Induction of a State of Iron Limitation in Uropathogenic *Escherichia coli* CFT073 by Cranberry-Derived Proanthocyanidins as Revealed by Microarray Analysis[∇]

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Transcriptional profiles of uropathogenic *Escherichia coli* CFT073 exposed to cranberry-derived proantho-cyanidins (PACs) were determined. Our results indicate that bacteria grown on media supplemented with PACs were iron deprived. To our knowledge, this is the first time that PACs have been shown to induce a state of iron limitation in this bacterium.

Urinary tract infection (UTI) refers to clinical signs and symptoms arising from the presence of microorganisms in the genitourinary tract. Furthermore, a wide range of UTI-related nosocomial pathologies may be caused by microbial contamination of invasive medical devices (15). The principal treatment intervention in patients with acute UTIs is oral therapy with antibiotics. The standard treatment for managing recurrent, uncomplicated UTIs in women is continuous or postcoital prophylaxis with low-dose antimicrobials or intermittent self-treatment with antimicrobials (2, 13, 23). However, the development of bacterial resistance to antibiotics has resulted in rising rates of clinical failure (2, 3, 17), emphasizing the need to develop alternate options for infection prevention and treatment.

For over 100 years, North American cranberries (*Vaccinium macrocarpon* Ait., family *Ericaceae*) have been linked to UTI prevention (4). It was initially believed that the acidity of cranberries reduced bacterial growth levels and hence reduced the incidence of UTIs (4). However, subsequent studies revealed that the pH of urine is not significantly altered by the consumption of cranberries (1, 16). Also, cranberry products have been proven to be nonbacteriostatic (27). Recent research suggests that cranberries prevent UTIs by hindering bacterial attachment to surfaces via phytochemicals known as proanthocyanidins (PACs) (5, 10–12, 19, 25). However, a mechanistic understanding of the way in which cranberry extracts alter bacterial adhesion is still lacking. Investigation of the bacterial response to cranberry PACs at the genome level may facilitate the unveiling of these mechanisms and identify the factors that may be involved. The purpose of the work presented here was to perform a genome-wide transcriptional analysis using microarray technology to determine how PACs affect gene expression of the uropathogen *Escherichia coli* CFT073 (28).

For this study, dry PACs (Marucci Center for Blueberry and Cranberry Research, Rutgers University) were solubilized in distilled, deionized water and sterilized by filtration. The average molecular mass of the PACs used was ~15 kDa. For the transcriptome analysis, the bacteria were grown in LB broth alone (controls) or in LB broth supplemented with PACs at a final concentration of 100 μg/ml. After 5 h of incubation at 37°C and 200 rpm, RNA was extracted by following the standard protocol with an RNeasy minikit (Qiagen). Figure 1 shows optical density versus time for the bacteria grown in the presence or absence of PACs and confirms that PACs, under these conditions, are not inhibitory of growth. Additionally, no inhibition of growth was observed for bacteria that were grown in the presence of any of the higher concentrations of PACs we tested, including a concentration as high as 1,600 μg/ml (data not included).

Affymetrix GeneChip *E. coli* genome 2.0 arrays were used to run the microarray analysis. Targets were prepared by following the prokaryotic target preparation protocol from Affymetrix (Affymetrix GeneChip expression analysis technical manual). Hybridization and chip analysis were performed by
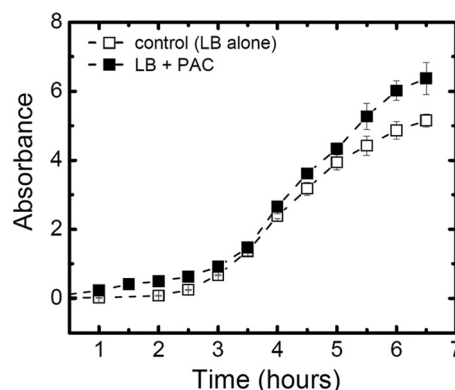

FIG. 1. Growth curves of cultures used for transcriptional profiling. *E. coli* CFT073 was grown in 500-ml flasks containing 150 ml of LB broth at 37°C in the presence (■) or absence (□) of 100 μg/ml cranberry-derived proanthocyanidins. The culture was harvested for RNA extraction 5 h after seeding.

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TABLE 1. *E. coli* CFT073 iron metabolism genes up- or downregulated in the presence of cranberry proanthocyanidins[a]

Gene	Gene product and/or function	Fold change	P value (<0.05)
Upregulated genes[b]			
ryhB	RyhB, a small regulatory RNA involved in downregulation of Fe storage proteins and nonessential Fe proteins when iron is limiting	13.83	1.6×10^{-10}
iucA	Aerobactin protein IucA	2.52	1.2×10^{-4}
iucB	Aerobactin protein IucB	2.54	3.4×10^{-5}
iucC	Aerobactin protein IucC	3.11	8.3×10^{-7}
iucD	Aerobactin protein IucD	5.1	3.2×10^{-8}
iutA	Outer membrane aerobactin receptor	3.95	3.2×10^{-7}
entA	Enterobactin system dehydrogenase	3.33	1.0×10^{-5}
entB	EntB, a subunit of apo-EntB	4.69	1.6×10^{-7}
entC	Isochorismate synthase EntC, enterobactin system	3.47	4.9×10^{-6}
entE	Enterobactin synthase subunit E	2.39	4.1×10^{-4}
entF	Enterobactin synthase subunit F, aryl carrier protein	5.41	6.1×10^{-9}
entH	Proofreading thioesterase in enterobactin biosynthesis	4.48	4.3×10^{-8}
fes	Ferric enterobactin esterase	3.64	7.9×10^{-8}
fepA	Outer membrane ferrienterobactin receptor precursor	2.41	1.0×10^{-6}
fepB	Iron enterobactin transporter periplasmic binding protein	2.15	1.2×10^{-5}
ybdZ	Part of the *fes-ybdZ-entF-fepE* transcriptional unit, an enterobactin synthesis and transport operon	5.98	7.7×10^{-8}
fhuA	Ferrichrome outer membrane transporter	2.25	4.6×10^{-6}
fhuE	Ferric rhodotorulic acid outer membrane transporter	2.65	3.9×10^{-6}
fhuF	Ferric iron reductase involved in ferric hydroximate transport	4.29	1.6×10^{-7}
tonB	Cytoplasmic membrane protein that provides energy for the import of iron-siderophore complexes; part of the TonB-ExbB-ExbD complex	2.1	1.5×10^{-4}
exbD	Part of the TonB-ExbB-ExbD complex	3.2	7.4×10^{-8}
chuX	ChuX, a ShuX-like protein, required for hemin utilization	4.68	1.9×10^{-7}
chuT	ChuT, a putative periplasmic binding protein; may be required for hemin utilization	3.06	5.5×10^{-6}
chuW	ChuW, a putative coproporphyrinogen III oxidase; may be required for hemin utilization	3.01	4.2×10^{-6}
chuY	Hypothetical protein	2.84	7.0×10^{-6}
chuU	Putative hemin permease	2.52	7.5×10^{-7}
chuV	Hypothetical protein highly similar to ATPases of iron ABC transporters	2.01	7.7×10^{-6}
chuA	ChuA, an outer membrane heme/hemoglobin receptor	3.88	4.5×10^{-6}
chuS	ChuS, a putative heme/hemoglobin transport protein; may be required for hemin utilization	3.76	4.0×10^{-6}
sitA	Iron transport system SitA protein	5.23	2.4×10^{-6}
sitB	SitB, an ATP-binding component of the iron transport system	3.07	1.6×10^{-4}
sitC	Inner membrane component of the iron transport system	2.10	9.1×10^{-4}
feoA	Ferrous iron transport protein A	2.98	1.8×10^{-7}
feoB	Ferrous iron transporter	2.32	2.9×10^{-6}
Downregulated genes[c]			
acnA	Aconitate hydratase 1, contains a $[4Fe-4S]^{2+}$ cluster	0.26	3.6×10^{-7}
ftnA	Ferritin iron storage protein FtnA	0.24	4.2×10^{-7}
sodB	Superoxide dismutase	0.27	1.5×10^{-8}

[a] Shown are *E. coli* CFT073 iron metabolism genes up- or downregulated in the presence of cranberry proanthocyanidins (100 µg/ml) as revealed by Affymetrix GeneChip *E. coli* genome 2.0 microarrays. Fold change refers to growth in LB broth supplemented with PACs relative to growth in LB broth alone.
[b] Upregulated genes, genes which are upregulated in the presence of PACs.
[c] Downregulated genes, genes whose expression is regulated by RhyB and which are downregulated in the presence of PACs.

using the Functional Genomics Platform at McGill University and Génome Québec Innovation Centre. Microarray data analysis was done using the FlexArray 1.4.1 software program (http://genomequebec.mcgill.ca/FlexArray/). The data were normalized using the robust multiarray average algorithm (RMA) (14). Comparison between the two data sets was done using the randomized variance model (RVM) with the cutoff being a 2-fold increase or decrease and with a P value of 0.05 (30). The expression level of each of 5,379 open reading frames (ORFs) was determined. Overall, 616 genes were found to be upregulated and 419 were found to be downregulated.

The microarray results revealed that the most upregulated gene was *ryhB* (13.8-fold increase) (Table 1). This gene encodes a small regulatory RNA that acts as a quick off-switch for a suite of dispensable iron storage and iron-using proteins when the metal becomes limiting, and therefore leaves more iron available for essential proteins (6). RyhB downregulates the expression of the *sdhCDAB* operon that encodes a succinate dehydrogenase, two genes that encode proteins in the tricarboxylic acid cycle, the *acnA* and *fumA* genes, two ferritin genes, the *ftnA* and *bfr* genes, and a gene for superoxide dismutase, the *sodB* gene (21, 26). Consistent with the upregulation of the *ryhB* gene, we observed the downregulation of the *acnA*, *ftnA*, and *sodB* genes in the culture exposed to PACs (Table 1). Additionally, 33 genes that encode proteins involved in iron uptake and assimilation were upregulated in the presence of PACs (Table 1). These include the *ent* genes, encoding enterobactin, the *iuc* genes, encoding aerobactin, and the *iutA*

gene, the aerobactin receptor. Also, the *fhu* genes, for ferrichrome and rhodotorulic acid transport, the *feo* genes, for ferric iron transport, the *chu* genes, required for hemin utilization, and the *sit* genes, which encode an iron transport system, were upregulated. Furthermore, the *tonb* and *exbD* genes that encode proteins that form the TonB-ExbB-ExbD complex, which provides energy for ferrisiderophore transport across the outer membrane, were upregulated. The observed changes in gene expression signify that *E. coli* adapts to the presence of PACs by reducing iron storage and upregulating iron acquisition systems. Taken together, our results indicate that cranberry PACs limit iron availability.

Additional genes of interest that were differentially regulated in the presence of PACs include the *fliC* gene, encoding the flagellar structural protein, which was downregulated 2.4-fold. On the other hand, the *flgL* gene, which encodes one of the two flagellar hook-filament junction proteins, FlgL, was upregulated 2.7-fold. The *fimA* gene, encoding the major subunit of the *E. coli* type 1 fimbriae, and the genes encoding proteins involved in biofilm formation, the *ymgA*, *ymgB* (*ariR*), and *ymgC* genes, were downregulated. Curli, chemotaxis, and other fimbrial or flagellar genes were not differentially regulated in the presence of PACs.

Further validation of the biological reproducibility of our array data was obtained by conducting reverse transcription-PCR (RT-PCR) to determine the relative quantities of selected transcripts in the presence and absence of PACs. Four genes were selected for this test, two (the *chuX* and *iucD* genes) that were observed to be upregulated during the microarray analysis and two (the *ftnA* and *sodB* genes) that were downregulated. Reverse transcription was performed with RNA taken from the same aliquots as those used in the microarray study. Three genes, the *arcA*, *dnaG*, and *grpE* genes, were used as a reference, and calculations were performed using the qBase package (9). The RT-PCR confirmed that in the presence of PACs, there was significant stimulation of the *chuX* and *iucD* genes, and the *ftnA* and *sodB* genes were downregulated (data not shown).

Additional confirmation of the iron-chelating capability of PACs was obtained by monitoring the formation of complexes between PACs and ferrous and ferric ions. Changes in the UV-visible spectrum were tracked as described by Guo et al. (7). Briefly, small aliquots of 1 mM Fe^{2+} or Fe^{3+} solutions were added sequentially to a 10 μM PAC solution and homogenized. Spectra were recorded after each iron addition using an 8453 Hewlett-Packard spectrophotometer (Hewlett-Packard Company, Palo Alto, CA); results are shown in Fig. 2A. Guo et al. (7) observed a peak near 372 nm, which was assigned to the π-π* transition in the B ring of quercitin, one of the phenolic phytochemicals present in cranberries. Binding of quercitin to Fe^{2+} or Fe^{3+} resulted in the disappearance of this band, while a new one appeared at 425 nm. Cranberry-derived PACs are a mixture of oligomers, each of which may exhibit several unique electronic transitions in the region near 372 nm. This can result in the disappearance of a distinctive narrow peak corresponding to a single π-π* transition in the B ring but the appearance of a broader peak that is less sensitive to small amounts of iron cations. The addition of Fe^{2+} to the PAC solution led to the appearance of new bands at 405 nm and 545 nm, consistent with the formation of PAC-Fe^{2+} complexes.
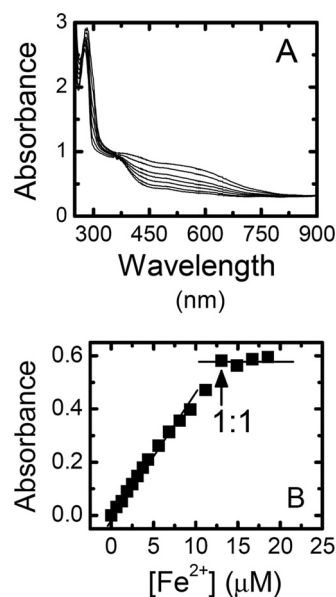

FIG. 2. (A) Spectra obtained for the titration of ~10 μM cranberry-derived proanthocyanidins (PACs) in 0.1 M HEPES buffer at pH 7.2 with (from bottom to top) 0, 2, 5, 7, 9, 11, and 16 μM Fe^{2+}. (B) Titration curve for the formation of the PAC-Fe^{2+} complexes. The absorbance of the complex at 545 nm minus the absorbance of the Fe^{2+}-free PACs (also at 545 nm) is plotted versus the Fe^{2+} concentration. 1:1 refers to the 1:1 PAC/Fe^{2+} complex.

To better understand the stoichiometry of complex formation between PACs and Fe^{2+}, the absorbance obtained for the iron-free PACs was subtracted from the absorbance at 545 nm, and this difference was plotted as a function of the corresponding total [Fe^{2+}] (Fig. 2B). A 1:1 PAC/Fe^{2+} complex was formed after the addition of 13 μM Fe^{2+} to ~10 μM PAC. This small disparity in the concentration equivalence between PACs and Fe^{2+} is attributable to the heterogeneity of the PAC solution. Identical experiments were carried out with Fe^{3+} cations, and comparable results were obtained, thus confirming complex formation between PACs and Fe^{3+} cations (data not shown).

Strains of uropathogenic *E. coli* (UPEC) have evolved multiple strategies that facilitate bacterial growth and persistence within the unfavorable setting of the host urinary tract. Relative to K-12 and commensal *E. coli* isolates, UPEC harbor more genes encoding iron acquisition systems (28). These systems enable UPEC to appropriate host iron stores (29) and have been shown to be highly expressed *in vivo* during human infection (8). Given that PACs induce a state of iron limitation in *E. coli* CFT073, it is possible that prevention of UTIs by cranberry PACs is partially the result of the extreme reduction in the amount of available iron to UPEC strains in the urinary tract. It is noteworthy that the absorption and metabolism of cranberry PACs by humans have not been thoroughly studied; however, reports from other laboratories have shown that proanthocyanidins and anthocyanins are mostly absorbed into the human circulatory system and transported into the urine in intact form (20, 22, 24). If further information about metabolites or induced compounds of PACs is published, research concerning the effects of these compounds on bacterial metab-

olism must be conducted in order to obtain a thorough understanding of their potential as agents for the prevention of bacterial adhesion.

This is the first study to investigate the effect that PACs have on the transcriptome of *E. coli* CFT073. Based on changes in gene expression, we found that PACs induce a state of iron limitation in this bacterium. Observation of the formation of PAC-Fe complexes confirmed that PACs act as iron chelators. A study by Lin et al. (18) reported that exposure of a nonuropathogenic *E. coli* strain to cranberry juice also resulted in the altered expression of iron metabolism genes. Future work will be oriented toward determining whether the ability of PACs to chelate iron is a factor that influences the anti-adhesive properties of these compounds and hinders biofilm formation.

Microarray data accession number. Array data were submitted to the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE25745.

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