Cranberry derivatives enhance biofilm formation and transiently impair swarming motility of the uropathogen *Proteus mirabilis* strain HI4320

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CHE O'MAY, OLIVIER AMZALLAG, KARIM BECHIR, and NATHALIE TUFENKJI*,

Department of Chemical Engineering, McGill University, 3610 University Street, Montreal, Quebec H3A 0C5, Canada

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* *Corresponding Author. Phone: (514) 398-2999; Fax: (514) 398-6678; E-mail: nathalie.tufenkji@mcgill.ca*

Abstract

Proteus mirabilis is a major cause of catheter associated urinary tract infection (CAUTI) emphasizing that novel strategies for targeting this bacterium are needed. Potential targets are *P. mirabilis* surface-associated swarming motility and the propensity of these bacteria to form biofilms that may lead to catheter blockage. We previously showed that the addition of cranberry powder (CP) to lysogeny broth (LB) medium resulted in impaired *P. mirabilis* swarming motility over short time periods (up to 16 h). Herein, we significantly expanded on those findings by exploring: (i) the effects of cranberry derivatives on biofilm formation of *P. mirabilis*; (ii) whether swarming inhibition occurred transiently or over longer periods more relevant to real infections (~3 days); (iii) whether swarming was also blocked by commercially available cranberry juices; (iv) whether CP or cranberry juices exhibited effects under natural urine conditions; and (v) the effects of cranberry on medium pH which is an indirect indicator of urease activity. At short time-scales (24 h), CP and commercially available pure cranberry juice impaired swarming motility and repelled actively swarming bacteria in LB medium. Over longer time periods more representative of infections (~3 days), the capacity of the cranberry material to impair swarming diminished and bacteria would start to migrate across the surface, albeit by exhibiting a different motility phenotype to the regular 'bull's-eye' swarming phenotype of *P. mirabilis*. This bacterium did not swarm on urine agar or LB agar supplemented with urea suggesting that any potential application of anti-swarming compounds may be better suited to settings external to the urine environment. Anti-swarming effects were confounded by the ability of cranberry products to enhance biofilm formation in both LB and urine conditions. These findings provide key insights into the long-term strategy of targeting *P. mirabilis* CAUTIs.

Keywords: *cranberry, urinary tract infection, Proteus mirabilis, motility, biofilm*

Introduction

The Gram-negative bacterium *Proteus mirabilis* is a key pathogen causing catheter-associated urinary tract infections (CAUTI), a common occurrence in long-term catheterised (> 28 days) individuals (Morris et al. 1999; Nielubowicz and Mobley 2010). *P. mirabilis* can colonise multiple types of urinary catheters (silver-coated latex, hydrogel-coated latex, silicone-coated latex and silicone) and form surface-associated communities called biofilms (Morris and Stickler 1998) which are problematic due to recalcitrance to antibiotics (Stickler 2008) and contributions to catheter blockage. Thus, there remains a need to identify alternative strategies for preventing and/or treating *P. mirabilis* CAUTI.

Some studies suggest that *P. mirabilis* can adhere to the catheter and migrate against the urine flow via a flagellum-dependent surface-associated motility called swarming (Armbruster and Mobley 2012; Jacobsen et al. 2008). *P. mirabilis* can swarm across commonly used catheter types including those made of silicone (Stickler and Hughes 1999) and hydrogel biomaterials (Sabbuba et al. 2002); however, the role of swarming in CAUTI is not clearly established. Swarming has been positively correlated with production of virulence factors, such as urease (discussed below) and haemolysins, contributing further to *P. mirabilis* pathogenesis (Allison et al. 1992a; Fraser et al. 2002). Thus, it follows that compounds limiting swarming motility could exert clinical benefits. Given that infections usually develop over extended time periods, it is important to assess whether anti-swarming compounds mediate such an effect transiently or over longer durations. Indeed, the majority of studies investigating anti-swarming effectors have limited this to 16-24 h. Moreover, to enhance clinical relevance, it is critical to determine the effects of anti-swarming compounds under urine-representative conditions.

A potential limitation of anti-swarming compounds is that they may contribute to enhanced biofilm formation. Indeed, inverse relationships between motile swarming and adherent biofilm phenotypes in *P. mirabilis* have been reported (Jacobsen and Shirtliff 2011; Pearson et al. 2008; Verstraeten et al. 2008). For example, the protein RsbA up-regulates biofilm formation and exopolysaccharide production (Liaw et al. 2004), while down-regulating swarming and urease production (Liaw et al. 2001; Liaw et al. 2004). Moreover, Jones and colleagues found that swarming defective mutants blocked catheters at a faster rate than wild-type strains (Jones et al. 2005). Thus, when investigating anti-swarming compounds and their potential implications, it is important to also consider the effects on biofilm formation. The influence of cranberry products on *P. mirabilis* biofilm formation has never been examined.

Swarming has been linked to enhanced *P. mirabilis* urease production, which is one of *P. mirabilis'* most potent virulence factors (Allison et al. 1992b; Gibbs and Greenberg 2011). Urease converts urea within the urine to ammonia and carbon dioxide, subsequently raising the pH, leading to the precipitation of calcium and magnesium phosphate crystals (Broomfield et al. 2009; Jones and Mobley 1988; Mobley and Hausinger 1989). Problematically, these crystals form complexes with *P. mirabilis*, which contributes to biofilm formation and catheter blockage (Mobley and Warren 1987; Rather 2005). Thus, from a clinical perspective, identification of compounds that mitigate swarming motility and impair urease production may be beneficial.

Previously, we reported that cranberry powder (CP) blocked *P. mirabilis* swarming motility over short time periods (up to 16 h) (McCall et al. 2013) and that *P. mirabilis* was unable to swarm across catheter materials impregnated with CP (Chan et al. 2013). The study herein significantly expanded on these findings to investigate: (i) the effects of cranberry derivatives on biofilm formation of *P. mirabilis*; (ii) whether swarming inhibition occurred transiently or long-

term (> 16 h); (iii) whether swarming was also blocked by commercially available cranberry juices (R.W. Knudsen cranberry juice and Ocean Spray diet cranberry juice); (iv) whether CP and cranberry juices exhibited effects under natural urine conditions; and (v) the effects of cranberry on medium pH which is an indirect indicator of urease activity.

Materials and Methods

Bacterial strains and media

Experiments were undertaken with *P. mirabilis* laboratory strain HI4320, originally isolated from the urine of an elderly, long-term-catheterized woman (Mobley and Warren 1987). Pure stock cultures were maintained at -80°C in 30% (v/v) frozen glycerol solution in 50% lysogeny broth (LB) (tryptone 10 g/L, yeast extract 5 g/L and NaCl 5 g/L). Frozen cultures were streaked onto low-swarm agar (LSW) and incubated (37 °C, 24 h) as swarming does not occur on this medium and thus permits the isolation of individual colonies (Belas et al. 1991). LSW consists of the following per L: tryptone 10 g, yeast extract 5 g, glycerol 5 mL, NaCl 0.4 g, agar 20 g, and a filter sterilised solution added after autoclaving to achieve an additional 2 μM $MgSO_4$, 0.4% v/v glycerol and 0.002% w/v nicotinic acid (Belas et al. 1991). A colony of bacteria was transferred from LSW into LB broth (15 mL in a 50 mL Falcon tube) to grow for experiments (37°C, 16 h, 200 rpm). Media used for the swarm, biofilm, and urease assays are described in the relevant sections. All media were prepared using deionized water (DI).

Cranberry juices and powder

Cranberry powder (CP) consisted of dehydrated, crushed cranberries (Atoka Cranberries, Canada), and was kept at room temperature and solubilized to achieve a stock concentration of 100 mg/mL in DI. Two commercially available cranberry drinks were used in these experiments: (i) R.W. Knudsen cranberry juice (CJK) (USA) that contained 100% reconstituted cranberry juice and, (ii) Ocean Spray diet cranberry juice (CJO) (USA) that contained water, natural flavours, pectin, citric acid, fumaric acid, ascorbic acid, sodium citrate, acesulfame potassium sucralose and colour. The pH of the cranberry juices was neutralized with 5M NaOH (using an Accumet AR20 pH meter) prior to filter sterilizing with a 0.45 µm filter (Millipore, Germany). Stock solutions were stored at 4°C protected from light and subsequently diluted into the relevant media to yield the final concentration that is reported in each experiment. Control media were also diluted accordingly with DI. Table S1 summarizes the composition of each cranberry product used at the dilution used in the experiments. Total fat, carbohydrate and protein contents are reported as these are the key components making up the total organic carbon (TOC) of the cranberry products.

Urine medium

Ethics approval to use human urine in experiments was obtained by the McGill University Institutional Review Board (IRB Study Number A01-M08-13B). The participant provided written informed consent to participate in this study that was approved by the IRB. Mid-stream urine samples were obtained from a healthy male volunteer with no recent (at least three month) history of UTI or antibiotic use and no recent (at least 16 h) consumption of cranberry or other fruit products. Urine was filter sterilised using a bottle top filter, pore size 0.45 µm (Fisher, Canada).

Samples were stored at 4ºC and used within one week. Each experiment was performed with different urine samples.

Swarming assays

Swarming assays were undertaken in Petri dishes (polystyrene, diameter of 86 mm) containing LB agar (2.0% w/v agar). For swarm assays on urine, filter sterilized urine was solidified to 1.5% w/v agar by combining 1:1 ratios of 3% w/v agar dissolved in urine (autoclaved) with urine that had been filter sterilised only (not autoclaved). The lower concentration of final agar on urine plates (1.5 % w/v) compared to that of LB (2.0 % w/v) was used so that only 50% of the urine would be autoclaved when diluted from 3% w/v agar (the highest agar concentration able to be made without agar setting problems). Media was supplemented with cranberry materials or urea (Sigma-Aldrich, Canada) as indicated.

Overnight grown cultures (single colony inoculated into 15 mL LB broth in a 50 mL Falcon tube, 37°C, 16 h, 200 rpm) were adjusted to an OD_{600} of 1.0, and a sterile inoculation needle was used to transfer bacteria onto the center of the agar surface. After incubation (37°C, humidified chamber), the diameters of the swarming motility zones were measured (in mm) at the indicated time intervals.

To determine whether isolated sources of DI/cranberry solutions would repel swarming bacteria, droplets (5 µL) or lines (100 µL) of DI/cranberry solutions were placed on control LB swarm agar plates at 2.5 cm distances from the point of bacterial inoculation. After incubation, it was recorded whether swarming was affected by the cranberry solutions.

Phase-contrast microscopy was used to obtain images of edges of the swarming or non-swarming bacteria when indicated (Olympus BX51 upright microscope, USA).

Biofilm assays

Biofilm formation was examined using 12×75 mm polystyrene and glass-culture tubes in LB broth or urine supplemented with cranberry solutions (CP, CJO, or CJK). Overnight grown cultures adjusted to an OD_{600} of 1.0 were diluted 1:100 (yielding approximately 10^7 CFU/mL) into fresh medium, and aliquots (300 µL) were transferred to triplicate tubes. After incubation (37ºC, 24 or 48 h), the tubes were gently rinsed three times with DI water prior to staining with 300 µL of 0.1% (w/v) crystal violet for 15 min at room temperature. After rinsing three times with DI water, the surface-distribution of the stained material was photographed using a digital camera (Canon PowerShot SD1300 IS Digital Elph), and the crystal violet was solubilised with 300 µL of 100% ethanol for 10 min with intermittent mixing of the tubes. Aliquots (200 µL) were transferred from tubes into a flat-bottom polystyrene microtitre plate and the absorbance of the solubilized stain was then measured at 570 nm in a microplate reader (TECAN Infinite M200 Pro; Tecan Group Ltd., Männedorf, Switzerland) to quantify biofilm development.

To directly observe the formed biofilm, biofilms were examined in angled 24-well plate assays as described elsewhere (Merritt et al. 2005). As per the microtitre plates above, aliquots of inocula (600 µL) were added to wells and plates were incubated on an angle so that the air-liquid interface transected the middle of the well base. After incubation, wells were rinsed and stained with crystal violet (600 µL), as per the microtitre plate assay. Biofilms were then visualised with an inverted microscope (Olympus Inverted IX71, 200× magnification, bright-field).

Bacterial growth assays

Growth assays were performed in LB broth by monitoring changes in the OD_{600} using flat-bottom polystyrene non-tissue culture treated 96 well plates (BD Falcon, USA). Bacterial inocula were prepared as per the biofilm assay. Aliquots (200 µL) of these cultures were transferred into triplicate wells and plates were incubated at 37°C, under shaking (orbital shaking 44.3 rpm) conditions within the microtitre plate reader (Tecan Infinite M200 Pro, Switzerland). Distilled water was placed in the outerwells of the plates to minimize evaporation effects (control experiments confirmed the insignificant evaporation of liquid from wells). OD_{600} measurements were recorded every 20 min for 24 h (from the bottom of wells). Growth yield at 24 h was also determined by serial dilutions (1:10) in phosphate buffered saline (PBS) and subsequent spot-inoculation (10 µL) to obtain the CFU/mL.

Urease activity

Urease activity was determined indirectly by measuring pH increases (Accumet AR20 pH meter) from inoculation time to 4 h later (37 ºC). Bacteria were inoculated into the indicated medium (5 mL in 15 mL polystyrene tubes) at the same dilutions used for the biofilm assay. Tubes without bacteria were used as negative controls.

Statistics

Where indicated, a two-tailed Student's *t* test was used to determine whether the presence of cranberry or urea resulted in any significant differences compared to when these materials were not present. A P value of <0.05 was considered significant.

Results

Cranberry derivatives transiently impair *P. mirabilis* swarming motility

The effects of CP and cranberry juices, CJO and CJK, on *P. mirabilis* swarming capacities were tested on LB plates (with 2% w/v agar (Fig. 1)). Under control conditions (no cranberry materials), *P. mirabilis* exhibited proficient swarming motility (> 60 mm diameter at 24 h) with the characteristic concentric ring (bull's-eye) phenotype at agar concentrations of 2.0% w/v (Fig. 1B) and 1.5% w/v (not shown). This phenotype incorporates active swarming phases interspersed with rings of non-swarming consolidation phases, where bacteria temporarily differentiate back into short cells (Armbruster and Mobley 2012). CP blocked 24 h motility from concentrations of 5 mg/mL, in agreement with our previous data (McCall et al. 2013). CJO did not significantly impair 24 h swarming at the concentrations tested (up to 10% v/v), while CJK significantly blocked swarming motility at the 10% v/v concentration ($P < 0.05$) (Fig. 1A & B), but not at the lower concentrations (≤ 5% v/v). The concentrations tested did not impair *P. mirabilis* bacterial growth levels (Fig. S1), and thus swarming inhibition is attributable to a different mechanism.

During incubations longer than 24 h (up to 6 days) (Fig. 1C), CP consistently blocked swarming up to day 3, but on day 4, bacteria emerged from the non-motile colony that proceeded to cover the agar surface. This surface-associated motility pattern differed to the regular concentric

ring swarming motif as the motility pattern appeared to lack the consolidation phase (Fig. 1D). However, if bacteria were taken from the edges of these motile zones lacking the consolidation phase and transferred onto fresh control or CP 10 mg/mL plates, they switched back to exhibiting the standard concentric ring motility pattern on control plates or the absence of short-term swarming on CP plates (Fig. 1D). This suggested that the phenotypic change seen during long-term incubation on cranberry plates was transient or that the compound(s) inhibiting motility had been degraded over the course of four days.

In the presence of CJK, a surface-associated motility was evident by the second day (Fig. 1C) that also lacked the consolidation phase (Table S2). By day 3, *P. mirabilis* had entirely covered the agar surface as a thin layer of bacteria with no consolidation rings, and thus, different from the regular swarming motility pattern. As was seen with CP, when bacteria were transferred from a CJK plate (day 5) to a new control swarm plate, normal swarming motility was restored (Table S2).

To verify whether the motility pattern on CP and CJK plates during extended incubations was not simply due to outgrowth from the central colonies, *P. mirabilis* was incubated on a medium non-conducive to swarming motility (LSW ± cranberry materials) for 5 days (37°C) (Belas et al. 1991). Under all conditions, colonies remained at the inoculation point and did not propagate across the agar surface (Table S2).

P. mirabilis cells are repelled by cranberry derivatives

As cranberry products were able to transiently inhibit *P. mirabilis* motility, it was next tested whether isolated cranberry sources would affect already actively swarming *P. mirabilis.* To do

this, cranberry solutions were applied to swarm plates in the form of droplets or lines (Fig. 2A, B, C). Swarming bacteria were undeterred by droplets (5 µL) of DI or CJO, but would consistently migrate around CP or CJK (Fig. 2A). Similarly, swarming bacteria were able to cross a line of DI or CJO that extended across the entire plate, but could not cross a line of CP or CJK (Fig. 2B). This effect was temporally dependent as it was seen after incubation for 24 h, but not after 48 h (Fig. 2C). Phase-contrast images of *P. mirabilis* on the swarm plates depicted finger-like projections at the active swarm front under control conditions. However, at the edges of CP and CJK droplets, bacteria had formed a smooth boundary (Fig. 2D).

P. mirabilis does not swarm on urine agar

The above assays were performed using standard laboratory conditions (LB); however, CAUTIs occur in the presence of urine, which has high concentrations of urea (~ 10 mg/mL) and we were interested to verify whether cranberry also mitigated swarming in urine conditions. However, when comparable swarming assays were conducted in the presence of clinically isolated human urine, *P. mirabilis* did not swarm and formed a non-motile smooth-edged colony at the inoculation point (Fig. 3A), even after 5 days incubation (data not shown). To test whether urea itself mitigated swarming motility, urea was supplemented to LB agar at concentrations of 0, 5, 10, 15 and 20 mg/mL. Urea significantly impaired *P. mirabilis* swarming motility at the concentrations tested and blocked motility at the metabolically relevant concentrations of 10 mg/mL and above (Fig. 3C). Thus, *P. mirabilis* did not exhibit swarming motility under the urine or urea conditions tested and we were not able to assess cranberry mediated swarming mitigation under such conditions.

P. mirabilis biofilm formation is enhanced by cranberry derivatives

While cranberry could mitigate swarming in LB broth, it is important to know the effects of cranberry on sessile biofilm development. Biofilms in media ± cranberry (equivalent concentrations to those used in the swarming assays) were formed on polystyrene (Fig. 4A) and glass (Fig. S2) tubes (24 and 48 h, 37°C, LB broth) and subsequently stained with crystal violet. Results were corrected for background staining of cranberry solutions in LB (with no bacteria) even though all conditions without bacteria exhibited negligible background staining (<0.1 AU at 570 nm). In LB broth alone, *P. mirabilis* formed little biofilm at 24 h and only moderate biofilm at 48 h (OD at 570 nm of ~0.5) in both polystyrene and glass tubes. However, in the presence of all the cranberry solutions, *P. mirabilis* formed significantly higher stained 24 and 48 h biofilms on both surfaces ($P < 0.05$), with biofilms being at least 3-fold higher at the maximum concentrations. As depicted in the images, biofilms predominantly formed at the air-liquid interface (Fig. 4), where oxygen levels would predominate.

Growth measurements (Fig. S1) showed that cranberry solutions enhanced OD measurements compared to control conditions suggesting that enhanced biofilm formation could be attributable to higher bacterial growth. However, in the presence of cranberry, a precipitate was observed that was very likely a cranberry-bacterial aggregates (data not shown) and these factors would undoubtedly influence the OD measurements (this precipitate was not observed in "bacteria only" controls in LB nor in "cranberry only" controls in LB). Thus, growth yields were also determined at 24 h by dilutions and spot-inoculations to obtain the CFU/mL. This technique revealed that there were no measurable differences in the CFU/mL (Fig. S1). This is in accordance with our earlier work demonstrating that CP did not significantly affect growth (by CFU/mL) at multiple time points for *P. mirabilis* (McCall et al. 2013) or for *P. aeruginosa* (O'May et al. 2012).

When experiments were conducted in urine (48 h), cranberry also caused an enhancement in biofilm formation in comparison to levels seen under control conditions (Fig. 4B). This suggested that the cranberry-mediated enhancement of biofilm formation is independent of the external medium. There were no significant differences in bacterial growth levels within the tubes as determined by CFU/mL ($2.0 \pm 0.1 \times 10^6$ CFU/mL).

An angled-biofilm assay in 12-well plates (LB broth) was undertaken to directly visualise the stained material at the air-liquid interface at 4, 24 and 48 h (Fig. S3). There was a higher level of stained material in the presence of cranberry compared to under control conditions. This stained material may include bacteria, cranberry, and cranberry-bacteria precipitants. Although these precipitants could contribute to the cranberry-mediated enhanced biofilm development, the images suggested that there is enhanced bacterial attachment in the presence of cranberry compared to under control conditions. Noteworthy, cranberry control solutions without bacteria exhibited virtually no background staining (data not shown).

Cranberry products do not alter short-term urease activity in broth cultures

Urease is a key virulence factor of *P. mirabilis* and has also been linked to swarming motility (Liaw et al. 2001; Liaw et al. 2004). Urease activity was not directly measured in this study; however, as an indirect measure of urease activity, we investigated whether cranberry would mitigate the urease-induced pH rise in the surrounding medium (Broomfield et al. 2009; Jones and Mobley 1988; Mobley and Hausinger 1989), by monitoring changes in the pH of (i) LB, (ii) LB + urea (10 mg/mL) and (iii) urine, ± cranberry solutions. The pH increase occurred to the same magnitude in the presence of cranberry solutions in comparison to that seen under control

conditions (Table 1), suggesting that cranberry did not impair the rise in pH that is indirectly associated with urease activity.

Discussion

The study herein found that cranberry products can transiently impair short-term swarming of *P. mirabilis* on swarming agar and actively swarming bacteria would migrate around isolated cranberry sources. Noteworthy, during longer-term incubations, *P. mirabilis* was able to overcome the impairment of surface-associated motility. Moreover, swarming impairment corresponded with an augmentation of biofilm development.

The authors' laboratory had previously demonstrated that CP blocked swarming motility at short time periods on LB-agar of *P. mirabilis* (16 h) (McCall et al. 2013) and the uropathogen *Pseudomonas aeruginosa* (up to 48 h) (Mittal et al. 2009; O'May and Tufenkji 2011). As CP consists of dehydrated, crushed cranberries, it was relevant to determine whether cranberry juices (CJO and CJK) actually consumed by the public, also exerted anti-swarming effects, albeit the levels of active cranberry components and different additives would differ between the cranberry materials. The study herein showed that pure cranberry juice, CJK, could block 24 h swarming motility at 10% v/v, whereas diet cranberry juice, CJO, did not have any observable effect on swarming motility at the concentrations tested (Fig. 1). The observable effects of CP and CJK may be linked to the greater dilution of the CJO, which has a lower TOC content than the other two products tested (Table S1). Thus, it would be expected that consumption of the pure juice would be more likely to exert effects compared to consuming a more dilute preparation, albeit the

concentrations used in this study would be higher than the concentrations that would reach the urinary tract post cranberry-juice consumption.

Importantly, both CP and CJK only transiently impaired swarming motility (24 h), and bacteria would emerge from the non-motile colony by at least day 3 and migrate over the agar surface. This is the first time that swarming inhibition has been assessed over longer-time periods (> 24 h), as other studies reporting anti-swarming compounds tested motility at < 24 h with examples being p-nitrophenylglycerol (Williams 1973), tannic acid (Smith 1975), resveratrol (Wang et al. 2006), 109(Z),139(E)-heptadecadienylhydroquinone (Liu et al. 2012) and selected saturated fatty acids (Liaw et al. 2004). This study demonstrates for the first time that a product arresting swarming at 24 h (CP, CJK) does not continue to do this at longer periods. Given that CAUTI is established over the long-term, it is important to consider how anti-swarming candidates modulate long-term behavior. It would be interesting to also determine whether the aforementioned compounds, or any future identified swarming inhibitors restrict *P. mirabilis* swarming motility only transiently (<24 h) or over a longer-term (>24 h). The cranberry-induced transient swarming inhibition could be attributable to a breakdown of the anti-swarming cranberry compound(s) or a gene-level response in the bacteria permitting an adaptation. These questions warrant future investigation as they will influence the potential use of anti-swarming effectors in longer-term applications (e.g., whether they can be embedded into a catheter and will degrade over time or whether a continuous supply will need to be administered).

It is noteworthy that during the extended incubations in CP or CJK the bacteria were able to migrate across the agar surface but they lacked the consolidation phase normally associated with swarming (Fig. 1). This motility type could be indicative of asynchronous swarming and alteration of the swarm cycle periodicity (Kearns 2010). Continuous motility without consolidation has also

been reported in the presence of glutamine on a minimal medium (Allison et al. 1993) and with crude extract from the red algae *Delisea pulchara* (Gram et al. 1996), but the mechanisms for this phenomenon remain unknown (Morgenstein et al. 2010). Characterisation of the gene expression phenotype of the emergent bacteria would be interesting, albeit beyond the scope of the current study.

Investigations of swarming under clinically relevant urine conditions showed that urine agar itself does not enable swarming motility and swarming on LB was impaired by urea (Fig. 3),. Although these experiments were conducted with different urine samples from the same volunteer, the lack of swarming on urine medium was consistently observed and is in agreement with other studies (Armbruster et al. 2013; van Asten and Gaastra 1999). Armbruster et al. (2013) suggested that the swarming impediment on urine agar was attributable to *P. mirabilis* urease converting urea to crystals causing a motility restriction. In agreement, a *P. mirabilis* mutant with inactive urease was able to migrate across the urine medium, albeit exhibiting a motility phenotype without consolidation rings (Armbruster et al. 2013).

Studies in murine-UTI models have also implied a lack of swarming in the urinary tract (Jansen et al. 2003; Pearson et al. 2011). This suggests that swarming behaviour differs substantially between LB and urine conditions. Elucidation of when swarming occurs in the context of CAUTI establishment by urease producing *P. mirabilis* could shed light on potential clinical applications of anti-swarming materials. *P. mirabilis* is suggested to colonise the catheter from the fecal flora (Mathur et al. 2005), and swarming plays a role in bacteria gaining entry to the host from the perineum (Nielubowicz and Mobley 2010). The application of anti-swarming materials to the perineal region is an area worth exploring. It would be of interest to determine

anti-swarming mediators in environments where proficient *P. mirabilis* swarming motility is occurring.

Although swarming inhibiting compounds could be beneficial in some settings, it is also relevant to assess effects on biofilm development as reduced *P. mirabilis* motility can correspond with enhanced biofilm development (Jacobsen and Shirtliff 2011; Pearson et al. 2011; Verstraeten et al. 2008). Indeed, this is consistent with our results. All cranberry products in this study enhanced *P. mirabilis* biofilm formation in both LB and urine (Figs. 4, S2, S3) suggesting the enhanced biofilm formation is independent of the external medium. It is noteworthy to reiterate that the biofilm experiments were also repeated with different urine samples from the same volunteer, but the observation of enhanced biofilm formation was consistent. In agreement, Morris and Sticker (2001) reported that *P. mirabilis*-induced catheter encrustation was higher in urine from individuals that had consumed cranberry juice in comparison to individuals that consumed an equivalent volume of water (Morris and Stickler 2001). This inverse link between biofilm formation and swarming motility capacities is consistent with other motile, biofilm-forming bacteria as demonstrated with CP and other tannins in *P. aeruginosa* (O'May et al. 2012) where inverse regulation is mediated by a c-di-GMP intracellular messenger (McDougald et al. 2011; Merritt et al. 2007). Thus, while targeting swarming could be an effective strategy for reducing colonisation, it is essential to consider effects on biofilm formation in such a treatment strategy.

In LB, cranberry derivatives (powder and pure juice) cause a transient inhibition and repulsion of *P. mirabilis* motility by unknown mechanisms; however, this is tempered by an increase in surface-attached biofilm, which is consistent with physiological behaviors observed in other motile, biofilm-forming bacteria. An ideal therapeutic intervention would prevent swarming motility long-term while concurrently being resistant to biofilm formation and impairing urease

activity. Although biofilm enhancing/swarming inhibition results herein are consistent with those observed in another motile, biofilm-forming bacteria (O'May et al. 2012), further studies with other clinical isolates of *P. mirabilis* would be beneficial. This study provides several insights into the long-term strategy of targeting *P. mirabilis* CAUTIs and gives rise to many questions that could further characterise cranberry effects on *P. mirabilis* behavior.

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Table 1. Effects of cranberry on pH changes in LB and urine (37°C)

		Without *P. mirabilis*		With *P. mirabilis*	
		0 h	4 h	0 h	4 h
LB	Control	7.03 ± 0.12[a]	7.00 ± 0.19	7.04 ± 0.15	6.13 ± 0.23
	CP[b]	7.08 ± 0.04	7.04 ± 0.04	7.06 ± 0.06	5.66 ± 0.39
	CJK	7.11 ± 0.01	7.08 ± 0.03	7.09 ± 0.06	5.77 ± 0.25
LB + urea	Control	7.06 ± 0.18	6.99 ± 0.18	7.11 ± 0.10	8.90 ± 0.12
	CP	7.09 ± 0.01	7.04 ± 0.01	7.05 ± 0.03	8.77 ± 0.15
	CJK	7.18 ± 0.04	7.11 ± 0.02	7.09 ± 0.10	8.86 ± 0.16
Urine	Control	6.68 ± 0.16	6.79 ± 0.17	6.69 ± 0.15	8.58 ± 0.15
	CP	6.71 ± 0.07	6.85 ± 0.01	6.32 ± 0.41	8.62 ± 0.15
	CJK	6.79 ± 0.06	6.91 ± 0.01	6.67 ± 0.16	8.82 ± 0.14

[a] Values shown are the mean ± SD from 3 separate experiments. Highlighted areas represent the urease-induced pH increase.

[b] CP and CJK are at concentrations of 10 mg/mL and 10% v/v, respectively.

Figures

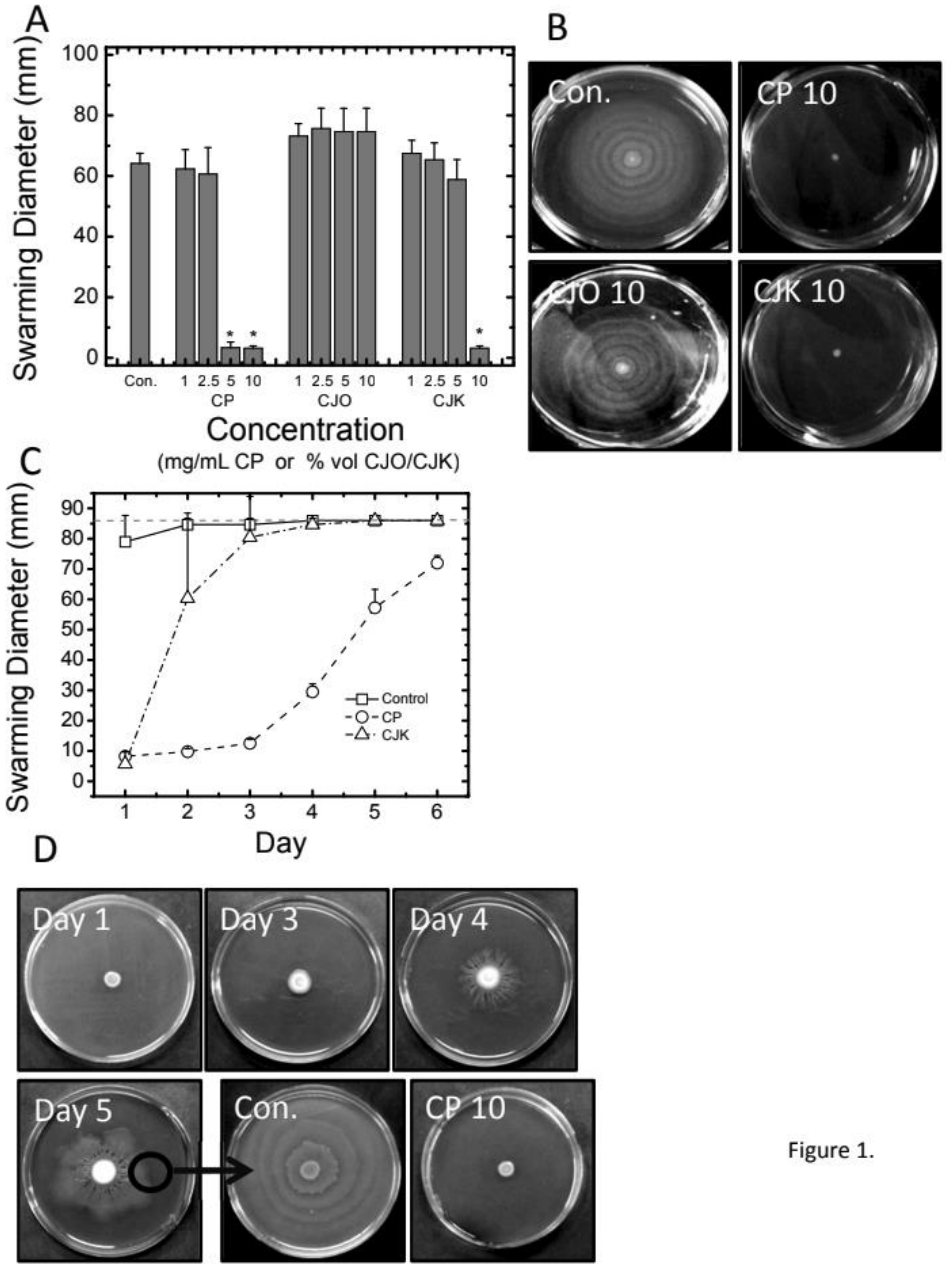

Figure 1.

Figure 1. Cranberry derivatives transiently impair P. mirabilis swarming motility. *Effects of cranberry powder and juices on P. mirabilis swarming motility (LB + 2.0% w/v agar, 37°C). (A) and (B) show 24 h data, while (C) and (D) show data over multiple days. Values shown denote the mean + SD of 3 experiments with duplicate plates per experiment. Dashed horizontal line at 86 mm in (C) corresponds with the maximum diameter of the Petri dish and hence maximum motility. Representative images of swarming zones at (B) 24 h and (D) days*

(1-5) in CP (10 mg/mL) when P. mirabilis (from day 5) was transferred to fresh control or CP plates and incubated (24 h).

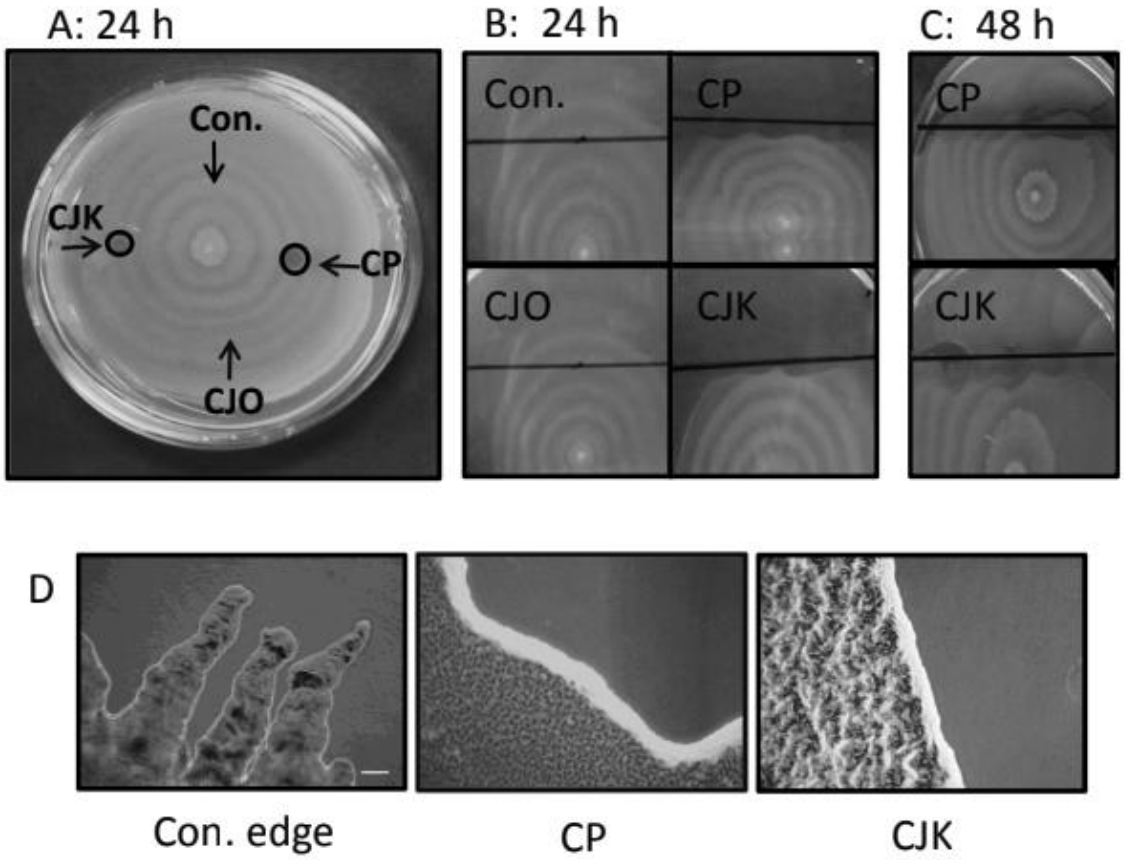

Figure 2.

Figure 2. Effects of isolated sources of cranberry materials on actively swarming P. mirabilis. *Cranberry and DI solutions were placed as 5 µL droplets in A (24 h) and 100 µL distributed over a line in B (24 h) and C (48 h). CP and CJO/CJK were applied at concentrations of 10 mg/mL and 10 % v/v, respectively. To enhance clarity, circles have been drawn around droplets indicating where P. mirabilis did not swarm. (D) Representative phase-contrast images of P. mirabilis at the edges of active swarm zones (Con.) and CP and CJK droplets. Bacteria were migrating from the lower left to the upper right corner. Scale bar denotes 100 µm and all images are at the same magnification.*

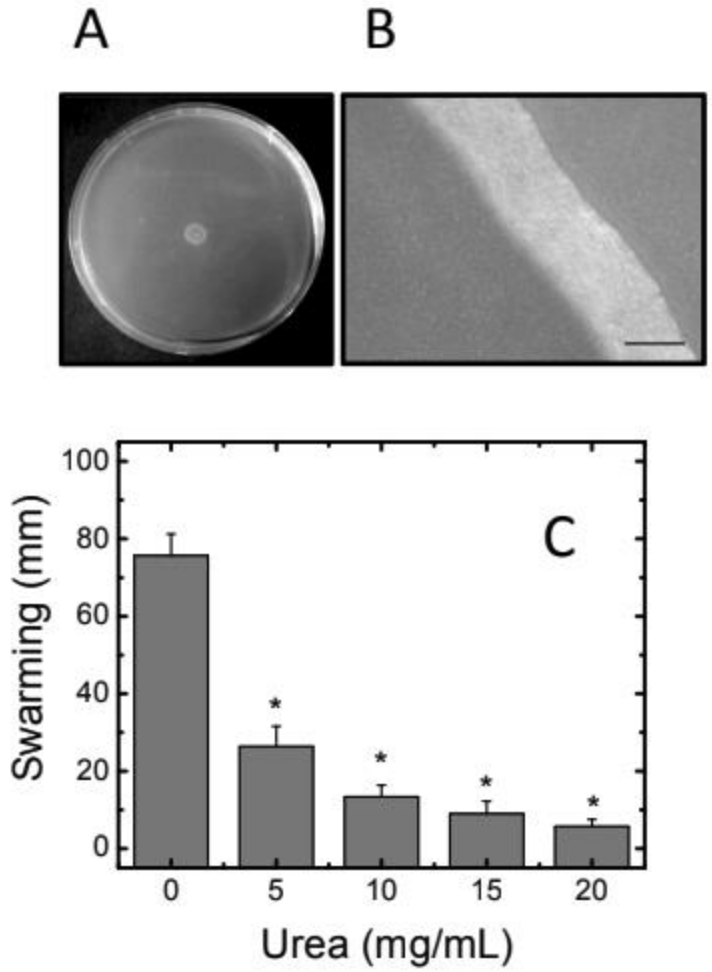

Figure 3.

Figure 3. P. mirabilis does not swarm in the presence of urine or urea. *(A) Representative image of P. mirabilis swarming motility (48 h) on urine solidified with 1.5% w/v agar. (B) Magnified portion of phase-contrast image of P. mirabilis colony wall. Scale bar denotes 100 μm. (C) Effect of urea concentrations on P. mirabilis swarming motility in LB agar. Values shown denote the mean + SD of three experiments with duplicate plates per experiment. * represents a statistically significant difference compared to controls (P < 0.05).*

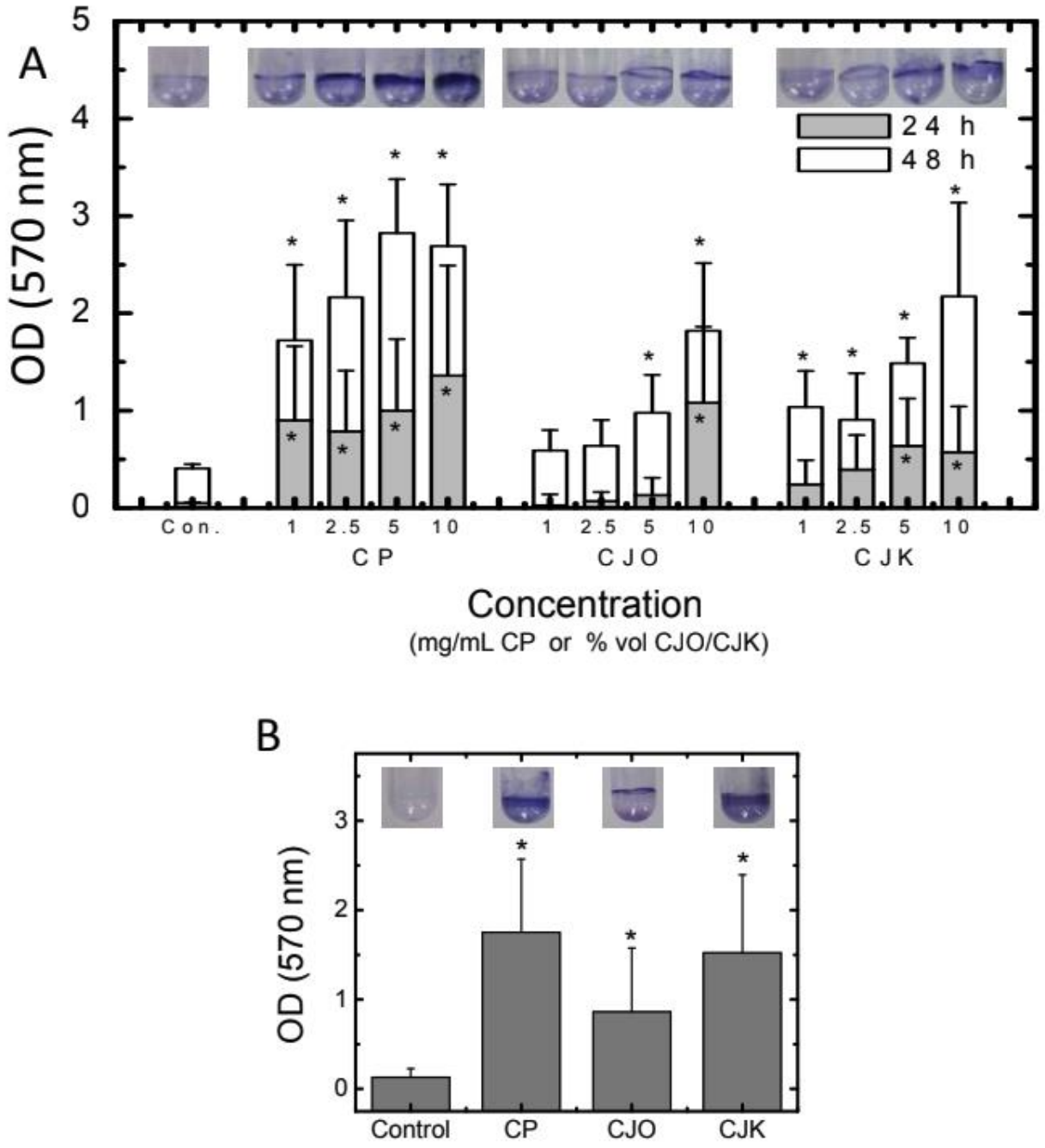

Figure 4.

Figure 4. P. mirabilis biofilm formation is enhanced in the presence of cranberry. *Biofilm formation (37°C) in polystyrene tubes in (A) LB, 24 h & 48 h and (B) urine, 48 h. Figure insets show representative biofilms stained at 48 h. Values shown denote the mean + SD of 3 experiments with triplicate tubes per experiment. * represents significantly enhanced biofilms in comparison to values seen under control conditions ($P < 0.05$). Cranberry addition without bacteria did not cause any background staining (data not shown).*