Cranberry impairs selected behaviors essential for virulence in *Proteus mirabilis* HI4320

Jennifer McCall, Gabriela Hidalgo, Bahareh Asadishad, and Nathalie Tufenkji

**Abstract:** *Proteus mirabilis* is an etiological agent of complicated urinary tract infections. North American cranberries (*Vaccinium macrocarpon*) have long been considered to have protective properties against urinary tract infections. This work reports the effects of cranberry powder (CP) on the motility of *P. mirabilis* HI4320 and its expression of flaA, flhD, and ureD. Our results show that swimming and swarming motilities and swarmer-cell differentiation were inhibited by CP. Additionally, transcription of the flagellin gene flaA and of flhD, the first gene of the flagellar master operon flhDC, decreased during exposure of *P. mirabilis* to various concentrations of CP. Moreover, using ureD–gfp, a fusion of the urease accessory gene ureD with gfp, we show that CP inhibits urease expression. Because we demonstrate that CP does not inhibit the growth of *P. mirabilis*, the observed effects are not attributable to toxicity. Taken together, our results demonstrate that CP hinders motility of *P. mirabilis* and reduces the expression of important virulence factors.

**Key words:** flagella-propelled motility, urease, urinary tract infection, cranberry.

**Résumé :** *Proteus mirabilis* est un agent étiologique d’infections compliquées des voies urinaires. On considère depuis longtemps que les canneberges d’Amérique du Nord (*Vaccinium macrocarpon*) recèlent des propriétés qui protègent contre les infections urinaires. Les présent travaux font état des effets de la poudre de canneberge (PC) sur la motilité de *P. mirabilis* HI4320 et sur son expression de flaA, flhD et ureD. Nos résultats démontrent que les motilités de nage et d’envahissement ainsi que la différenciation cellulaire des cellules envahissantes sont inhibées par la PC. De plus, la transcription du gène de la flagelline flaA, et de flhD, le premier gène de l’opéron directeur flhDC, subit une baisse lorsque *P. mirabilis* est exposé à diverses concentrations de PC. En outre, l’emploi de ureD–gfp, une fusion du gène auxiliaire de l’uréase ureD et de gfp, permet de mettre en évidence l’inhibition de l’expression de l’uréase par la PC. Étant donné que nous démontrons que la PC ne ralentit pas la croissance de *P. mirabilis*, les effets observés ne peuvent être attribués à une toxicité. Pris dans leur ensemble, nos résultats démontrent que la PC entrave la motilité de *P. mirabilis* et diminue l’expression de facteurs de virulence importants. [Traduit par la Rédaction]

**Mots-clés :** motilité par propulsion flagellaire, uréase, infection des voies urinaires, canneberge.

**Introduction**

The urinary tract is one of the most common sites of bacterial infection in humans (Foxman 2002), and the enteric bacterium *Proteus mirabilis* is one of the most frequent causative agents of complicated urinary tract infections (UTIs) (Mobley and Belas 1995; Nielubowicz and Mobley 2010). Furthermore, because *P. mirabilis* is urease-positive, individuals suffering from UTIs caused by this bacterium may suffer from catheter obstruction and kidney and bladder stones (Griffith et al. 1976).

*Proteus mirabilis* differentiates from a short swimmer cell (2–4 μm) that possesses 6 to 10 peritrichous flagella into an elongated mor-phototype (up to 80 μm) that possesses thousands of flagella during swarming (Mobley and Belas 1995). Swarmer cells migrate in rafts of cells connected by flagella (Gibbs and Greenberg 2011). After an initial migration, the swarmer cells stop their movement and consolidate into swimmer cells (Gibbs and Greenberg 2011). Following a brief pause, the swimmer cells elongate again and swarming resumes (Gibbs and Greenberg 2011). Macroscopically, the repeated cycles of migration followed by consolidation result in the characteristic bull’s eye pattern observed during swarming of *P. mirabilis* on hard agar surfaces (Gibbs and Greenberg 2011). Even though swimmer cells are rarely observed during urinary tract ascension (Jansen et al. 2003), it has been shown that *P. mirabilis* cells are capable of swimming across the surface of urinary cathe-

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the formation of crystalline biofilms that block catheters and form kidney and bladder stones (Griffith et al. 1976; Mobley and Warren 1987). Urease production appears to be one of the reasons why Proteus species cause more severe histological damage than Escherichia coli infections (Johnson et al. 1993; Salyers and Whitt 1994; Mobley 1996).

The standard management approach for UTIs is therapy with antibiotics (Prais et al. 2003; Nielsubowicz and Mobley 2010); yet, the current rise in antibiotic resistance among uropathogens (Prais et al. 2003; Foxman 2010; Gupta 2003) has spurred the interest in the development of nonantibiotic, prophylactic strategies. Cranberry (Vaccinium macrocarpon) is a fruit native to North America that has traditionally been used for the treatment and prophylaxis of UTIs. However, in spite of recent advances (Howell et al. 1998; Liu et al. 2006; Eydelnant and Tufenkji 2008; Pinzón-Arango et al. 2009; Tufenkji et al. 2010), a mechanistic understanding of the effects of cranberry materials on uropathogen behavior is still lacking.

Recent studies by our laboratory have begun to explore the effects of cranberry materials on pathogen gene expression and phenotype as well as infectivity (Harmidy et al. 2011; Hidalgo et al. 2009; Tufenkji et al. 2010), a mechanistic understanding of why cranberry materials hinder uropathogenic E. coli to assess if, as for the other bacterial species that we have studied so far, cranberry hinders motility and virulence. Given that the expression of virulence genes, the ability to invade human cells, and an increased resistance to antibiotics have been linked to swarmer cells (Fraser and Hughes 1999; Rather 2005; Pearson et al. 2010; Tremblay and Désilez 2010), this research is of importance. Other materials have been reported to inhibit the swelling motility of P. mirabilis (Snyder and Lichstein 1940; Gillespie 1948; Kopp et al. 1966; Smith 1975; Firehammer 1987; Liaw et al. 2000). However, this is the first time that the response of P. mirabilis to the presence of cranberry has been assessed.

Materials and methods

Bacterial strain, growth conditions, and media

Proteus mirabilis HI4320, isolated from the urine of a long-term-catheterized woman, previously described (Mobley and Warren 1987), sequenced and annotated (Pearson et al. 2008), was the test bacterium used in this study. Cultures were routinely grown at 37 °C in standard Luria–Bertani (LB) medium with rotary shaking (150 r/min) or on LB solidified with 1.5% agar. CP (Canneberges Atoka Cranberries, Canada) was solubilized in distilled, deionized water at 10 mg/mL. Aliquots (200 μL) of each cell suspension were transferred to 96-well plates and incubated at 37 °C. At selected time points, 10 μL aliquots of cell suspensions in each well were serially diluted (in LB), and a final volume of 100 μL was used to inoculate low-swarm (LSW–) agar plates for determination of CFU counts (following overnight incubation at 37 °C). Each condition was set up in triplicate on different days. At 2 time points (10 and 16 h), 5 μL aliquots of cell suspensions for each treatment (0, 5, and 10 mg/mL CP) were extracted from the 96-well plate and used to evaluate the effect of CP on bacterial cell integrity using a Live/Dead BacLight kit (Invitrogen).

Motility assays

Swimming and swarming motility assays were conducted in LB broth solidified with either 0.3% or 2.0% agar, unless noted. CM55 blood agar base (Remel Inc., Kansas) and LSW– agar (Belas et al. 1991) were also used where indicated.

Plates containing CP at concentrations ranging from 0 to 10 mg/mL for swimming and swimming motility assays were prepared. Swimming plates were seeded with 5 μL of an overnight culture using a sterile inoculating needle. Swarming plates were inoculated by spotting 1 μL of a 16 h culture at the center of the dishes. All plates were incubated at 37 °C for 16 h. Three independent experiments were conducted for each assay with quadruplicates of each condition in each experiment. For imaging purposes, swimming plates containing CP at 0 or 10 mg/mL and 100 μg/mL ampicillin were inoculated with P. mirabilis HI4320 pBAC001 (Jansen et al. 2003) and incubated for 16 h. Bacteria from the active swarming edge were harvested and imaged using a Zeiss LSM 5 Exiter Confocal Laser Scanning Microscope with a 60× oil immersion objective.

RNA extraction, cDNA synthesis, and comparative qPCR

An overnight culture of P. mirabilis HI4320 was used to inoculate swim plates containing CP at 0, 1, 5, and 10 mg/mL as described above. The plates were incubated at 37 °C for 16 h, after which bacteria from the edge of the swim front were harvested with a sterile tip. Total RNA was extracted using Direct-zol RNA Mini-Prep (Zymo Research Corporation) following the manufacturer’s instructions. After elution, nucleic acid concentrations and quality were determined by spectrophotometry using an Eppendorf BioPhotometer Plus. Expression of target genes was quantified using 2-step qRTPCR analysis that first required 1000 ng of total RNA to synthesize cDNA using oligo (dT)12-18 (Invitrogen), 10 mmol/L dNTP mix (10 mmol/L of each dATP, dGTP, dCTP, and dTTP) (Invitrogen), and M-MLV Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions. Next, the equivalent of 2 ng of total RNA were loaded with TaqMan Universal PCR Master Mix (Applied Biosystems) per well, and qRT-PCR was processed with ABI Prism 7900 HT (Applied Biosystems). Results were analyzed with SDS software, version 2.2 (Applied Biosystems). Data were normalized to the endogenous reference gene rpoA (RNA polymerase A) and analyzed by 2−ΔΔCT method (Livak and Schmittgen 2001). Primer sequences were obtained from Pearson et al. 2008). The qPCR experiment was repeated in independent trials on 3 separate days.

Urease reporter assay

Fluorescence of the ureD-gfp reporter was quantified as described by Zhao et al. (1998). Briefly, an overnight culture of the strain was diluted 1:1000 in LB containing ampicillin (50 mg/mL) and CP at 0, 1, 5, and 10 mg/mL and was incubated for 3 h. The bacteria were then induced with urea (0 to 500 mmol/L) and harvested after 3 h of additional growth. Before fluorimetry, induced bacteria were washed twice in phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4, and 0.24 g of KH2PO4 per litre (pH 7.4)), and the fluorescence (excitation wavelength of 470 nm and emission wavelength of 510 nm) and OD560 were measured with a TECAN Infinite M200 Pro. For each condition, the fluorescence was divided by the OD560.

Statistics

Where indicated, a 2-tailed Student’s t test was used to determine whether the presence of CP resulted in any significant differences compared with the absence of CP.

Results and discussion

Previous work conducted by our group demonstrated that cranberry materials hinder uropathogenic E. coli (UPEC) motility by downregulating the transcription of the flagellin gene flIC.

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The importance of *P. mirabilis* as an etiological agent of complicated UTIs (Nielubowicz and Mobley 2010) prompted us to study whether cranberries would also hinder the motility of this bacterium and alter its gene expression.

The majority of compounds that have been shown to inhibit the swarming motility of *P. mirabilis* have toxic effects; some of these compounds include sodium azide (Snyder and Lichstein 1940), triclosan (Firehammer 1987), and chloral hydrate (Gillespie 1948). p-Nitrophenylglycerol, which also inhibits the swarming motility of this uropathogen, is one of the few substances that is not growth-inhibitory (Kopp et al. 1966; Liaw et al. 2000). It is noteworthy that cranberries contain tannic acids (Howell 2007), which have also been shown to inhibit the swarming of several *Proteus* species (Smith 1975). Our laboratory and that of others have shown that cranberry does not inhibit the growth of *E. coli* (Sobota 1984; Hidalgo et al. 2011a) or *Pseudomonas aeruginosa* PA01 (O’May and Tufenkji 2011; O’May et al. 2012). However, the effect of cranberry on the growth of *P. mirabilis* has not been examined. Preliminary experiments using spectrophotometry revealed that *P. mirabilis* growth curves based on an OD_{600} were influenced by precipitation of CP at 10 mg/mL. Thus, the effect of CP on *P. mirabilis* growth was determined using CFU counts on LSW*. Figure 1 shows that CP does not have growth inhibitory effects on *P. mirabilis* HI4320 at 5 and 10 mg/mL CP for a period up to 18 h. For predetermined dilutions, statistically equal numbers of CFUs were counted independently of the CP concentration. The effect of CP (at 5 and 10 mg/mL) on bacterial cell membrane integrity was also evaluated at 10 and 16 h of growth in LB medium using the commercial BacLight kit. Fluorescence microscopy imaging of BacLight-labeled samples revealed no red fluorescing cells at both time points, indicating that CP did not cause damage to the cell membrane (data not shown). Our results confirm that CP, at the concentrations tested, is not toxic to *P. mirabilis* and underscores the potential role of cranberry as a non-bacteriostatic agent in the prevention and treatment of UTIs.

Next, we set out to evaluate whether CP would impair *P. mirabilis* swimming and swarming motilities. Figures 2A and 2B are representative images of swimming experiments and evidence that CP inhibited this type of motility. Figure 2C shows that exposure to 10 mg/mL CP completely blocked swimming motility, even after 16 h of incubation. CP at 5 mg/mL caused only a temporal decrease in the swimming motility because after 16 h of incubation the cultures had swum the full diameter of the plate.

The swarming motility of *P. mirabilis* was also inhibited following CP exposure. Figures 3A–3D depict *P. mirabilis* swarm colonies on plates supplemented with CP at 0, 1, 5, and 10 mg/mL, respectively. As may be appreciated in those images, the characteristic *P. mirabilis* bull’s-eye swimming pattern was observed clearly under control conditions and at 1 mg/mL CP. This pattern became less distinct when 5 mg/mL CP was added to the medium and disappeared at 10 mg/mL CP. It was also observed, as evidenced in Figs. 3A–3D and on the chart on Fig. 3E, that CP inhibits swarming in a dose-dependent manner. Furthermore, a CP concentration of 10 mg/mL completely blocked swarming of the bacteria. The effect of the growth medium on *P. mirabilis* swarming was tested by preparing CM55 blood agar base plates. CM55 is a rich medium typically used to assess swarming of *P. mirabilis* (Senior 1978; Gibbs et al. 2011). In agreement with the previous experiments, CP at 10 mg/mL completely blocked the swarming motility (Fig. 3F). It has been reported that lower agar concentrations facilitate swarming while higher agar concentrations result in slower, shorter swarm phases (Rauprich et al. 1996). In fact, 6% agar plates were traditionally used by hospital bacteriologists to inhibit the swarming of *Proteus* during clinical testing (Holman 1957). For this reason, we decided to test whether varying the concentration of agar would alter the inhibitory effect of CP on swarming. LB agar plates containing CP at 0 or 10 mg/mL and agar concentrations between 1.2% and 3.0% were prepared, and as may be seen in Fig. 3G, CP at 10 mg/mL inhibited bacterial motility at all the agar concentrations tested. To assess cell elongation, a swarming motility requirement (Rather 2005), fluorescent bacteria were harvested from the active swarming edge of LB agar plates containing no CP (control) or 10 mg/mL CP and imaged by confocal microscopy. It is evident from Figs. 3H and 3I that the bacteria growing in...
the presence of CP did not elongate. Together, these results highlight the strong inhibitory effect that CP has on swarming, even under the most favorable conditions for this kind of motility. To confirm that the observed impairment of motility was not a result of mutant selection following exposure to CP, an additional experiment was conducted whereby *P. mirabilis* was grown in LB containing 10 mg/mL CP and then used to inoculate normal LB agar plates. When these cells were compared with cells that had been grown under control conditions (grown in LB medium alone), we did not observe any difference in swarming motility. No impairment of motility was noted even after 2 subcultures in CP-supplemented LB. This result implies that the antimotility effect is conditional on the bacteria being in a CP-rich environment (data not shown).

A recent publication by Nielubowicz and colleagues (Nielubowicz et al. 2010) reported that Zn-starved *P. mirabilis* displayed reduced swimming and swarming motilities and produced less flaA transcript and flagellin protein. Swarming motility was restored by the addition of Zn2+ and, to a lesser degree, Fe2+ (Nielubowicz et al. 2010). Our group previously determined the transcriptional profile of UPEC during growth on cranberry-derived proanthocyanidins (cPACs) and showed that bacteria grown on media supplemented with cPACs were Fe-deprived (Hidalgo et al. 2011b). Additionally, we confirmed the Fe-chelating capability of cPACs by monitoring the formation of complexes between cPACs and Fe2+ and Fe3+ ions (Hidalgo et al. 2011b). Our laboratory has also shown that CP and cPACs reduce UPEC motility by downregulating transcription of the flagellin gene (Hidalgo et al. 2011a). It would then follow that the reduced motility of *P. mirabilis* on CP might result from the chelation of Fe or Zn ions by CP. This hypothesis was tested by preparing LB plates with CP at 10 mg/mL and ZnSO4 at 0, 250, 500, and 1000 μmol/L. Contrary to expectations, swarming motility was not restored by the addition of Zn.

![Image of swarming motility plates](image_url)

**Fig. 3.** Effect of cranberry powder (CP) on swarming motility of *Proteus mirabilis*. Swarming motility was assayed in hard agar plates by spotting 1 μL of a 16 h *P. mirabilis* culture and incubated at 37 °C for 16 h. Representative images of swarming motility plates are shown for (A) the control (0 mg/mL CP), (B) 1 mg/mL CP, (C) 5 mg/mL CP, and (D) 10 mg/mL CP. (E) Swarm radii at 16 h postinoculation in Luria–Bertani (LB) medium. (F) Swarm radii at 16 h postinoculation in CM55 blood agar base. (G) Effect of agar concentration on inhibition of swarming motility by CP at 10 mg/mL. Error bars represent the standard deviation of triplicate samples. Asterisks (*) indicate statistical significance at *p* < 0.001. (H) and (I) Representative images of *P. mirabilis* pBAC001 cells harvested from the active swarming edge of (H) control (LB alone) and (I) 10 mg/mL CP plates imaged with a confocal microscope.
Some earlier reports have attributed specific health benefits of the cranberry to the fraction consisting of A-type cPACs (Howell 2007). However, in a study with the uropathogen \textit{E. coli} CFT073 (Hidalgo et al. 2011a), we observed a stronger impairment of motility with the whole CP than with the isolated cPAC fraction, which supports the hypothesis that cranberry-derived biomolecules other than just cPACs can exhibit bioactivity against the uropathogen. Thus, in this study, we focused on investigating the effect of the whole cranberry fruit in the form of CP. Future studies in our laboratory will be aimed at fractionating the whole CP in an effort to identify the most bioactive fractions. Finally, it is important to note that the results presented herein were obtained with a single clinical isolate of \textit{P. mirabilis} and may not necessarily reflect the response of all clinical \textit{P. mirabilis} isolates to cranberry.

Preliminary experiments conducted in our laboratory with another clinical isolate of \textit{P. mirabilis} (namely, strain BB2000) show that CP blocks swarming motility of this other strain (C. O’May, N. Tufenkji, unpublished data). The effect of cranberry in vivo remains to be determined, but the importance of our results is underscored by the current rising rates of bacterial antibiotic resistance as cranberry could provide a nonantimicrobial strategy to target clinical infections.

**Acknowledgements**

This research was supported in part by NSERC (Natural Sciences and Engineering Research Council of Canada), the CRC (Canada Research Chair) Program, the Wisconsin Cranberry Board, and the Cranberry Institute. Gabriela Hidalgo received financial support from FQRNT (Fonds québécois de la recherche sur la nature et les technologies) and Jennifer McCall was supported by FRSQ (Fonds de la recherche en santé du Québec). Canneberges Atoka Cranberries provided CP, and H. Mobley (University of Michigan) provided \textit{P. mirabilis} HI4320, pBAC001, and the ureD–gfp reporter. The authors thank Karine Gibbs (Harvard) and Che O’May (McGill) for helpful discussions and technical support.

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