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Biological and Physicochemical Wastewater Treatment Processes Reduce the Prevalence of Virulent *Escherichia coli*

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Effluents discharged from wastewater treatment plants are possible sources of pathogenic bacteria, including *Escherichia coli*, in the freshwater environment, and determining the possible selection of pathogens is important. This study evaluated the impact of activated sludge and physicochemical wastewater treatment processes on the prevalence of potentially virulent *E. coli*. A total of 719 *E. coli* isolates collected from four municipal plants in Québec before and after treatment were characterized by using a customized DNA microarray to determine the impact of treatment processes on the frequency of specific pathotypes and virulence genes. The percentages of potentially pathogenic *E. coli* isolates in the plant influents varied between 26 and 51%, and in the effluents, the percentages were 14 to 31%, for a reduction observed at all plants ranging between 14 and 45%. Pathotypes associated with extraintestinal pathogenic *E. coli* (ExPEC) were the most abundant at three of the four plants and represented 24% of all isolates, while intestinal pathogenic *E. coli* pathotypes (IPEC) represented 10% of the isolates. At the plant where ExPEC isolates were not the most abundant, a large number of isolates were classified as both ExPEC and IPEC; overall, 6% of the isolates were classified in both groups, with the majority being from the same plant. The reduction of the proportion of pathogenic *E. coli* could not be explained by the preferential loss of one virulence gene or one type of virulence factor; however, the quinolone resistance gene (*qnrS*) appears to enhance the loss of virulence genes, suggesting a mechanism involving the loss of pathogenicity islands.

Fecal contamination is the major source of pathogenic microorganisms, including *Escherichia coli*, in wastewater (1). Wastewater treatment processes are designed to reduce the concentration of contaminants, including pathogens, in the effluent before discharge to receiving water bodies; however, many wastewater treatment plants (WWTPs) discharge such effluents without disinfection. Consequently, nondisinfected effluents could still contain high proportions of pathogenic bacteria, thus presenting a threat to public health (2).

E. coli, which has been widely used as a fecal contamination indicator in aquatic environments, is normally considered nonpathogenic; however, some strains can be pathogenic. This was demonstrated by the Walkerton (Ontario, Canada) drinking water *E. coli* O157:H7 outbreak in 2000, which resulted in 2,300 reported illness cases and 7 deaths (3, 4). Based on their virulence properties and the clinical symptoms of the host, pathogenic *E. coli* isolates can be classified into two major groups: intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC) (5, 6). IPEC isolates can be further divided into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (5, 6). ExPEC isolates are grouped into three main pathotypes: uropathogenic *E. coli* (UPEC), meningitis-associated *E. coli* (MNEC), and septicemia-causing pathogenic *E. coli* (SEPEC) (5, 6).

Previous environmental surveys to determine the natural prevalence of pathogenic *E. coli* relied on the detection of genes by PCR (2, 7, 8) and colony hybridizations (9, 10) or on their phylogenetic classification (groups A, B1, B2, C, D, and E) (11, 12). At least 567 virulence genes (VGs) have been reported for *E. coli* (13), which can be grouped into 78 virulence factors (VFs) and include functions such as adhesins, toxins, capsules, secretion systems, iron uptake systems, and invasins (14–18). Therefore, the identification of specific VG subsets can indicate the likelihood of a disease pathotype (19–21). Given the large number of genes to be tested for accurate pathotyping, a customized DNA microarray has been developed (16, 22–24), allowing a more comprehensive environmental survey of pathogenic *E. coli* isolates to be performed (9, 20–22, 25). Microarray-based studies at several locations around the Great Lakes found that 26 to 28% of *E. coli* isolates were ExPEC and that 2 to 5% were IPEC, with the locations most influenced by urban discharges (wastewater and runoffs) harboring higher proportions of isolates with defined pathotypes (20, 21). Thus, a significant proportion of environmentally isolated *E. coli* strains are potentially pathogenic. The term "potentially pathogenic" is used here, as a direct demonstration of the disease-causing ability in animals or humans was not done.

To our knowledge, only three studies have examined the proportion of potentially pathogenic *E. coli* isolates in municipal WWTPs. One study that detected the presence of Shiga-like toxin II genes (stx_2 genes) by PCR did not find any of their 1,520 isolates to be positive and found that only 1 influent sample and no effluent samples were positive when DNA was extracted directly from the wastewater samples (26). Another study used integrase genes to genotype 973 isolates and found that 71.5% of the 109 inte-

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TABLE 1 Treatment capacities and types of treatment processes employed in the WWTPs studied[a]

Plant	Population served in 2010	Avg flow (m^3/day)	HRT (h)	SRT (days)	Treatment process
Activated sludge					
AS1	60,000	65,000	20	7	Conventional activated sludge without primary settling
AS2	50,000	45,000	13	4–5	Conventional activated sludge with primary settling
Physicochemical					
PC1	59, 000	44,000	2.69	NA	Coagulation, with aluminum sulfate (alum) with organic polymer addition
PC2	280,000	240,000	1.87	NA	Coagulation, with aluminum sulfate (alum) with organic polymer addition

[a] HRT, hydraulic retention time; SRT, solids retention time; NA, not applicable.

grase-positive isolates contained at least one of the 11 VGs tested, suggesting a high proportion of potentially pathogenic *E. coli* isolates in this group (2). The proportion of integrase-positive isolates decreased after treatment, with 75% being isolated from the influent and 15% being isolated from the effluent. In a third study, isolates obtained at various tertiary treatment stages within municipal WWTPs were assessed for 12 VGs (27). No IPEC-associated VGs were found, but 60% contained at least one ExPEC VG. Although good initial steps, those studies did not directly evaluate the impact of primary or secondary treatment processes on the frequency of VGs or pathotypes.

The objective of this study was to investigate the impact of biological (specifically activated sludge [AS]) and physicochemical (PC) wastewater treatment processes on the frequency and distribution of *E. coli* pathotypes and VGs in treated municipal wastewater effluents. Isolate libraries were analyzed by using a DNA microarray capable of detecting 195 virulence or virulence-related genes as well as 96 antimicrobial resistance genes (22, 28). Our results suggest a possible link between enhanced VG loss and the quinolone resistance peptide synthesis gene (*qnrS*) during wastewater treatment.

MATERIALS AND METHODS

Wastewater sampling and isolation of *E. coli*. Four municipal WWTPs located near Montreal, Canada, were selected for this study. Of the four treatment plants, two, designated AS1 and AS2, used a biological (activated sludge) treatment process, and the other two, designated PC1 and PC2, used physicochemical treatment processes. A description of the treatment processes and treatment capacities of the four WWTPs is given in Table 1. Samples were obtained between 20 May and 4 August 2009 (summer) from the influent (after bar screening and grit removal) and effluent (prior to disinfection) channels of each plant by submersing sterile 1-liter containers. The influent and effluent samples from a given plant were collected within minutes of each other and from the center of the respective channels, to exclude material accumulated on the walls. The samples were immediately placed on ice in a closed cooler, transported to the laboratory, and stored at 4°C until testing (within 24 h). Samples were analyzed for pH, turbidity, chemical oxygen demand (COD), and suspended solids (SS), according to standard methods (29). The mean particle size (PS) was obtained by using a Lasentec M100 F particle system characterization monitor (Lasentec, Redmond, WA). *E. coli* cells in the influent and effluent of each treatment plant were enumerated by using a membrane filtration (0.45 μm) technique (29). Briefly, filters were incubated at 44.5°C in mFC agar with 5-bromo-6-chloro-3-indolyl glucuronide (BCIG) (catalog no. CM1111; Oxoid Ltd., England) plates. *E. coli* colonies (identified as blue) were then picked at random and streaked onto Luria-Bertani (LB) agar consisting of 1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar to obtain pure *E. coli* isolates. The confirmation of *E. coli* was done by growing colonies on Chromocult agar (*E. coli* identified by a blue streak) and further testing by using Kovac's reagent (EMD Chemicals, Germany) for the indole test (*E. coli* identified by pink color development). Between 92 and 100 *E. coli* isolates were collected from the influents and effluents of each plant (a total of 765 isolates); of these, 83 to 93 *E. coli* isolates from each influent and effluent sample from each treatment plant (a total of 719) were randomly selected for DNA microarray analysis.

DNA extraction and labeling. DNA was extracted from 1-ml samples of *E. coli* cells cultured overnight in LB broth, washed once with distilled deionized water (ddH_2O), and resuspended in 175 μl of ddH_2O. The suspension was boiled at 95°C for 10 min and centrifuged at 13,000 × *g* for 3 min, and 150 μl of the supernatant containing extracted DNA was removed for labeling by using the BioPrime DNA labeling kit (Invitrogen Life Technologies, Burlington, Ontario, Canada), according to a protocol described previously by Hamelin et al. (21). The labeling reaction was carried out with a total volume of 50 μl containing 10 μl of extracted DNA, 10 μl of random primer solution, 22.5 μl of ddH_2O, 0.5 μl of high-concentration DNA polymerase (Klenow fragment) (40 U/μl), 5 μl of a deoxynucleoside triphosphate mix (1.2 mM dATP, 1.2 mM dGTP, 1.2 mM dTTP, and 0.6 mM dCTP in 10 mM Tris [pH 8.0] and 1 mM EDTA), and 2 μl of 1 mM Cy5-dCTP (GE Healthcare, Little Chalfont, United Kingdom). Labeling reactions were performed in the dark at 37°C for 3.5 h, and the reaction was then stopped by the addition of 5 μl of 0.5 M Na_2-EDTA (pH 8.0) to the mixture. The labeled samples were purified by using Pure Link PCR purification kits (Invitrogen Life Technologies), according to the instructions provided by the manufacturer. The amount of incorporated fluorescent Cy5 dye was quantified by scanning the purified DNA samples at wavelengths from 200 to 700 nm and calculating the result by using the Internet-based Percent Incorporation Calculator (http://www.pangloss.com/seidel/Protocols/percent_inc.html).

Microarray hybridization, imaging, and analysis. The oligonucleotide probes (70-mers) used in the DNA microarray of this study were developed and validated previously (22, 28). The current microarray probe assembly is dubbed MaxVir1.0. It contains 306 probes targeting different alleles and versions of 195 virulence or virulence-related genes; 96 antibiotic resistance gene probes; 4 positive-control probes, *tnaA* (tryptophanase), *uidA* (β-glucuronidase), *lacY* (lactose permease), and *lacZ* (β-galactosidase); and 2 negative controls derived from the green fluorescent protein of *Aequoria victoria* and chlorophyll synthase from *Arabidopsis thaliana*. A complete list of probe names can be found in Table S1 in the supplemental material, and their sequences can be found in supplemental material reported previously (22, 28).

The hybridization protocol used for this study was described previously (20, 21). Briefly, microarrays were prehybridized at 50°C for 60 min under a Lifterslip coverslip (Erie Scientific Company, Portsmouth, NH), using a SlideBooster hybridization workstation (model SB800; Advalytix, Germany), with 60 μl of prewarmed (37°C) digoxigenin (DIG) Easy Hyb

TABLE 2 Wastewater characteristics at the WWTPs studied at the time of sampling

Treatment plant	Source	Value for wastewater characteristic[a]						
		Temp (°C)	pH	Turbidity (NTU)	SS (mg/liter)	COD (mg/liter)	Mean particle size (μm)	*E. coli* count (CFU/100 ml)
Activated sludge								
AS1	Influent	13.0	7.2	80.0	163	434	10.4	4×10^6
	Effluent	13.8	7.2	20.0	20.5	73.4	18.4	3×10^5
AS2	Influent	9.9	7.2	62.5	181	306	10.9	5×10^6
	Effluent	10.0	7.7	9.5	11.0	46.3	13.4	5×10^4
Physicochemical								
PC1	Influent	13.0	7.6	39.5	96.0	131	11.4	2×10^6
	Effluent	13.1	7.2	7.22	8.77	38.5	7.6	1×10^5
PC2	Influent	16.7	7.8	30.5	27.3	80.6	5.4	2×10^6
	Effluent	16.0	7.4	9.5	12.0	34.9	7.1	8×10^5

[a] SS, suspended solids; COD, chemical oxygen demand; NTU, nephelometric turbidity units.

buffer (Roche Diagnostics, Laval, Quebec, Canada) supplemented with 5% (vol/vol) bovine serum albumin (1 mg/ml; New England BioLabs Inc., Beverly, MA). After prehybridization, the slides were dipped in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and air dried. Hybridization was carried out overnight at 50°C under a Lifterslip coverslip (18 by 18 mm) in a SlideBooster workstation. One microgram of Cy5-labeled genomic DNA was dispensed into each microarray by resuspending dried DNA in 15 μl of DIG Easy Hyb buffer supplemented with 0.1 μg/μl single-stranded salmon sperm DNA (Sigma-Aldrich, Ontario, Canada) after denaturing for 5 min at 95°C. After hybridization, Lifterslip coverslips were removed by dipping the slides in a solution containing 0.1× SSC and 0.1% (wt/vol) SDS (sodium dodecyl sulfate). Posthybridization washes were performed at 37°C: two washes with 0.1× SSC and 0.1% SDS for 10 and 5 min, respectively, and one last wash with 0.1× SSC for 5 min. The microarrays were finally air dried.

Microarray slides were scanned with a ScanArray Lite fluorescent microarray analysis system (Perkin-Elmer, Mississauga, Ontario, Canada). The acquisition of fluorescent spots was performed by using ScanArray Express software (Perkin-Elmer, Foster City, CA). Fluorescent spot intensities were quantified by using ImaGene v8.0 (BioDiscovery Inc., El Segundo, CA). Intensities were normalized to control for variations between hybridizations using invariant background (buffer) spots. Normalized spot intensities were then divided by the average background intensity to create signal-to-noise ratios. Spots with a signal-to-noise ratio of >3 were considered positive. The binary positive/negative results were then analyzed.

Phylotyping and pathotyping of *E. coli* isolates based on VGs. The phylotyping of *E. coli* isolates within the four main groups A, B1, B2, and D was performed by using two genes, *chuA* and *yjaA*, and a DNA fragment, namely, TSPE4.C2 (19). *E. coli* isolates were classified into defined pathotypes according to the set of VGs or markers which they contain (22, 28). The complete list of probes, genes, and rules used to determine the various pathotypes can be found in Table S1 in the supplemental material. For ExPEC pathotypes, 37 genes were used for pathotyping (the minimum numbers of VGs detected to identify a pathotype were 5 for UPEC, 6 for SEPEC, 6 for MNEC, and 3 for incomplete ExPEC), and an additional 8 genes associated with ExPEC pathotypes were monitored. IPEC pathotyping relied on 56 genes (the minimum numbers of VGs detected to identify a pathotype were 1 for STEC, 3 for DAEC, 9 for EHEC, 7 to 8 for EPEC, 7 for EAEC, and 5 for ETEC), and an additional 46 IPEC VGs were monitored. Finally, 26 VGs that are common to several pathotypes and 27 not-classified VGs were also monitored.

Statistical analysis. Microarray data were analyzed by using the log-linear model for frequencies (30), with the computations performed by the CATMOD procedure in the SAS statistical software (SAS Institute, Cary, NC). The null hypotheses for the test were that the wastewater treatment process has no impact on the proportion of virulent *E. coli* isolates (i.e., the frequencies in the influent and effluent are not different, forming 2 [virulent versus not virulent]-by-2 [influent versus effluent] contingency tables) and that the different plants (or processes) did not affect the observed impacts (forming a 2-by-2-by-[4 plants or 2 processes] contingency tables). A similar approach was used to test the frequency distributions of each gene. However, only the overall difference between the influent and effluent samples (2-by-2 contingency tables) was evaluated for all the isolates or isolates containing *qnrS* genes. Note that for these tests to be performed, a VG must have been present in more than five isolates or more than four isolates also containing a *qnrS* gene. The difference in the phylogenetic distribution among pathotype groups and nonpathogenic strains was also tested by using the log-linear model approach (2 [pathotype versus nonpathogenic]-by-4 [phylogroups] contingency tables). Because of the complex flow patterns within WWTPs, making it difficult to match exactly the influent and effluent samples, the test statistics were evaluated at a P value of ≤0.10. Finally, the difference in the average frequencies of ExPEC and IPEC genes was tested by a permutation t test using the MULTTEST procedure in the SAS statistical software (SAS Institute, Cary, NC).

RESULTS

Wastewater characteristics. Of the four WWTPs, two treatment plants employed the conventional AS process, and the other two treatment plants used a PC process consisting of flocculation-coagulation-sedimentation with the addition of aluminum sulfate (alum) and polymer. The influent and effluent characteristics of the four WWTPs are summarized in Table 2. *E. coli* reduction counts were 1.1 to 2.0 logs for the AS treatment plants and 0.4 to 1.3 logs for the PC treatment plants. From the wastewater quality data, it was observed that AS plants reduced the concentrations of turbidity, SS, and COD by 75 to 84%, 87 to 94%, and 83 to 85%, respectively, whereas for the PC plants, the reductions were 68 to 83%, 56 to 91%, and 57 to 71%, respectively. These performances are typical for each of these types of treatment processes (31).

Frequency and distribution of potentially pathogenic *E. coli*. Pathogen quantification is necessary to evaluate the impact of the wastewater treatment processes on the prevalence of virulent *E. coli*. In the current study, potentially pathogenic *E. coli* strains were identified by detecting in isolates the subsets of VGs associated with different pathotypes (see Table S1 in the supplemental material) by using microarray hybridization. The proportions of pathotypes in the *E. coli* isolate population were compared between the corresponding influent and effluent samples with the

TABLE 3 Frequency and distribution of virulent *E. coli* isolates from the influents and effluents of the WWTPs[c]

	No. (%) of *E. coli* isolates from plant								Statistical test of variation in frequency[b]		
	Activated sludge				Physicochemical					Difference in Inf/Eff changes	
	AS1		AS2		PC1		PC2		Overall Inf/Eff change	Between plants	Between processes
Pathotype[a]	Inf	Eff	Inf	Eff	Inf	Eff	Inf	Eff			
IPEC											
ETEC	1	1	0	0	5	0	0	0	−	−	−
STEC	6	5	4	4	24	16	1	3	−	−	−
Subtotal	7	6	4	4	29	16	1	3	−	−	−
ExPEC											
UPEC	11	10	29	25	16	16	12	7	+	−	−
SEPEC	2	0	0	0	0	0	0	0	−	−	−
Incomplete ExPEC	9	1	3	2	7	5	9	5	++	−	−
Subtotal	22	11	32	27	23	21	21	12	++	−	−
Double pathotypes[d]	5	4	3	3	16	11	0	0	−	−	−
Total virulent[e,f]	24 (26)	13 (14)	33 (36)	28 (31)	46 (51)	26 (29)	22 (27)	15 (16)	++	−	−
Total avirulent	67	77	58	62	45	64	61	78	++	−	−
Total	91	90	91	90	91	90	83	93	NA	NA	NA

[a] See Table S1 in the supplemental material for details for determining the pathotypes.
[b] −, $P > 0.10$; +, $P < 0.10$; ++, $P < 0.05$.
[c] Inf, influent; Eff, effluent; NA, not applicable.
[d] Double pathotypes are defined as *E. coli* isolates that contained VGs of both STEC and UPEC (36 isolates), STEC and incomplete ExPEC (5 isolates), or ETEC and incomplete ExPEC (1 isolate) pathotypes. The double-pathotype isolates are reported in their respective pathotype groups; their number should be removed from the sum of IPEC and ExPEC isolates to obtain the total number of virulent isolates.
[e] The percentage of total virulent *E. coli* isolates was calculated as (total number of pathotypes/total number of *E. coli* isolates) × 100.
[f] The percentage reductions between influent and effluent samples, which were calculated as 100 − (percentage of pathotypes in effluent/percentage of pathotypes in influent) × 100, were 45% for AS1, 14% for AS2, 43% for PC1, and 39% for PC2.

null hypothesis that pathotype frequency remains the same. The percent reductions of the proportions of pathogenic *E. coli* between the influent and effluent were 14 to 45% and 40 to 45% for the AS and PC plants, respectively (Table 3). The overall average reduction in pathogenic *E. coli* (36%) was significant ($P < 0.05$); however, the differences in reductions between plants or between process classes (AS or PC) were not significant ($P > 0.10$). A similar reduction was suggested by a previous study of municipal WWTPs (2).

Both IPEC and ExPEC were present in all samples (Table 3). Among IPEC isolates, only toxigenic pathogenic *E. coli* pathotypes such as ETEC and STEC were detected, with STEC being the more abundant of the two pathotypes (for pathotype-defining gene subsets used in this study, see Table S1 in the supplemental material). The relative frequencies of IPEC pathotypes (including the double-pathotype isolates classified as both IPEC and ExPEC) were 8% or lower in the plant influents, except for PC1 (i.e., one plant sampled on a specific day), where it reached 32%. Together, the ExPEC pathotypes were typically more abundant than the IPEC pathotypes, and they accounted for 24% or more of the isolates in the plant influent samples. Among ExPEC pathotypes, UPEC and incomplete ExPEC were detected in all samples, with UPEC being predominant (8 to 32% for UPEC compared to 1 to 11% for incomplete ExPEC). Overall, the proportions of both of these pathotypes were significantly reduced ($P < 0.05$) between influents and effluents, but the difference in the reduction between plants was not significant ($P > 0.10$) (Table 3). The only other ExPEC pathotype detected was SEPEC, which was detected in a single sample (only 2% of isolates). Finally, portions of the isolates (3 to 18%) from three of the treatment plants were classified under multiple pathotypes, mainly either STEC-UPEC or STEC-incomplete ExPEC (only one isolate was classified as ETEC-incomplete ExPEC).

Phylogenetic distribution of *E. coli* isolates. ExPEC isolates with a single pathotype belonged predominantly to groups B2 (80%) and D (13%), which were significantly ($P < 0.05$) higher than the prevalences for the other two groups (3 to 4%), and their phylogenetic distribution was significantly ($P < 0.05$) different

TABLE 4 Phylogenetic distribution of *E. coli* isolates

Pathotype[c]	No. (%)[a] of *E. coli* isolates per phylogroup					Result of test of distribution difference from avirulent isolates[b]
	A	B1	B2	D	Total	
Single IPEC						
ETEC	2 (33)	1 (17)	0 (0)	3 (50)	6	ND
STEC	11 (50)	8 (36)	1 (5)	2 (9)	22	−
Subtotal	13 (45)	9 (31)	2 (7)	5 (17)	29	−
Single ExPEC						
UPEC	3 (3)	1 (1)	83 (83)	13 (13)	100	++
SEPEC	0 (0)	0 (0)	2 (100)	0 (0)	2	ND
Incomplete ExPEC	1 (3)	5 (14)	20 (57)	9 (26)	35	++
Subtotal	4 (3)	6 (4)	105 (77)	22 (16)	137	++
Double pathotype						
STEC-UPEC	0 (0)	1 (3)	34 (94)	1 (3)	36	++[d]
STEC-incomplete ExPEC	1 (20)	0 (0)	2 (40)	2 (40)	5	ND
ETEC-incomplete ExPEC	0 (0)	0 (0)	1 (100)	0 (0)	1	ND
Subtotal	1 (2)	1 (2)	37 (88)	3 (7)	42	++[d]
Total virulent	18 (9)	16 (8)	143 (69)	30 (14)	207	++
Total avirulent	271 (53)	186 (36)	24 (5)	31 (6)	512	

[a] Percentages were calculated with respect to a given phylogroup.
[b] −, $P > 0.10$; ++, $P < 0.05$; ND, not determined due to low frequencies.
[c] Single means a single-pathotype classification, and double means a double-pathotype classification.
[d] Distributions of these groups were not different ($P > 0.1$) from that of the subtotal single-pathotype ExPEC group.

from that of avirulent strains (Table 4). Similarly, the phylogenetic distribution of the double-pathotype isolates (ExPEC-IPEC) was significantly different ($P < 0.05$) from that of avirulent strains but not from that of single-pathotype ExPEC isolates ($P > 0.1$) (Table 4). Conversely, the majority of IPEC isolates with a single pathotype (93%) was found to be distributed among three phylogenetic groups (groups A, B1, and D), without a significant ($P > 0.1$) difference from the distribution of avirulent isolates (Table 4). These results clearly support the correlation between ExPEC pathotyping and phylogenetic grouping (mainly groups B2 and D) for isolates from municipal wastewater, as was observed previously (27).

Frequency of ExPEC and IPEC genes. Many more isolates were classified as ExPEC (169 isolates) than as IPEC (70 isolates) (Table 3), but is the frequency of ExPEC VGs also higher than that of IPEC VGs? As the occurrence of lateral gene transfer in surface waters is suspected (32), this question is important for an understanding of the role of municipal wastewater discharges for the influx of VGs in the environment. The IPEC isolates tended to carry fewer VGs (10 to 53 VGs) than the ExPEC isolates (26 to 63 VGs), although not all the VGs detected in one isolate were associated with its pathotype classification. Of the 71% of strains not classified within a pathotype, only 7% contained fewer than 14 VGs, while 84% contained 15 to 34 VGs, demonstrating a high VG load for the entire isolate collection. When genes related to specific pathotypes were analyzed, 43 of 45 ExPEC VGs and 44 of 102 IPEC VGs were detected in more than five isolates (the threshold for inclusion in the analysis). The ExPEC VGs occurred significantly ($P < 0.1$) more frequently (average, 78 isolates) than the IPEC-specific VGs (average, 37 isolates [note that the four genes listed for both ExPEC and IPEC in Table S1 in the supplemental material were not included in this comparison]). Therefore, both ExPEC isolates and ExPEC-related VGs were more abundant than IPEC isolates and VGs.

Differences in specific gene frequencies between influents and effluents. Our data suggested a reduction in the frequencies of ExPEC pathotypes between the influents and effluents of the WWTPs (Table 3). In order to determine if specific VGs and, eventually, cellular functions were associated with this decrease in frequency, the frequency differences between influent and effluent samples were also analyzed for each VG, whether or not they were present in an isolate classified as being virulent. The approach here relies on the hypothesis that if a VG is lost from a virulent strain, making it avirulent, the overall frequency of that VG will decrease. Furthermore, this VG is more likely lost from both virulent and avirulent strains if it influences strain selection in the wastewater treatment system or if it is carried on unstable genetic elements. Conversely, if a given VG is not affected by the treatment system, its frequency should stay constant between the influent and the

effluent (as a virulent strain carrying this gene could be changed into an avirulent one by the loss of other VGs).

On average, the frequencies of a total of 38 of the 121 VGs with more than 5 observations were found to decrease significantly ($P < 0.1$) from the influent to the effluent (see Table S2 in the supplemental material), most of which (33 VGs) decreased in at least 3 of the 4 plants. These genes are of interest since they could explain the average significant reduction in the proportions of pathogenic *E. coli* observed at all plants. The VGs were categorized under 9 virulence factor functions (e.g., adherence, capsule, and iron acquisition), with a 10th category encompassing miscellaneous functions (see Table S1 in the supplemental material). The 38 VGs showing a significant overall decrease in frequency fell in seven of these categories (see Table S2 in the supplemental material). The categories "locus of enterocyte effacement" and "type III secretion systems" did not have any genes that were significantly reduced in frequency between the influent and the effluent samples. These categories contain genes associated mainly with IPEC isolates other than STEC and were found in relatively low abundances, which reduces the power of the test. The other category lacking VGs with significantly reduced frequencies through the wastewater treatments was "colicins and microcins." The majority of the genes in this group, however, showed a reduction in frequency that was statistically insignificant ($P > 0.1$). Focusing on the VGs used to recognize the main pathotypes detected in this study (UPEC, incomplete ExPEC, and STEC), all the function categories contained VGs with frequencies significantly reduced by wastewater treatment (Table 5). These data suggest either that several functions are selected against by the treatment processes or that groups of genes are lost simultaneously. The latter case could arise because these genes are associated with each other and because the function of one of the genes is counterselected, or they are carried together on unstable mobile genetic elements such as pathogenicity islands (PAIs), plasmids, or prophages.

Confirmation of the exact mechanism explaining the loss of genes through the wastewater treatments is beyond the scope of this study; however, there are indications in the data that would lend support to one mechanism. It was reported previously that in some UPEC strains, PAIs can be unstable. In those studies, lower temperatures and the presence of ciprofloxacin (a quinolone) at subinhibitory concentrations enhanced the loss of PAIs above the background level of spontaneous deletions (33, 34). Such results suggest a relationship between pathotype or gene reductions and the detection of integrases and transposases or quinolone resistance genes (*qnrS1* or *qnrS2*). When considering only the *qnrS*-carrying isolates (147 strains), the overall pathotype reduction increased significantly ($P < 0.05$) from 36% to 52% (Table 5). The presence of integrase and transposase genes did not show a similar association (data not shown). Looking for the same association at the gene level, 33 of the 38 genes that were significantly reduced in frequency had more than four simultaneous occurrences with *qnrS* genes (see Table S2 in the supplemental material), which allowed for statistical testing. Of these 33 genes, 23 (70%) showed increased overall reductions in frequency, although only 10 were significant ($P < 0.1$). The data on the pathotype and gene frequencies suggest that the presence of *qnrS* in a strain somehow enhanced the loss of VGs.

DISCUSSION

Impact of wastewater treatment processes on the proportion of pathogenic *E. coli*. High percentages (between 26 and 51%) of potentially virulent *E. coli* were detected in municipal WWTP influents. Variations in pathogenic *E. coli* levels in the influents for the four treatment plants may be due to differences in source wastewater quality since each plant receives wastewater from diverse sources, including industrial, residential, and recreational sources (27). Levels of pathogenic *E. coli* in the WWTP effluents varied from 14 to 31%, with an average observed reduction of 36%. The dominant pathotype was ExPEC in both influents and effluents (Table 3). Notably, the level of virulent *E. coli* in lakes and rivers using an earlier microarray version ranged from 29 to 32% and was also dominated by ExPEC pathotypes (20, 21), thus making the level of potentially pathogenic *E. coli* in natural waters comparable to that in wastewater samples.

All treatment plants tested showed a reduction of pathogenic *E. coli* levels, although the differences between plants or processes were not significant (Table 3). This result is intriguing given the different types of treatment systems studied. On the one hand, PC treatment is the simple coagulation and flocculation of suspended solids by aluminum sulfate (alum) or ferric chloride and polyelectrolytes. Such plants are characterized by a short residence time (a few hours) of the wastewater and flocculated solids (Table 1). On the other hand, AS treatment is a secondary biological treatment in which suspended solids, including bacterial biomass, are recycled and accumulate to several grams per liter, concentrations much higher than those in PC plants (31). While the wastewater residence time is also on the order of hours, the residence time of the solids is typically 7 to 15 days. Thus, if the mechanism driving the reduction of pathogenic *E. coli* is the same for both processes, it would have to be fast acting.

It has been shown that the removal kinetics of *E. coli* by AS treatment systems is biphasic, with an initial rapid adsorption to the floc followed by slower protozoan grazing (35). Although removal by floc incorporation would also occur in PC treatment, removal by grazing would not occur, as protozoa are not present. Because *E. coli* is essentially absent from AS microbial communities, as it is seldom detected, even in large 16S rRNA pyrosequencing libraries (36, 37), it seems unlikely that *E. coli* isolates found in the effluent were released by AS flocs, as they presumably originated from the influent. Thus, the few hours of wastewater retention in both treatment systems could support the growth of *E. coli* for only a few generations at best.

A possible mechanism for the enhanced removal of pathogenic *E. coli* is preferential adsorption to the flocs of both WWTP types. For example, the presence of adhesins or capsules could positively affect adsorption. One possible manifestation of this phenomenon could be the preferential loss in the *E. coli* population of VGs associated with given functions. This, however, was not the case, as significant reductions in frequencies were observed for genes in 7 of the 10 virulence factor categories (see Table S2 in the supplemental material) and in all categories used to determine the pathotypes most frequently observed (UPEC, incomplete ExPEC, and STEC) (Table 5). However, this mechanism cannot be ruled out, as VGs from several functional categories have been observed together on the same PAIs (34, 38).

Another mechanism could be the direct loss of the PAIs themselves. Previous studies have shown that some UPEC PAIs are unsta-

TABLE 5 Gene-by-gene analysis of the overall reductions in frequencies of virulence genes associated with the most abundant pathotypes and in isolates containing the quinolone resistance gene (*qnrS*) between the influents and the effluents[b]

Descriptor or virulence gene	Pathotype definition (group no.)[a]			Positive isolates						Isolates with quinolone resistance gene		
	Incomplete ExPEC	UPEC	STEC	Total no. of isolates	No. of isolates in Inf	No. of isolates in Eff	% Inf/Eff change	Significant reduction[c]	No. of plants with reduction	Total no. of isolates	% Inf/Eff change	Significantly lower trend[c,d]
Isolates												
Total				719	356	363	NA	NA	NA	147	−26.4	NA
Pathogenic				207	125	82	−35.7	++	4	53	−52.1	++
Virulence factors/genes												
Adherence[e]												
f165(1)A	1			26	11	15	33.7	−	1	5	100.0	−
focA	1			21	13	8	−39.6	−	3	5	−66.7	−
focG	1			50	30	20	−34.6	+	4	5	−66.7	−
papA	1	1		515	271	244	−11.7	++	4	122	−7.4	−
papC	1	1		121	65	56	−15.5	−	2	25	−25.0	−
papG	1	1		141	80	61	−25.2	+	3	40	−11.1	−
pixA	1	1		7	4	3	−26.4	−	1	1	ND	ND
sfaA	1			16	8	8	−1.9	−	1	1	ND	ND
sfaD	1			68	41	27	−35.4	−	3	12	−4.8	−
sfaHII	1			34	21	13	−39.3	−	4	5	−66.7	−
Capsule												
kpsM-II	2	2		189	110	79	−29.6	++	4	44	−44.1	−
kpsM-III	2	2		15	8	7	−14.2	−	3	2	ND	ND
Iron acquisition or transport systems												
fyuA	3	3		233	138	95	−32.5	++	4	54	−38.7	−
iron	3	3		100	55	45	−19.8	−	3	17	−6.7	−
irp1	3	3		255	151	104	−32.5	++	4	58	−44.7	−
irp2	3	3		260	154	106	−32.5	++	4	58	−44.7	−
iucD	3	3		94	55	39	−30.5	++	4	26	−60.0	++
iutA	3	3		98	57	41	−29.5	++	3	26	−60.0	++
sitA	3	3		267	152	115	−25.8	++	4	58	−49.2	+
sitD	3	3		270	151	119	−22.7	++	4	58	−49.2	++
Hemolysins and other toxins[e]												
cnf1		4		73	44	29	−35.4	+	3	17	−44.4	−
hlyA		4		83	47	36	−24.9	−	3	19	−22.2	−
sat		4		97	56	41	−28.2	−	4	34	−36.2	−
vat		4		156	88	68	−24.2	++	4	36	−48.7	−
stx_1 (A, B)			1	61	35	26	−27.1	−	2	29	−18.5	−

[a] Rules to classify isolates under pathotypes as defined in Table S1 in the supplemental material. At least one gene from each number group needs to be present for an isolate to be classified under a given pathotype.

[b] Inf, influent; Eff, effluent; NA, not applicable; ND, not determined due to insufficient observations. Criteria were a virulence gene frequency of >5 isolates and a *qnrS* frequency of >4 isolates.

[c] −, $P > 0.10$; +, $P < 0.10$; ++, $P < 0.05$.

[d] Statistical test of reduction (negative influent/effluent change) among *qnrS*-positive isolates being greater (more negative) than the reduction among *qnrS*-negative ones. For details on gene descriptions, see Table S1 in the supplemental material.

[e] The genes *gafD*, *cnf2*, and stx_2 (A, B, tA, tB) in these categories were not tested because they were not abundant enough (frequency of ≤5). For details on gene descriptions, see Table S1 in the supplemental material.

ble at lower temperatures and are lost at a rate of $\sim 10^{-5}$ per CFU (33, 34), which may correlate with conditions found in wastewater treatment systems as well as in natural freshwater. Another study demonstrated that ciprofloxacin (an antibiotic of the quinolone family) at subinhibitory concentrations increases the loss of virulence genes in UPEC isolates by a few orders of magnitude (33). EHEC-associated genes also seem to be affected by the presence of quinolones at subinhibitory concentrations, as norfloxacin induces the transcriptional activity of prophages carrying *stx* genes in a strain of *E. coli* O157:H7, leading to the release of phages in the growth medium (39).

In the current study, the presence of a quinolone resistance gene (*qnrS*) enhanced the loss of specific genes though the treatment processes (Table 5; see also Table S2 in the supplemental material). The typical MICs for sensitive environmental *E. coli* isolates are ~1 to 10 μg/liter for ciprofloxacin and ofloxacin and 15 μg/liter for norfloxacin (40, 41). These three quinolones were detected in the effluents of AS plants in Canada at median concentrations of 0.118, 0.094, and 0.050 μg/liter for ciprofloxacin, ofloxacin, and norfloxacin, respectively, and at maximum concentrations as high as 0.400 and 0.506 μg/liter for ciprofloxacin and ofloxacin, respectively (42). A commonly reported 90% removal rate of these antibiotics (43) places the median and maximum influent concentrations of ciprofloxacin and ofloxacin at these plants between 0.9 and 5.1 g/liter, which is close to the MIC for susceptible *E. coli*. These concentrations had been observed in WWTP influents in other countries (43). At sub-MIC levels, significant limitations on *E. coli* growth and cell division (filamentous phenotype) can occur (39), suggesting that growth in WWTPs may be impacted by the presence of quinolone antibiotics. However, because *qnrS* activity usually increases the MICs by 8 to 128 times (44), the growth of strains carrying this gene in WWTPs should not be affected.

Hence, our data, in combination with data reported previously, suggest a mechanism for the loss of VGs common to both types of treatment plants, namely, the loss of PAIs that could be enhanced by low temperatures and/or the presence of quinolones in wastewater. Further studies are needed to confirm this mechanism, but if substantiated, it could point to complex interactions between competing public health policies: one aimed at protecting the public by reducing infectious disease agents and the other aimed at protection by reducing antibiotic usage.

Dominance of UPEC among the pathogenic isolates. Except for one treatment plant (PC1), ExPEC pathotypes (12 to 35%) were more abundant than IPEC pathotypes (1 to 8%) (Table 3), with UPEC isolates representing the majority of the ExPEC pathotypes. Other studies of municipal WWTPs also showed trends similar to those observed here for ExPEC and IPEC pathotypes in the influents and effluents of these plants (2, 26, 27). A recent Australian watershed study suggesting that >50% of surface water *E. coli* isolates likely originated from municipal wastewater discharges (32) prompts the question, "Are the pathotype frequencies in plant effluents observed here similar to pathotype occurrences in surface waters?" Two studies of the Great Lakes also found a predominance of ExPEC pathotypes with low levels of IPEC (20, 21). PCR and colony hybridization-based studies also found a higher proportion of isolates carrying ExPEC VGs than isolates carrying IPEC VGs in both freshwater (10, 45) and coastal waters (9, 46). Taken together, WWTPs and surface water studies suggest that concentrations of ExPEC pathotypes and VGs are higher in the environment than those of IPEC.

Are these observations an artifact of *E. coli* isolation, or are they the result of the ecophysiology of ExPEC and IPEC pathotypes? In the current work and in most of the cited studies (except references 45 and 46), *E. coli* strains were isolated at 44.5°C on media containing bile salts as a selective agent. Although early studies of *E. coli* O157:H7 isolates suggested that isolates of this important verotoxigenic EHEC serotype could not grow at 44.5°C (47, 48), more recent studies of multiple verotoxic strains (including O157 and non-O157 strains) showed that growth at this temperature was commonly achieved in nonselective media (49–51). Hence, the combination of bile salts and a temperature of 44.5°C may inhibit growth. Furthermore, the reported proportion of isolates in collections capable of growing under these conditions varied between 33% and 75% (50–52). From these data, it would appear that the magnitude of the IPEC recovery bias (maximum 33% recovery) does not explain the average gap in the abundances of IPEC and ExPEC isolates at the AS1, AS2, and PC2 plants (25 IPEC isolates versus 135 ExPEC isolates). Finally, the results obtained at the PC1 plant show that the protocol can recover high levels of STEC isolates. Therefore, our data suggest that ExPEC strains are naturally much more abundant than IPEC strains in municipal wastewater, while other studies suggested the same for surface waters.

The predominance of ExPEC in municipal wastewaters may be due to differences in the ecophysiologies of IPEC and ExPEC pathotypes in the human intestine, presumably the origin of most of the *E. coli* isolates from municipal wastewaters. While IPEC will cause intestinal diseases for which the human body has developed immune defenses, ExPEC would be considered a commensal organism by the gastrointestinal immune system and would be allowed to grow normally, as would any other avirulent *E. coli* isolate. Furthermore, an increasing body of literature suggests that ExPEC VGs are factors that promote intestinal colonization. Studies that compared the levels of ExPEC and IPEC VGs in isolates recovered from stool samples of healthy humans found >31% and <5% of these isolates to carry ExPEC and IPEC VGs, respectively (10, 53). Studies of specific clones showed that dominant or resident clones have more ExPEC VGs than minor or transient ones (54–57). Finally, a study using *E. coli* strain 536 (UPEC) in which the seven PAIs had been deleted showed that the wild-type strain had a marked advantage for intestinal colonization in a mouse model, while the PAI-deleted mutant had a competitive advantage in laboratory batch cultures (58). Together, these data support the hypotheses that ExPEC pathogenicity is a by-product of intestinal colonization mechanisms (59) and that VGs are present at relatively high frequencies in commensal *E. coli* flora inhabiting the human intestine, thus explaining the differences in frequencies between ExPEC and IPEC strains and VGs observed in this study.

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