic effect of the two compounds was observed (Fig. 1c).

To get rid of toxic substances and waste products, bacteria have efficient efflux systems. Efflux pumps occur both as single- and multi-component systems (Lee *et al.*, 2000). Upon inhibition of these efflux pump systems, the intracellular concentration of toxic substances can reach critical levels, resulting in stress and/or killing of cells (Marquez, 2005; Piddock, 2006). Efflux pump inhibitors (EPIs) have attracted a great deal of attention due to their ability to block some types of antibiotic resistance in bacteria (Marquez, 2005; Piddock, 2006); the EPIs known to block multidrug resistance pumps are of particular interest. Thioridazine (TZ; Fig. 1a) is a well-known EPI (Piddock, 2006). In a recent study, we demonstrated that TZ is an efficient inhibitor of biofilm formation in *E. coli* and *Klebsiella pneumoniae* (Kvist *et al.*, 2008). These

Fig. 1. (a) Molecular structures of the plant components TA and EA and of the EPI TZ used in the biofilm experiments. (b) Biofilm formation in FBA minimal medium of *E. coli* strains VR50 and F18 alone (control) or in the presence of TZ, EA and TA. Asterisks indicate significant reduction compared with the control (paired two-tailed *t* test of at least three independent experiments; **P* < 0.05, ***P* < 0.01). Bars indicate standard deviation. (c) Biofilm formation of *E. coli* UTI strain VR50 (black bars) and the two knockout mutants VR50 $\Delta yggA$ (shaded bars) and VR50 $\Delta aaeX$ (white bars) in the presence of TZ, EA and TA. In all cases, except for TA, the two knockout mutants showed significant reduction in biofilm formation compared with VR50 grown with the same compound (paired two-tailed *t* test of at least four independent experiments; *P* < 0.01). Bars indicate standard deviation.

results suggest the use of EPIs as novel anti-biofilm agents. Biofilm formation in the presence of TZ was also monitored. TZ was purchased from Sigma and a stock solution (10 mg ml⁻¹) was prepared in

water and filter sterilized. The MIC was determined as described for EA and TA, and the concentration used in the biofilm experiments (40 μ g ml⁻¹) was set to 1/5 of the MIC. TZ reduced biofilm formation in

VR50 and F18 by ~20 % and ~60 %, respectively (Fig. 1b), at 1/5 MIC; MIC values for both strains were 200 μ g ml⁻¹. With this in mind, we wanted to test the effect of TA and EA in combination with

TZ. In the case of strain VR50, the biofilm-reducing effect of EA was significantly enhanced in the presence of TZ, i.e. from 22 % to 78 % (Fig. 1b). For both *E. coli* strains tested, the combination of TZ with either TA or EA reduced biofilm formation by between 73 % and 89 % (Fig. 1b).

Two knockout mutants of VR50 with deletions in transport/efflux systems, i.e. VR50 Δ yggA and VR50 Δ aaeX (Hancock *et al.*, 2007), showed significant reduction in biofilm formation in FBA minimal medium (35–37 %). These mutants have previously been shown to have decreased biofilm formation (Hancock *et al.*, 2007). In the presence of EA, the mutants showed a significantly greater reduction in biofilm formation, 78–80 %, compared with the wild-type (Fig. 1c). In the presence of TA, however, the mutants showed no significant difference compared with wild-type. Despite the fact that no synergistic effect of the two plant acids on biofilm formation by the wild-type could be observed, the very different effects of the plant compounds on the knockout mutants suggest that they act by different mechanisms on biofilm formation (Fig. 1c).

The mechanism responsible for the antibacterial effects of tea and other plant compounds is somewhat elusive; plant secondary metabolites, to a large extent polyphenols, show bactericidal effects as a result of damage to the cell membrane (Maeyama *et al.*, 2005). However, we observed no growth reduction of bacteria growing in liquid cultures with the concentrations used for the biofilm experiments. Therefore, the mechanism by which EPIs potentiate the use of antibiotics (*vide supra*) does not necessarily apply to the effect observed in the case where the EPI TZ was used in combination with our plant compounds.

In conclusion, we have presented results indicating that secondary metabolites, such as TA and EA, from plants act as anti-biofilm agents. Such substances are worthy of attention in the fight against bacterial infections. Contrary to classical antibiotics that generally kill bacteria, the plant compounds reported on here rather seem to modify the behaviour of bacteria, i.e. they abolish biofilm formation but have no or little effect on planktonic bacteria. In effect, they can be considered as lifestyle-modifying drugs. Hopefully, such compounds might not be as prone as classic antibiotics to the development of bacterial resistance.

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Akiyama, H., Fujii, K., Yamasaki, O., Oono, T. & Iwatsuki, K. (2001). Antibacterial action of several tannins against *Staphylococcus aureus*. *J Antimicrob Chemother* **48**, 487–491.

Costerton, J. W., Stewart, P. S. & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–1322.

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerg Infect Dis* **8**, 881–890.

Ferrières, L., Hancock, V. & Klemm, P. (2007). Biofilm exclusion of uropathogenic bacteria by selected asymptomatic bacteriuria *Escherichia coli* strains. *Microbiology* **153**, 1711–1719.

Hamilton-Miller, J. M. T. (1995). Antimicrobial properties of tea (*Camellia sinensis* L.). *Antimicrob Agents Chemother* **39**, 2375–2377.

Hancock, V., Ferrières, L. & Klemm, P. (2007). Biofilm formation by asymptomatic and virulent urinary tract infectious *Escherichia coli* strains. *FEMS Microbiol Lett* **267**, 30–37.

Kvist, M., Hancock, V. & Klemm, P. (2008). Inactivation of efflux pumps abolishes bacterial biofilm formation. *Appl Environ Microbiol* **74**, 7376–7382.

Lee, A., Mao, W., Warren, M. S., Hoshino, K., Okumura, R., Ishida, H. & Lomovskaya, O. (2000). Interplay between efflux pumps may provide either additive or multiplicative effects on drug resistance. *J Bacteriol* **182**, 3142–3150.

Lewis, K. (2001). Riddle of biofilm resistance. *Antimicrob Agents Chemother* **45**, 999–1007.

Maeyama, R., Kwon, I. K., Mizunoe, Y., Anderson, J. M., Tanaka, M. & Matsuda, T. (2005). Novel bactericidal surface: catechin-loaded surface-erodible polymer prevents biofilm formation. *J Biomed Mater Res A* **75**, 146–155.

Marquez, B. (2005). Bacterial efflux systems and efflux pumps inhibitors. *Biochimie* **87**, 1137–1147.

Myhal, M. L., Laux, D. C. & Cohen, P. S. (1982). Relative colonizing abilities of human fecal and K-12 strains of *Escherichia coli* in the large intestines of streptomycin-treated mice. *Eur J Clin Microbiol* **1**, 186–192.

Piddock, L. J. V. (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin Microbiol Rev* **19**, 382–402.

Roos, V., Nielsen, E. M. & Klemm, P. (2006). Asymptomatic bacteriuria *Escherichia coli* strains: adhesins, growth and competition. *FEMS Microbiol Lett* **262**, 22–30.