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Culture-Independent Analysis of Fecal Enterobacteria in Environmental Samples by Single-Cell mRNA Profiling

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A culture-independent method called mRNA profiling has been developed for the analysis of fecal enterobacteria and their physiological status in environmental samples. This taxon-specific approach determines the single-cell content of selected gene transcripts whose abundance is either directly or inversely proportional to growth state. Fluorescence in situ hybridization using fluorochrome-labeled oligonucleotide probes was used to measure the cellular concentration of *fis* and *dps* mRNA. Relative levels of these transcripts provided a measure of cell growth state and the ability to enumerate fecal enterobacterial cell number. Orthologs were cloned by inverse PCR from several major enterobacterial genera, and probes specific for fecal enterobacteria were designed using multiple DNA sequence alignments. Probe specificity was determined experimentally using pure and mixed cultures of the major enterobacterial genera as well as secondary treated wastewater samples seeded with pure culture inocula. Analysis of the fecal enterobacterial community resident in unseeded secondary treated wastewater detected fluctuations in transcript abundance that were commensurate with incubation time and nutrient availability and demonstrated the utility of the method using environmental samples. mRNA profiling provides a new strategy to improve wastewater disinfection efficiency by accelerating water quality analysis.

Fecal enterobacteria inhabit the human gastrointestinal tract and exhibit key biochemical properties distinguishing them from other members of the *Enterobacteriaceae*. Members of the genus *Escherichia*, typically *Escherichia coli*, are the major representatives of fecal enterobacteria. Efficacious disinfection and release of secondary treated municipal wastewater relies on culture-based methods that depend on the use of selective enrichment conditions to quantitate fecal enterobacterial content. Due to the volume of treated water and the time required to conduct standard tests, water release can precede the outcome of test results and treatment interventions must be applied retroactively. Despite this limitation, temporal averaging of test results enables domestic wastewater treatment plants to meet regulatory requirements. Direct assessment of fecal bacterial content in wastewater by culture-independent methods would reduce the time required to assess water quality and enable mechanistic studies to be conducted on wastewater disinfection.

Fluorescent in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes is a powerful tool for cultivation-independent analysis of the structure and dynamics of complex microbial communities (14). While extremely useful for taxonomic identification, rRNA-based techniques rarely provide information about the in situ behavior of bacteria within their respective ecosystems. Since cellular rRNA content ranges only severalfold over the extremes of bacterial growth rate, its utility for physiologic investigation is inherently limited (14, 17, 19). In addition, nongrowing stationary-phase bacterial cells in environmental samples can be difficult or impossible to detect using 16S rRNA probes because cellular rRNA content can become depleted and fall below the limit of detection (2, 19, 20). The application of techniques that allow in situ visualization of other targets such as mRNAs whose abundance varies in response to environmental perturbation and that reach levels sufficient for detection could overcome this limitation and permit studies on in situ physiological activities. While this has been recognized as a desirable goal, the low concentration and short half-life of most bacterial mRNAs contribute to the paucity of reports on the in situ detection of mRNA (7, 27). Methods developed to overcome these problems include hybridization with digoxigenin-labeled synthetic antisense transcripts (7, 27), in situ PCR (8), and in situ reverse transcription PCR (26); however, these methods have not seen widespread acceptance because they are relatively complicated to perform, particularly with environmental samples (24).

Growth state-regulated proteins and their transcripts provide an alternative target for the investigation of bacterial physiologic status in situ. Fis and Dps have been used previously for studies on enterobacteria (18, 23). Fis is an 11-kDa DNA binding protein (9, 10) that plays a critical role in coordinating rRNA synthesis with growth (15). Levels of Fis correlate directly with growth rate (3, 4). Dps is a 19-kDa DNA binding protein that is important in stationary-phase stress physiology (1, 11). Dps abundance is inversely correlated with growth rate and it ranges over 100-fold in cellular concentration between the extremes of stationary phase and rapid growth (16, 22). The occurrence, phylogenetic conservation, and regulation of synthesis of both of these proteins among the major enterobacterial genera have been described previously (23).

Recent efforts using a method called protein profiling (23) investigated several relationships between enterobacterial community physiology and wastewater processing at the single-

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cell level (18). The intracellular concentrations of the growth-related protein Fis and the stationary-phase protein Dps were analyzed by epifluorescence microscopy in uncultivated cells by the use of enterobacterial group-specific polyclonal fluorochrome-coupled antibodies. Enterobacterial single-cell community protein profiles were found to be distinct for different types of biological treatment. Trickling filter wastewater harbored enterobacterial communities containing levels of Fis that were elevated relative to those present in submerged aeration basin wastewater. Pure culture studies demonstrated that Fis/Dps ratios were directly proportional to disinfection efficiency, and culture-based disinfection measurements of replicate wastewater samples corroborated these findings. Together, these data demonstrated that wastewater processing can strongly influence disinfection efficiency by perturbing enterobacterial community physiology. A key drawback of the protein-profiling method, however, is the inability of the method to distinguish among individual enterobacterial taxa. Taxonomic differentiation is of particular importance in studies of wastewater enterobacterial communities in which the fecal enterobacterial subgroup is of most concern. To remedy this limitation, efforts to measure the levels of the *fis* and *dps* mRNAs in uncultivated single cells by the use of taxon-specific probes were undertaken.

MATERIALS AND METHODS

Bacterial strains and cultivation. The *E. coli* K12 strains used were PBL500 strains, as described previously (21). Strains PBL931 (Δ*fis*::Tn*10*) and PBL664 (Δ*dps*) were constructed as described previously (23). PBL931 was a *fis* mutant strain constructed by homologous recombination by the use of a phage-encoded mutant allele of *fis* as described previously (23). Recombinants devoid of integrated phage were verified by PCR after successive cycles of purification of single colonies on Luria-Bertani (LB) agar plates containing 0.5% deoxycholic acid and then on LB agar plates containing tetracycline (25 μg/ml). Cell densities in the culture media and wastewater were monitored spectrophotometrically using a model 50 Cary spectrophotometer at a wavelength of 600 nm. The media used were LB (12), A1 (Difco), and Endo (BBL) agar. Most-probable numbers were determined as described previously (5). Additional bacterial strains used to examine the specificity of mRNA probing were subcultured from overnight cultures at a ratio of 1:100 in LB medium, and samples were removed thereafter for analysis.

Source and treatment of wastewater samples. Wastewater samples were collected from a municipal treatment plant serving a population of 200,000. The wastewater in this facility is treated primarily with an activated-sludge process. Secondary treated wastewater samples were taken from the effluent of the final clarifiers. To examine fecal enterobacterial physiology following prolonged incubation or nutrient amendment, 1-liter volumes of secondary treated wastewater were shaken at 200 rpm and 25°C on a G-33 shaker (New Brunswick, Edison, N.J.). Nutrient supplementation was accomplished by the addition of tryptone (0.01% [wt/vol]). For mRNA profiling of wastewater samples, samples obtained from the wastewater treatment plant were treated immediately with paraformaldehyde and then transported to the laboratory. Grab samples for probe specificity reconstruction experiments consisted of 90-ml volumes collected in 125-ml sterilized bottles containing 10 ml of 40% (wt/vol) paraformaldehyde. A total of 10^8 *E. coli* cells in either exponential phase or stationary phase were added to these samples to achieve a ratio of wastewater cells to *E. coli* of 1:1. Culturable fecal enterobacterial content of these samples was 10^6 CFU/ml and was determined using replicate unfixed samples that were chilled and transported to the laboratory for analysis.

Isolation and characterization of *fis and dps* enterobacterial orthologs. Enterobacterial genomic DNA was recovered using a DNeasy tissue kit (QIAGEN). The *fis* and *dps* genes were acquired by PCR using primer pair 5′-TATACCAT GGTCGAACAACGCGTAAAT TCTGAC-3′ and 5′-TATACTCGAGGTTCA TGCCGAATTTTTTCAAT-3′ for *fis* and primer pair 5′-TACCGCTAAATTA GTTAAATCAAAAGC-3′ and 5′-CGATGTTAGACTCGATAAACCACAGG A-3′ for *dps*. PCR employed a program of 95°C for 1 min, 55°C for 2 min, and 75°C for 3 min for 30 cycles and final extension at 75°C for 5 min. The PCR products were sequenced using a Beckman/Coulter CEQ2000XL eight-capillary DNA sequencer and dye-terminator chemistry.

TABLE 1. Oligonucleotide probe sequences

Probe	Sequence
*fis*1	5′-Fluo-GTTCTACTTCAGCCAGTACCAGCTC-3′[a]
*fis*2	5′-Fluo-TTACCTGATCCTGAGAGTTAACGGTAG-3′
*fis*3	5′-Fluo-CACGGTTGATGCCCATCATCAGCGCAG-3′
*fis*4	5′-Fluo-GTTCTTCAGTGCCTGTTTAACCGAGTC-3′
*fis*5	5′-Fluo-ATTCACATCCTGACCATTCAGTTGAGC-3′
dps	5′-Cy3-GCCATGGTATCCAGATGATCGAT-3′
ENT	5′-Cascade Blue-CATGAATCACAAAGTGGTAAGCGCC-3′

[a] Fluo, fluorescein label.

Parts of *Proteus vulgaris* and *Serratia marcescens dps* genes were acquired by PCR using degenerate PCR primer pair 5′-CTIATIACIAARCAIGCICAYTG GAA-3′ and 5′-TCIATRAACAAIAGRAAYTTRTC-3′. In this case, PCR employed a program of 95°C for 1 min, 45°C for 1 min, and 75°C for 1 min for 50 cycles and final extension at 75°C for 5 min. Primers were used at 100 pmol/μl with 2.2 mM magnesium chloride. The degenerate PCR products were cloned into plasmid pCR4-TOPO by the use of a TOPO TA cloning kit (Invitrogen). Inserts were sequenced using phage M13 forward and reverse primers. Inverse PCR was used to obtain the remainder of the *P. vulgaris* and *S. marcescens dps* gene sequences. BamHI and BsrGI were used to digest *P. vulgaris* and *S. marcescens* genomic DNA, respectively, and digested genomic DNAs were ligated using phage T4 DNA ligase. The ligated genomic DNA was then used as a template for first-round PCR using primers 5′-TCACAGTGTACAAGATCAT TT-3′ and 5′-AATAGCAACGCCTCCCAATTG-3′ for *P. vulgaris* and primers 5′-CCACAGCGTGCAAGAGCACCT-3′ and 5′-ATCGTTCACCACCTGTAC GGT-3′ for *S. marcescens*. In this case PCR employed a program of 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min for 30 cycles, with a final extension at 72°C for 10 min. First-round PCR products were purified using a PCR purification kit (QIAGEN). The purified PCR products were then used as templates for second-round PCR using primers 5′-GCGGTCGTCGCGAATGATATT-3′ and 5′-TAAGTGTTCAACTAGCGTGGT-3′ for *P. vulgaris* and primers 5′-CCGA AGTGGAAGACGAAGACA-3′ and 5′-AAGGTGTCCTGATGCTCGAT-3′ for *S. marcescens*.

Oligodeoxynucleotide probes. Probes used in this study are shown in Table 1. The *fis* probes were purchased prelabeled with fluorescein (Invitrogen). The *dps* probe was purchased prelabeled with Cy3 (IDT). The 16S rRNA probe specific for members of the *Enterobacteriaceae* was 5′-CATGAATCACAAAGTGGTA AGCGCC-3′ (13) and was purchased prelabeled with Cascade Blue (Invitrogen).

Microscopy sample preparation and probing procedures. Cell fixation and gelatin-subbed slide preparation were performed as described previously (23). Fixed cells were applied to 0.1% (wt/vol) gelatin–0.01% (wt/vol) $CrK(SO_4)_2$-treated slides, dried at 37°C, and then dehydrated by successive rinses in 50, 80, 98, and 100% ethanol for 3 min. Dried slides were then simultaneously treated with 50 pmol of *fis* (*fis*1 to *fis*5), *dps*, and ENT probes, respectively, suspended in 20 μl of 0.9 M NaCl–50 mM Na_2HPO_4 (pH 7.0)–0.1% (wt/vol) sodium dodecyl sulfate–0.5 mg of yeast tRNA/ml–10× Denhardt's solution, and incubated for 2 h in a humidified chamber at 46°C in the dark. Unless otherwise indicated, formamide was used in hybridization buffers at 20% (vol/vol). Slides were washed in 0.9 M NaCl–50 mM Na_2HPO_4 (pH 7.0)–0.1% (wt/vol) sodium dodecyl sulfate for 20 min at 46°C in water for 10 min at room temperature and then dried at 37°C in the dark. Fluorescent bleaching was minimized using a ProLong kit (Molecular Probe). Slides were sealed by treatment with nail polish and maintained in the dark until examined. Probe specificity was also examined using hybridization buffers containing a range of formamide concentrations (10 to 50% [vol/vol]).

Probe specificity. Probe specificity was assessed initially using pure cultures of individual enterobacterial genera. Subsequent analysis of specificity employed mixed samples of pure cultures containing *E. coli* combined at a ratio of 1:1 with a mixture of equal parts of species of six major enterobacterial genera: *Salmonella*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Serratia*, and *Proteus*. Probe specificity using secondary treated wastewater was assessed by seeding wastewater with the same mixture of enterobacterial genera. Seeded cells were prepared using exponential-phase or stationary-phase cultures as indicated in the text and were fixed with paraformaldehyde prior to use. Cells were counted from five fields of image for each sample and constituted a total of 500 cells. Approximately 100 cells were examined for each data point obtained from five fields of view for each

TABLE 2. Sequence mismatches between oligonucleotide probes and *E. coli fis* and *dps*

Species and ATCC strain name	*fis* accession no.	No. of sequence mismatches with *fis* probe					*dps* accession no.	No. of sequence mismatches with *dps* probe *dps*1
		*fis*1	*fis*2	*fis*3	*fis*4	*fis*5		
E. coli K12 MG1655	gb AE000405.1	0	0	0	0	0	gb AE000405.1	0
S. flexneri 25875	gb AY174074	0	0	0	0	0	NC_004337.1	0
S. enterica serovar Typhimurium 700720	gb AE008856.1	0	0	1	0	2	gb AE008856.1	2
K. pneumoniae	gb AF040380.1	0	1	1	0	2	gb AY195736	3
E. cloacae 11439	gb AY174075	1	1	2	0	2	gb AY195735	7
C. freundii 33128	gb AY191361	0	1	1	0	3	gb AY191364	5
P. vulgaris 13315	gb AY191362	3	6	5	4	5	gb AY191365	10
S. marcescens 13880	gb AY191363	1	2	3	1	4	gb AY191366	5

sample prepared for microscopy. Data on the fecal enterobacterial growth state composition of secondary treated wastewater were analyzed statistically to estimate how representative each field of view was.

Micrograph analysis. Measurements of fluorescent intensity were performed as described previously (18, 23). The levels of fluorescent emission from fluorescein-, Cy3-, and Cascade Blue-labeled oligodeoxynucleotide probes were detected using a Microphot epifluorescence microscope (AX70; Olympus Co.), a charge-coupled device camera (DE1-750 Optronic), and Olympus AX-RFSL2 filter sets for fluorescein, Cy3, and Cascade Blue. Images were captured using Optronic image capture software. Cells were counted, and fluorescence was quantitated using Image-1 image analysis software on a single-cell basis. mRNA levels were determined by measuring the fluorescent intensity of the corresponding fluorochrome-labeled oligodeoxynucleotide probes.

Phylogenetic analysis. Phylogenetic distance analysis of *fis* or *dps* sequences from the major enterobacterial genera was performed using PHYLIP 3.37c software (6). Nearly full-length sequences including 297 nt for *fis* orthologs and 504 nt for *dps* orthologs were employed for sequence alignments. *fis* and *dps* were aligned using CLUSTAL W software (25) in Wisconsin package version 10.2 (Genetics Computer Group). Sequences from the following strains were used to construct the tree: *E. coli* K12 MG1655, *Shigella flexneri* 2a.str.301(ATCC 25875), *Salmonella enterica* serovar Typhimurium LT2, *Klebsiella pneumoniae* (ATCC33495), *Enterobacter cloacae* (ATCC 11439), *Citrobacter freundii* (ATCC 33128), *P. vulgaris* (ATCC13315), and *S. marcescens* (ATCC 13880). SEQBOOT was used to generate 100 bootstrapped data sets. Distance matrices were calculated with DNADIST using the default options. A total of 100 unrooted trees were inferred by neighbor-joining analysis of the distance matrix data by using NEIGHBOR software. Bias introduced by the order of sequence addition was minimized by randomizing the input order. The most-frequent branching order was determined with CONSENSE software.

Nucleotide sequence accession numbers. All new sequences for enterobacterial *fis* and *dps* genes have been deposited in GenBank, and accession numbers are indicated in Table 2.

RESULTS

Design, target specificity, and sensitivity of oligodeoxynucleotide probes. The *fis* and *dps* gene probes were designed in an attempt to create molecules that would specifically target *E. coli* and its closest relatives. The DNA sequences of the *fis* and *dps* genes were obtained from public databases and by cloning and sequencing the genes from selected strains. In several cases, *P. vulgaris* and *S. marcescens* sequence information required cloning the genes by the use of a combination of PCR with degenerate primers and inverse PCR to obtain flanking sequences. Multiple sequence alignments were then prepared, and the regions that were the most distinct between *E. coli* and other enterobacterial genera were selected for probe design (Table 1). The potential mismatches between these regions and the probes for the various organisms are shown in Table 2.

The concentration of *fis* mRNA present in exponentially growing *E. coli* cells was below the limit of detection when using a single *fis* probe after normalization for nonspecific hybridization. Instead, five *fis* oligodeoxynucleotide probes, all similarly labeled, were required for quantitative detection of *E. coli* cells in exponential phase. Even combinations of three of the five probes were insufficient for cell detection. In contrast, *dps* mRNA in stationary-phase cells was present in greater abundance and could be detected using a single *dps* probe.

The target specificity of mRNA detection by FISH was examined initially using physiologic and genetic controls (Fig. 1). *fis* mRNA was evident only in exponentially growing cells with a wild-type allele of *fis* and not in stationary-phase cells or in exponentially growing cells with a disrupted allele of *fis* (Fig. 1A to F). Dps mRNA was evident only in stationary-phase cells with a wild-type allele of *dps* and not in exponentially growing cells or stationary-phase cells with a disrupted allele of *dps* (Fig. 1G to L).

The role of hybridization stringency was examined for *fis* probes, the *dps* probe, and a 16S rRNA probe specific for *Enterobacteriaceae* species called ENT (13). FISH was conducted on *E. coli* cells harvested from exponential-phase or stationary-phase cultures by the use of a range of formamide concentrations from 0 to 50% in 10% increments, and the amount of fluorescence was determined (Fig. 2). The levels of fluorescence were highest under the conditions in which the *fis* and *dps* genes are expressed; however, background (nonspecific) fluorescence was also apparent. To distinguish between specific and nonspecific fluorescence as a function of increasing hybridization stringency, fluorescence values were normalized for levels produced when the target transcript was largely absent. For both the *fis* and *dps* mRNA, the normalized values demonstrated that fluorescence emission was quenched as formamide concentrations increased. In contrast, fluorescence emission for the ENT probe (adjusted for growth state differences) was relatively insensitive to increasing formamide concentrations. Formamide concentrations ranging from 10 to 20% (vol/vol) depending on the sample and the extent of signal quenching were employed for subsequent experiments using the *fis* and *dps* probes.

Probes for *fis*, *dps*, and 16S rRNA could be used simultaneously to evaluate mRNA content and taxon identity (Fig. 3). *E. coli* cells from exponential-phase or stationary-phase cultures were probed with all the probes, and fluorescence was examined at selected wavelengths. No interference, bleeding, or quenching was apparent, indicating that the three fluorochromes constituted a suitable set for mRNA analysis.

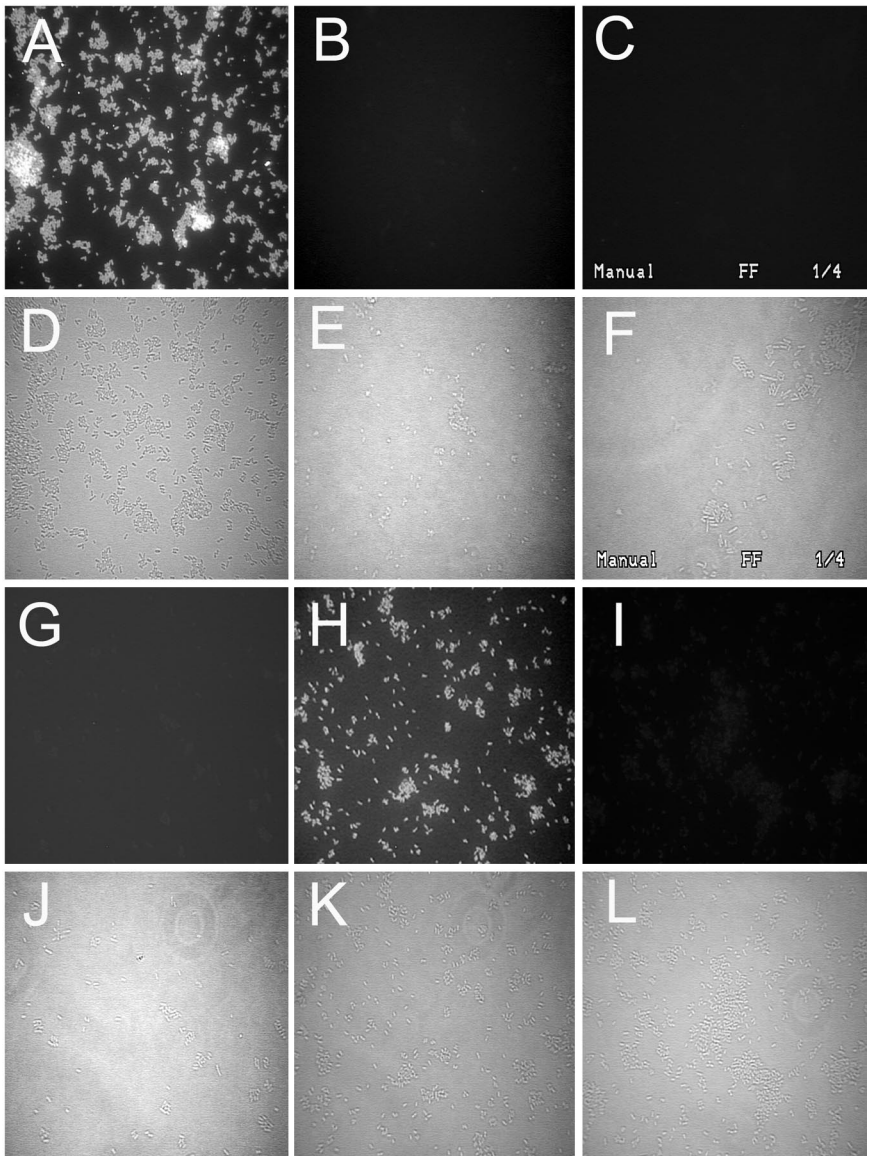

FIG. 1. Detection of *fis* and *dps* mRNA in *E. coli* by FISH. The results of detection of *fis* (A to F) and *dps* (G to L) are shown. (A to C) Fluorescent micrographs of cells probed with fluorescein-labeled oligonucleotide probes complementary to *fis* mRNA. Wild-type cells in early exponential phase (A), wild-type cells in stationary phase (B), and PBL931 cells (lacking *fis*) in exponential phase (C) are shown. (D to F) Bright-field images matching those shown in panels A to C. (G to L) Fluorescent micrographs of cells probed with a Cy3-labeled oligonucleotide probe complementary to *dps* mRNA. Wild-type cells in exponential phase (G), wild-type cells in stationary phase (H), and PBL646 cells (lacking *dps*) in stationary phase (I) are shown. (J to L) Bright-field images matching those shown in panels G to I.

mRNA profiling during a batch culture growth cycle. The concentrations of Fis and Dps protein and *fis* mRNA are dynamic and have been shown by both Western and Northern blot analysis to change in accordance with alterations in growth state (1, 3, 4). The relationship between these trends and the pattern of fluorescence produced by single cells individually probed for *fis* and *dps* mRNA was therefore examined (Fig. 4). Fluorescence with the *fis* probes reached maximal levels near the midpoint of the exponential phase of growth. Thereafter fluorescence decreased, reaching undetectable levels at the onset of stationary phase. Fluorescence with the *dps* probe remained nearly undetectable during exponential growth but increased rapidly to reach peak values after the onset of stationary phase. At times thereafter, fluorescence with the *dps* probe decreased to about 50% of peak values and remained at this level for an additional 8 h in the stationary phase before declining further. Cells exhibiting simultaneous fluorescence with *fis* and *dps* probes were not apparent. For both the *fis* probes and the *dps* probe, the total variation in fluorescence ranged over a minimum of 10-fold at the extremes of growth state. The variation observed between individual cell levels at single time points was typically less than 15%. These findings parallel those from previous studies conducted at the single-cell level (23) and using bulk methods of analysis (3, 4, 16, 22, 23) and indicate that fluorescence is directly proportional to mRNA concentration.

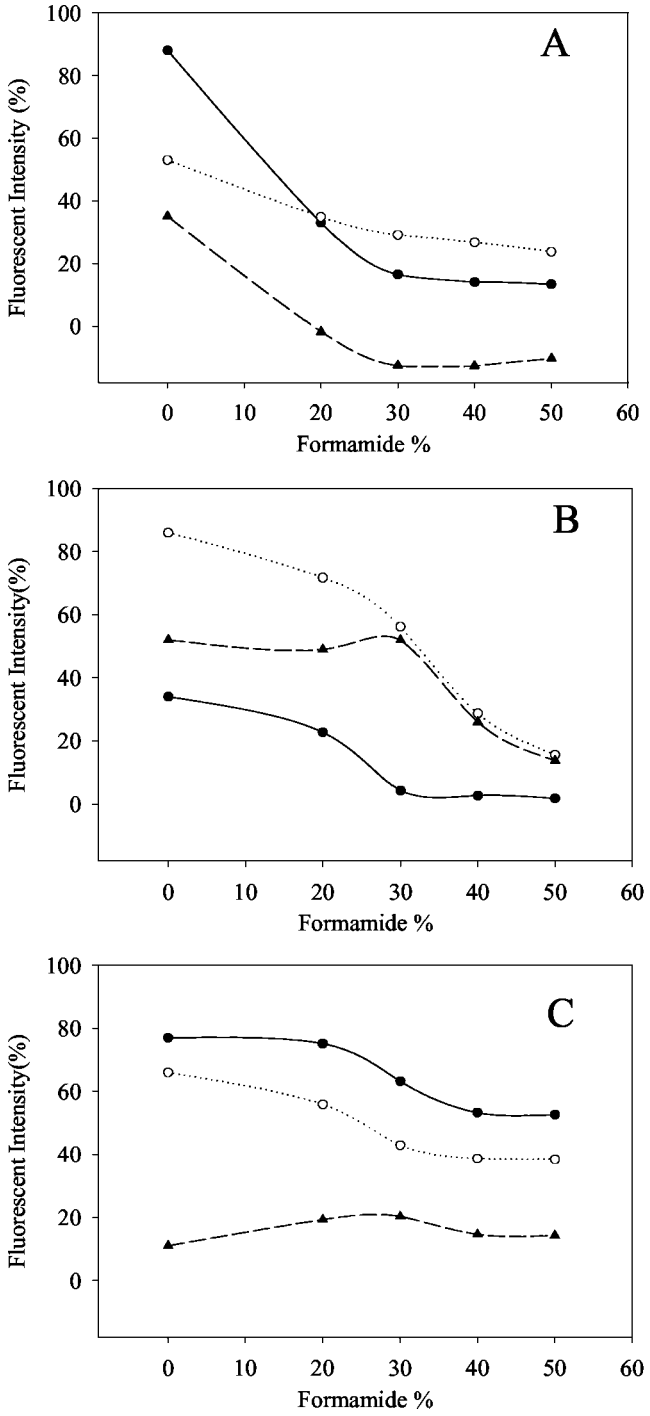

FIG. 2. Influence of hybridization stringency on fluorescence emission. *E. coli* cells in either exponential phase (closed circles) or stationary phase (open circles) were probed separately with the *fis* probes, the *dps* probe, or the 16S rRNA ENT probe. The differences in the levels of fluorescence between the two growth states are indicated (closed triangles). Fluorescence emission was examined at a range of formamide concentrations. (A) *fis*-probed cells; (B) *dps*-probed cells; (C) 16S rRNA ENT-probed cells.

Phylogenetic specificity of the mRNA probes. To investigate the utility of the *fis* and *dps* probes for analysis of enterobacterial community samples, FISH studies were conducted, first using pure cultures of the major genera and subsequently using mixtures of pure cultures. *K. pneumoniae*, *E. cloacae*, *C. freundii*, *P. vulgaris*, *S. enterica* serovar Typhimurium, and *S. marcescens* cells were examined separately using *fis* probes and the *dps* probe (Table 3). Samples of *Shigella* were not included, as this organism encodes *fis* and *dps* orthologs with sequences identical to those of *E. coli*. Faint fluorescence was observed for *S. enterica* serovar Typhimurium in exponential phase but not in stationary phase. No fluorescence was observed for any other strains under either set of growth conditions. These results are consistent with the degree of complementarity between the oligonucleotide probe sequences and the taxon-specific *fis* and *dps* gene sequences (Table 2). Probe specificity was investigated further by determining the numbers of fluorescent cells in mixed samples of pure cultures containing *E. coli* combined at a ratio of 1:1 with a mixture of equal parts of the other major enterobacterial genera. The mean values for the number of fluorescent cells detected using either the *fis* probes (47.05 ± 8.54) or the *dps* probe (48.23 ± 9.85) agreed closely with the number of *E. coli* cells that had been added to the samples (data not shown).

The utility of the *fis* probes or the *dps* probe for the detection of fecal enterobacteria in environmental samples requires that they function in a taxon-specific manner. Since the *Enterobacteriaceae* comprise over 50 genera and a correspondingly larger number of species, an exhaustive experimental analysis to establish taxonomic specificity was not pursued. As an alternative, phylogenetic comparisons of enterobacterial *fis* and *dps* orthologs from the major genera were conducted to establish the evolutionary relationships between these genes among members of the *Enterobacteriaceae* (Fig. 5). In both cases, *fis* and *dps* ortholog distribution followed the pattern of taxonomic relationships generally accepted for these enterobacterial genera; *E. coli* and *Shigella* orthologs were tightly clustered, while *Proteus* orthologs were the most distant. Comparisons of the evolutionary distances between selected sequences indicated that both enterobacterial genes are significantly more divergent among this group of organisms than their corresponding 16S rRNA sequences. The maximum divergence value relative to *E. coli* for *fis* was 13.1% (330 nucleotides [nt]), for *dps* was 29.9% (504 nt), and for 16S rRNA was 7% (1,400 nt).

mRNA profiling of secondary treated wastewater. The mRNA profiling procedure was then used to examine fecal enterobacteria in environmental samples consisting of secondary treated municipal wastewater. Fixed cell samples were simultaneously probed with the *fis*, *dps*, and 16S rRNA probes and examined at individual wavelengths and under bright-field conditions. Since the incidence of enterobacteria within the wastewater microbial community is low (approximately 10%), different fields of view within single samples were examined to assess the frequency of cells detected by either type of probe. In these samples, the *fis* probes detected 29.37% (± 4.96%) of the cells detected by the 16S rRNA ENT probe whereas the *dps* probe detected 2.75% (± 2.52%) of those detected by the 16S rRNA ENT probe. All cells detected by *fis* and *dps* probes were also detected using the 16S rRNA ENT probe.

To better understand the specificity of these probes in waste-

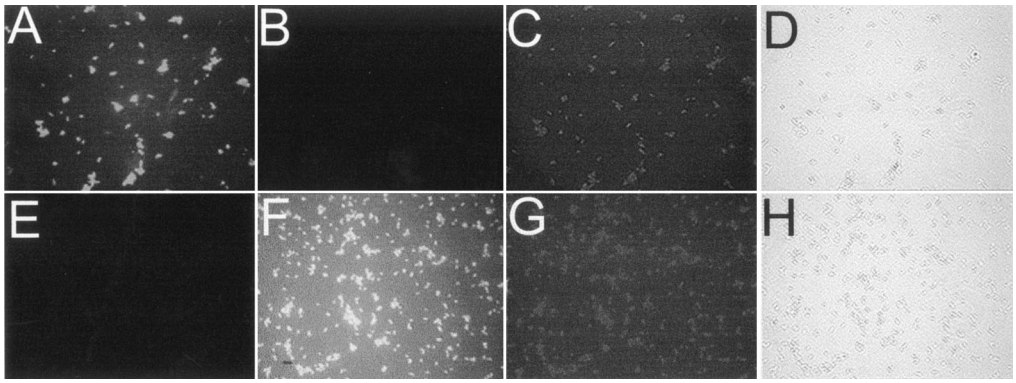

FIG. 3. Simultaneous probing of exponential- and stationary-phase *E. coli* with *fis*, *dps*, and 16S rRNA ENT probes. Fluorescent images of a single field of cells in exponential phase (A to D) or stationary phase (E to H) were captured and examined at three wavelengths and under bright-field illumination. (A and E) *fis* probes; (B and F) *dps* probe; (C and G) 16S rRNA ENT probe; (D and H) matching phase-contrast bright-field images.

water samples, known numbers of *E. coli* in either exponential phase or stationary phase were seeded into wastewater samples obtained from secondary clarifiers prior to sample analysis. Relative to the number of cells detected using the 16S rRNA probe (ENT), the *fis* probes detected 62.22% (± 11.58%) whereas the *dps* probe detected 51.24% (± 7.29%).

To determine whether the pattern of fluorescence detected in probed samples accurately reflected fecal enterobacterial growth state, the effect of prolonged incubation with and without nutrient supplementation was examined. Secondary treated wastewater samples were chilled, transported to the laboratory, and allowed to incubate at ambient temperatures with and without addition of tryptone (0.01% [wt/vol]). Samples were removed periodically and probed with the *fis*, *dps*, and 16S rRNA probes, and the percentages of cells detected by the *fis* or *dps* probes as a fraction of those detected by the 16S rRNA probe were determined (Fig. 6). Initial values indicated that 30.82% ± 11.48% of the cells detected by the 16S rRNA probe were also detected by the *fis* probes whereas only 11.48% ± 6.91% were detected by the *dps* probe. After 10 h of additional incubation and entry of the culture into stationary phase, as indicated by plateauing of cell density measurements, the fraction of cells detected by the 16S rRNA and *fis* probes dropped to 4.49% ± 6.67% whereas that of cells detected by the *dps* probe rose to 37.40% ± 6.67% (Fig. 6A). In cultures that were nutrient supplemented, however, the pattern was reversed and the fraction of cells detected by both the 16S rRNA and *fis* probes increased with continued incubation whereas that detected by the *dps* probe decreased (Fig. 6B). These results demonstrate that the mRNA profiling method

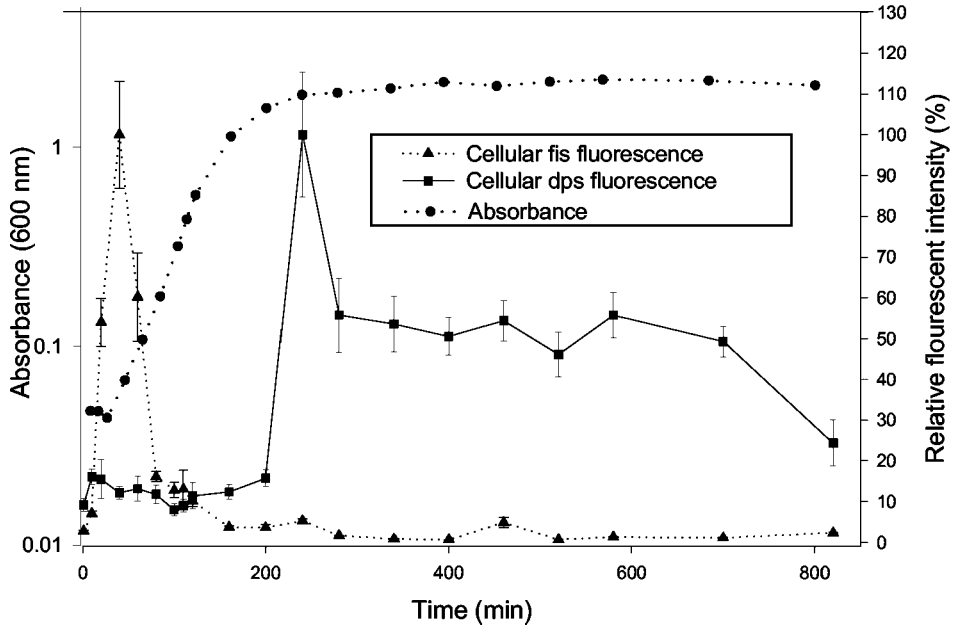

FIG. 4. Quantitation using FISH of *fis* and *dps* mRNA in *E. coli* during the growth cycle. Symbols: closed circles, cell density (A_{600}); closed squares, relative fluorescence of *dps*; closed triangles, relative fluorescence of *fis* mRNA. Error bars indicate the variations in relative fluorescence emission observed between replicate samples.

TABLE 3. Relative fluorescent intensities of cells probed with *fis* and *dps* probes

Species	Fluorescent intensity in indicated phase[a]			
	Exponential		Stationary	
	fis	*dps*	*fis*	*dps*
E. coli	+	−	−	+
S. enterica serovar Typhimurium	±	−	−	−
K. pneumoniae	−	−	−	−
E. cloacae	−	−	−	−
C. freundii	−	−	−	−
P. vulgaris	−	−	−	−
S. marcescens	−	−	−	−

[a] +, strong fluorescence; ±, faint fluorescence; −, no fluorescence.

could detect physiological changes in the endogenous fecal enterobacterial community in secondary wastewater.

DISCUSSION

Single-cell detection of *fis* and *dps* mRNA levels with complementary fluorochrome-labeled oligonucleotide probes was used to measure the physiological growth status of uncultivated fecal enterobacterial cells in secondary treated municipal wastewater. Cells in unamended secondary treated wastewater samples occurred in both growing and nongrowing states, and a subset of these responded to the addition of nutrients. The successful application of this method relies in part on the natural abundance of the target mRNAs, which can approximate levels achieved by 16S rRNA. For this reason, signal amplification using coupled enzymes or other strategies was unnecessary for detection of single-cell fluorescence. This method can therefore be used to determine the identity and the growth state of fecal enterobacteria during secondary wastewater disinfection (23).

The mRNA profiling method was effective in detecting fecal enterobacterial cells despite the overwhelming dominance of other taxa in water samples. Consequently the new method represents an improvement over previous culture-independent strategies that were inherently limited by the use of group-specific enterobacterial fluorochrome-coupled antibodies (18, 23). As the samples employed in this study underwent filtration to remove macroscopic particles, the remaining microbial communities were necessarily planktonic and particle-associated organisms were not examined. The availability of small nucleic acid-based probes, however, may enable studies to address the composition and physiologic status of particle-associated fecal enterobacteria in wastewater samples.

Not all cells detected in secondary wastewater with the enterobacterial 16S rRNA probe were detected using either the *fis* or *dps* probes; approximately 50% remained unaccounted.

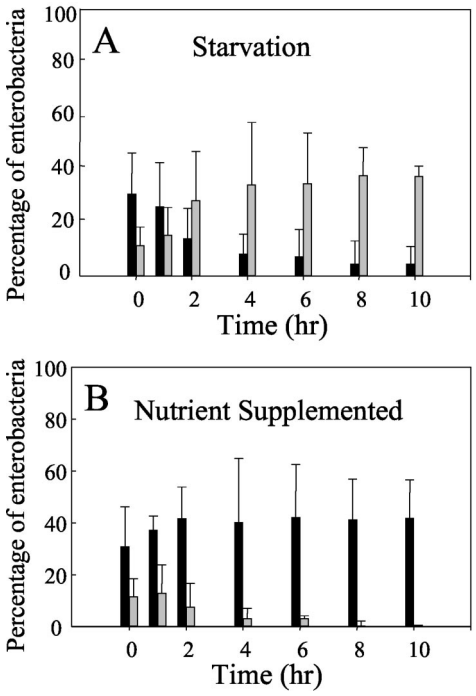

FIG. 6. Quantitative mRNA profiling of secondary treated wastewater. Quantitation of numbers of cells with detectable levels of *fis* or *dps* mRNA as percentages of the total number of cells with detectable levels of 16S rRNA is shown. (A) Unsupplemented secondary treated wastewater; (B) tryptone-supplemented secondary treated wastewater. Cells detected with the *fis* probes (black bars) and cells detected with the *dps* probe (grey bars) are shown.

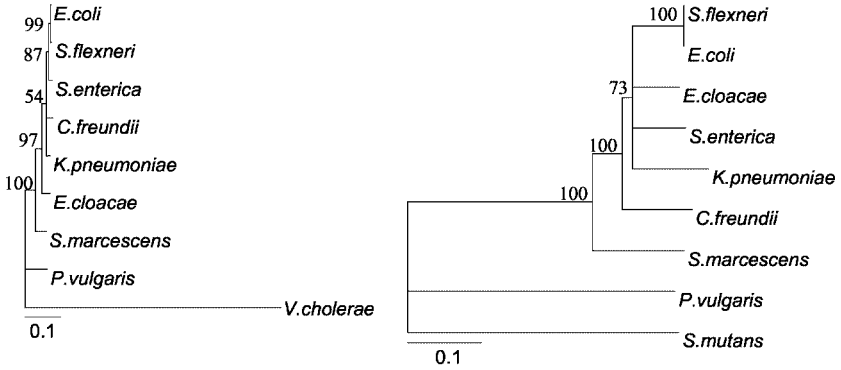

FIG. 5. Phylogenetic trees of enterobacterial *fis* and *dps* orthologs. (Left) Distance tree of *fis*. The outgroup was *V. cholerae*. (Right) Distance tree of *dps*. The outgroup was *S. mutans*.

Reconstruction experiments using either pure cultures of individual taxa, mixed cultures of individual taxa, or seeded environmental samples verified that the oligonucleotide probes are specific for fecal enterobacteria. Therefore, the remaining 50% of cells in unseeded wastewater that were detected by the enterobacterial group-specific probe are most likely to have been nonfecal enterobacteria. Note that culture-based methods used to examine wastewater taxonomic composition provide lower values for *E. coli* in such water samples. Perhaps this difference is associated with the use of specific enrichment methods that inhibit the growth of fecal enterobacteria present in an injured or dormant state (23). Despite these methodologic differences, it is likely that the values obtained by FISH are representative of unmanipulated communities; however, the absolute abundance of fecal enterobacteria detected using mRNA profiling can only be determined with techniques that ensure the complete recovery of cells during sample analysis.

The magnitude of divergence observed for the *fis* and *dps* genes of the major enterobacterial genera suggests that other members of the *Enterobacteriaceae* unexamined in this study are likely to harbor *fis* and *dps* orthologs sufficiently different to be distinguished during FISH analysis. Thus, the data presented here suggest that *fis* and *dps* constitute useful taxonomic indicators for distinguishing members of the *Enterobacteriaceae* by FISH analysis and could be used to derive information about their physiologic status in environmental samples.

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