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Extracellular DNA in Single- and Multiple-Species Unsaturated Biofilms

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The extracellular polymeric substances (EPS) of bacterial biofilms form a hydrated barrier between cells and their external environment. Better characterization of EPS could be useful in understanding biofilm physiology. The EPS are chemically complex, changing with both bacterial strain and culture conditions. Previously, we reported that *Pseudomonas aeruginosa* unsaturated biofilm EPS contains large amounts of extracellular DNA (eDNA) (R. E. Steinberger, A. R. Allen, H. G. Hansma, and P. A. Holden, Microb. Ecol. 43:416–423, 2002). Here, we investigated the compositional similarity of eDNA to cellular DNA, the relative quantity of eDNA, and the terminal restriction fragment length polymorphism (TRFLP) community profile of eDNA in multiple-species biofilms. By randomly amplified polymorphic DNA analysis, cellular DNA and eDNA appear identical for *P. aeruginosa* biofilms. Significantly more eDNA was produced in *P. aeruginosa* and *Pseudomonas putida* biofilms than in *Rhodococcus erythropolis* or *Variovorax paradoxus* biofilms. While the amount of eDNA in dual-species biofilms was of the same order of magnitude as that of of single-species biofilms, the amounts were not predictable from single-strain measurements. By the Shannon diversity index and principle components analysis of TRFLP profiles generated from 16S rRNA genes, eDNA of four-species biofilms differed significantly from either cellular or total DNA of the same biofilm. However, total DNA- and cellular DNA-based TRFLP analyses of this biofilm community yielded identical results. We conclude that extracellular DNA production in unsaturated biofilms is species dependent and that the phylogenetic information contained in this DNA pool is quantifiable and distinct from either total or cellular DNA.

Biofilm extracellular polymeric substances (EPS) are a complex mixture of hydrated polymers that serve various purposes (59), including nutrient (62) and water (46) retention and protection from toxins such as antibiotics (52, 53) and pollutants (61). EPS is often described as polysaccharide, although the model organism in biofilm studies, *Pseudomonas aeruginosa*, has long been known to excrete large amounts of DNA (19). Extracellular DNA (eDNA) has previously been shown to be essential for saturated biofilm stability during the early stages of biofilm growth (58). We recently reported that extracellular DNA was continuously present in unsaturated *Pseudomonas aeruginosa* biofilms (51) and was maximally 50% more abundant than cellular DNA. Since unsaturated biofilms are not subjected to hydrodynamic shear, the structural roles for eDNA in this context are questionable. The eDNA may also enhance gene transfer (36) and provide nutrition during oligotrophic conditions (14). However, little is yet known about the universality, composition, and persistence of eDNA, particularly in natural and multiple-species environments, so little more than speculation about its purpose is possible at the moment. These concerns should be addressed to improve our understanding of biofilm physiology; they also could have implications for culture-independent assessments of microbial communities.

Culture-independent techniques have allowed scientists to catalog and compare microbial communities of such diverse natural environments as oligotrophic waters (11, 24), marine and freshwater sediments (57, 60), and surface and subsurface soils (2, 10, 13, 25, 27, 30), as well as artificial environments such as water distribution pipes and sewage treatment plants (12, 20, 48). The most commonly used culture-independent techniques for microbial community analysis depend on the isolation and amplification via PCR of conserved genes, such as those encoding 16S rRNA (15). Two approaches to isolating microbial community DNA from environmental samples were developed almost 20 years ago—the direct extraction approach pioneered by Ogram et al. (38) and the indirect extraction approach pioneered by Holben et al. (21)—but debate as to which is superior continues today (17, 45). The direct extraction approach is often preferred because it generally yields more DNA with fewer steps (17). However, this approach will also extract eDNA along with cellular DNA. The original procedure of Ogram et al. (38) included extra washing steps to remove the eDNA prior to cell lysis, and some investigators continue to consider eDNA (for examples, see references 2, 16, 33, and 55) in their extraction protocol. However, others (for examples, see references 27, 30, 47, and 63) don't, which could be attributed to the widespread assumption that eDNA in natural, complex environments is of low abundance (37).

If little eDNA is produced and degradation is rapid, then the amount of eDNA found in natural environments will be small. However, soil concentrations of eDNA are as high as 2 μg/g of dry soil (37), and eDNA can comprise more than 70% of the total DNA pool in marine sediments (9). The actual amount of eDNA may be even higher since the majority of eDNAs may not be released even with multiple sequential extractions (29). While eukaryotic DNA (18) and dissolved DNA (42) are quickly degraded by environmental nucleases, bacterial DNA (2) and recombinant genes of bacterial origin (40) may persist

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outside cells as PCR-amplifiable fragments for months or longer. Binding to sediments (1, 9, 29), clays (3), and humic acids (8) can protect eDNA from degradation by nucleases, preserving its ability to transform cells (1, 8) and be amplified by PCR (2, 3). The DNA binding appears to result from interaction with a large number of low-specificity binding sites (43) which favors the retention of large DNA fragments. Extraction of only eDNA yields a unique community profile quite different from that of bacterial cells extracted from the same environment (2).

In this paper, we assayed single- and dual-species bacterial biofilms for relative eDNA content to determine the universality of eDNA production and the persistence of eDNA in dual-species biofilms. To gain insight into the composition of eDNA, we compared eDNA with cellular DNA isolated from *P. aeruginosa* using randomly amplified polymorphic DNA (RAPD). Finally, we investigated the proportional effects of eDNA versus cellular DNA on culture-independent assessments of microbial community composition and diversity. Our findings reveal the strain specificity of eDNA abundance and the unpredictable accumulation of eDNA in dual-species biofilms. Further, eDNA has a distinct signature of 16S rRNA genes relative to cellular and total biofilm DNA.

MATERIALS AND METHODS

Bacterial strains and chemicals. Four strains of bacteria were used in culturing biofilms. *P. aeruginosa* strain PG201 (Urs Ochsner, University of Colorado) and *Pseudomonas putida* strain mt-2 (Gary Sayler, University of Tennessee—Knoxville) are both gram-negative soil isolates. *Rhodococcus erythropolis* was provided by Joe Lepo (University of West Florida) and is a mucoid gram-positive environmental isolate. *Variovorax paradoxus* is a gram-negative environmental isolate (22) that appears to be a vadose zone specialist (22, 25). All strains were archived in 70% Luria broth–30% glycerol at −80°C. The specific growth rate constants of the strains in liquid culture (10% tryptic soy broth [TSB], 30°C, 200 rpm) were 0.387/h for *P. aeruginosa*, 0.397/h for *P. putida*, 0.150/h for *R. erythropolis*, and 0.197/h for *V. paradoxus*.

All chemicals, unless otherwise specified, were reagent grade or higher (Sigma-Aldrich, St. Louis, MO).

Biofilm growth and harvesting. Biofilms were cultivated for three purposes: to quantify the abundance of eDNA, to compare the composition of cellular DNA to eDNA via RAPD analysis, and to quantify the impact of eDNA on the culture-independent (terminal restriction fragment length polymorphism [TRFLP]) analysis of a biofilm bacterial community. For eDNA quantification and TRFLP analysis, biofilms were cultured using the method of Auerbach et al. (4) on Nuclepore polyester membranes (13-mm diameter, 0.1-μm pore, 6-μm thickness; Whatman, Clifton, NJ) overlaying 10% tryptic soy agar (3 g; TSB, 15 g Bacto Agar per liter [both from Difco, Fisher Scientific, Pittsburgh, PA]). To recover larger quantities of DNA for RAPD analysis, *P. aeruginosa* strain PG201 was cultured similarly on larger polyester Nuclepore membranes (47-mm diameter, 0.1-μm pore size) over Luria broth agar plates for 5 days. Prior to inoculation, membranes were sterilized (70% ethanol for 2 min), air dried, and then transferred with sterile forceps to the solid medium surface. Small membranes were spaced equally in a pentagon formation centered in the petri dish to facilitate similar zones of nutrient availability during growth. Inocula were prepared from suspensions of single-strain bacterial cultures (10% TSA at 30°C for 3 days) in sterile 0.9% NaCl solution. To facilitate the equal representation of species in mixed inocula, the densities (by absorbance, 600 nm) of the suspensions were equalized by adjusting them with sterile 0.9% NaCl. For single-species biofilms, each membrane was inoculated with 4 μl of suspension onto the center of each membrane. For multiple-species biofilms, 2 μl of each inoculum was deposited onto the center of each membrane into the same droplet so that dual-species membranes received a total of 4 μl of inoculum and biofilms composed of all four species received a total of 8 μl of inoculum. As with previous experiments, the inocula comprised less than 1% of the final assayed biofilm (50). Uninoculated sterile controls were prepared in the same manner. The petri dishes were sealed with Parafilm to prevent drying and incubated at 30°C in the dark. Biofilms grow radially and concentrically away from the point of inoculation (51).

TABLE 1. Sequences of 10-mer RAPD primers[a]

Primer name	Sequence (5′ to 3′)
208	ACGGCCGACC
228	GCTGGGCCGA
241	GCCCGAGCGG
270	TGCGCGCGGG
272	AGCGGGCCAA
275	CCGGGCAAGC
277	AGGAAGGTGC
287	CGAACGGCGG

[a] Data from reference 31.

After 120 h, three membranes were harvested for each species combination and for the sterile control. The process of biofilm harvesting and EPS separation was based on the method proposed by May and Chakrabarty (35). Membranes were lifted from the agar surface with biofilm intact using sterile forceps. Each biofilm was dispersed by vortexing the membrane in 1 ml of 0.9% NaCl solution containing 10 mM EDTA for small membranes and 3 ml of 0.9% NaCl–10 mM EDTA solution for large membranes. The 0.9% NaCl has a lower water potential (−0.71 MPa) than the growth medium (~0 MPa) to prevent cell lysis during harvesting. EPS and cells were separated by centrifugation (12,000 × *g* for 30 min at 4°C) and stored at −20°C until analysis. Based on the size of the *P. aeruginosa* genome (6.3 Mbp [54]) and the number of cells remaining in the biofilm (no more than 0.0001%), contaminating cellular DNA comprises approximately 0.01% of the total DNA in the EPS.

DNA extraction, quantification, and purification. The thawed cell pellets of the single- and dual-species biofilms were resuspended in 500 μl of 1 N NaOH and heated at 80°C for 1 h to complete lysis. The NaOH was then neutralized with 500 μl of 1 N HCl. For RAPD and TRFLP, DNA was released from thawed cell pellets by bead beating in sterile 0.9% NaCl for 30 s using 0.5 g of 100-μm sterile glass beads to prevent possible sequence degradation. The DNA in the lysates and the eDNA in the supernatant were quantified using Picogreen fluorometry (Molecular Probes, Eugene, OR) with calf thymus DNA as a standard. DNA was quantified prior to purification because of possible purification bias.

Both cellular DNA and eDNA were purified and concentrated by extracting twice with an equal volume of 25:24:1 phenol-chloroform-isoamyl alcohol and then precipitating with 1/10 volume of 3 M potassium acetate and 2/3 volume of isopropanol. After centrifugation (10 min, 4°C, 10,000 × *g*), the pellet was washed with 100% ethanol and air dried. The dried DNA pellet was resuspended in 20 μl of 10 mM Tris-Cl, pH 8.5. The concentration and purity of the purified DNA were determined spectrophotometrically by the absorbance ratio A_{260}/A_{280}. Purified DNA solutions were adjusted to 25 ng/ml with 10 mM Tris-Cl, pH 8.5.

RAPD analysis of *P. aeruginosa* DNA. To infer the composition of eDNA, RAPD was performed on *P. aeruginosa* cellular and eDNA according to the method of Mahenthiralingam et al. (32). Eight different 10-mer primers (Table 1) previously shown to produce reproducible and distinguishable RAPD profiles with *P. aeruginosa* (32) were obtained from Qiagen (Valencia, CA). Each 25-μl reaction contained 40 ng of genomic DNA, 40 pmol of oligonucleotide, 1 U *Taq* polymerase (Qiagen), 250 μM (each) deoxynucleoside triphosphates (Qiagen), 3 mM $MgCl_2$, and 5 μl Q solution (Qiagen) in PCR buffer (Qiagen). Amplification was done in a PCRSprint thermocycler (Hybaid US, Franklin, MA) as follows: (i) 4 cycles, each consisting of 5 min at 94°C, 5 min at 36°C, and 5 min at 72°C, (ii) 30 cycles, each consisting of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C, and (iii) a final extension step of 10 min at 72°C.

The RAPD products were separated by gel electrophoresis on a 2% agarose gel in 0.5% Tris-borate-EDTA using a Mini-Sub Cell GT system (Bio-Rad, Hercules, CA) at 100 V (approximately 25 mA) for 45 min. The gel was subsequently stained with ethidium bromide and photodocumented. Band intensity was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Pairwise comparisons of lanes were performed based on the presence or absence of bands, as identified by the ImageQuant software. Comparisons were also made by comparing the brightness along linear paths of equal length in each lane. The intensities were plotted against the location, and the correlation coefficient was calculated using the correlation tool of the Data Analysis ToolPak in Microsoft Excel 2000.

TRFLP analysis. 16S rRNA genes were amplified from purified DNA using eubacterial primers 8F Hex (fluorescently labeled 5′AGAGTTTGATCCTGGC

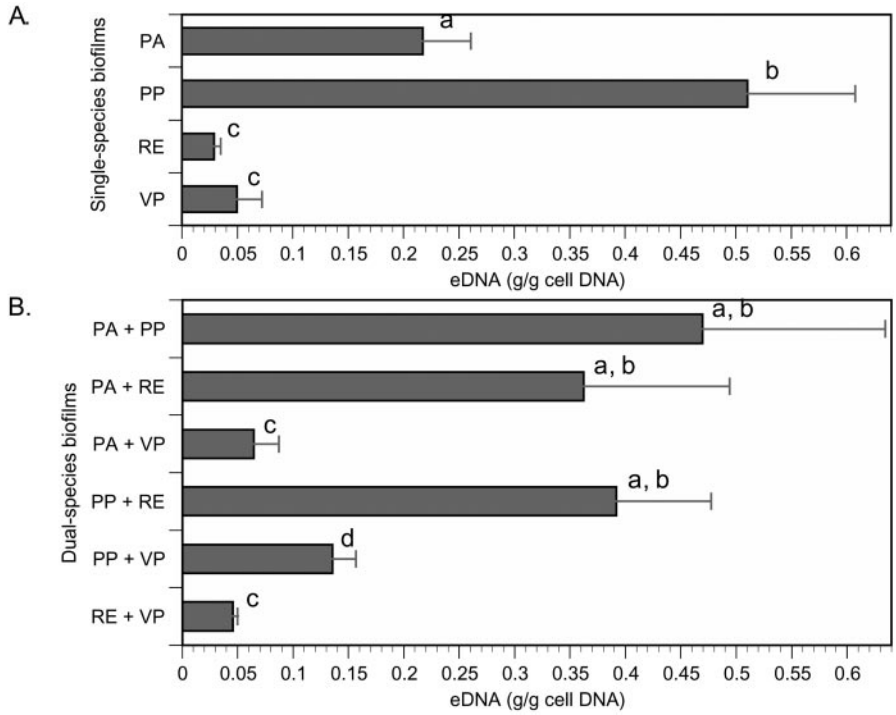

FIG. 1. Average eDNA found in single-species biofilms (A) and multiple-species biofilms (B). The eDNA has been normalized to cellular DNA. The species' names have been abbreviated as follows: PA, *P. aeruginosa*; PP, *P. putida*; RE, *R. erythropolis*; VP, *V. paradoxus*. Error bars represent 1 standard error of each mean. For single-species biofilms, $n = 7$. For multiple-species biofilms, $n = 3$. Bars with different letters are significantly different ($P < 0.05$).

TCAG [28]) and 1389R (5′ACGGGCGGTGTACAAG [39]). Each 100-μl reaction contained 75 ng template, 2.5 U *Taq* polymerase, 200 μM (each) deoxynucleoside triphosphates, 50 pmol DNA primers, 2.2 mM $MgCl_2$ (including 1.5 mM from the reaction buffer), 1× reaction buffer and Q mix (Qiagen, Valencia, CA), and 2 mg/ml bovine serum albumin (Promega, Madison, WI). Amplification was done in a PCRSprint thermocycler (Hybaid US) according to the following hot-start protocol: (i) denaturing for 4 min at 95°C, extension for 1 min at 58°C, and annealing for 90 s at 72°C, followed by the addition of the *Taq* mixture, (ii) 28 cycles of 45 s at 95°C, 45 s at 58°C, and 90 s at 72°C, and (iii) a final elongation for 10 min and 45 s at 72°C. After amplification, PCR product was purified using QIAquick PCR purification columns (Qiagen, Valencia, CA). Amplicons were then restricted using HhaI (New England Biolabs, Beverly, MA) in a 20-μl reaction containing 300 ng purified PCR product, 1 U HhaI enzyme, 1× reaction buffer, and 0.1 mg/ml bovine serum albumin overnight at 37°C. Following restriction, the enzyme was denatured (65°C for 20 min). Samples were desalted using QIAquick nucleotide removal columns (Qiagen, Valencia, CA) and eluted into distilled water. The lengths of the fluorescently labeled fragments were determined with an Applied Biosystems Instruments (Foster City, CA) Prism 3100 genetic analyzer. Peak areas for replicated peaks were normalized by total replicated peak area, and unreplicated peaks were not included in subsequent data analysis. Shannon-Weaver diversity and evenness indices were calculated according to the method of Brodie et al. (7). Principal components analysis (PCA) and correspondence analysis of shared peaks were performed using PC-Ord (MjM Software Design, Gleneden Beach, OR). Both types of ordination were performed to verify that problems such as normalization bias (23) and the horseshoe effect (41) were minimized. In order to assess variability in the TRFLP profiles, independent replicates for total DNA ($n = 8$), cellular DNA ($n = 7$), and eDNA ($n = 4$) were conducted from the PCR step forward. Due to the similarity of the total DNA and cellular DNA profiles, TRFLP analysis was replicated twice more for these two pools to strengthen the statistical significance of their interpretation.

RESULTS

Production and persistence of eDNA. Four species (*P. aeruginosa*, *P. putida*, *R. erythropolis*, and *V. paradoxus*) were tested for the ability to produce eDNA in single- and dual-species biofilms. Both strains of *Pseudomonas* produced high levels of eDNA, averaging 0.22 ± 0.04 g of eDNA/g of cellular DNA for *P. aeruginosa* and 0.51 ± 0.10 g of eDNA/g of cellular DNA for *P. putida* (Fig. 1A), although *P. putida* produced significantly more ($P = 0.017$) than *P. aeruginosa*. Both *V. paradoxus* and *R. erythropolis* produced very small but statistically similar ($P > 0.10$) amounts of eDNA (0.05 ± 0.02 and 0.029 ± 0.006 g of eDNA/g of cellular DNA, respectively) which was significantly less eDNA ($P \leq 0.005$) than either of the *Pseudomonas* species. Of the total DNA in the *Pseudomonas* biofilms, 17 ± 3% and 32 ± 4% were extracellular for *P. aeruginosa* and *P. putida*, respectively. eDNA represented less than 5% of the total DNA for *R. erythropolis* and *V. paradoxus*.

The levels of eDNA in the dual-species biofilms (relative to the concentration of cellular DNA) were similar to the levels of eDNA observed in single-species biofilms (Fig. 1) but were not necessarily additive or quantitatively predictable from the composite eDNA from individual strains. Combining the two high eDNA-producing strains (*P. aeruginosa* and *P. putida*) led to a high eDNA content not significantly different ($P > 0.1$) from either *P. aeruginosa* or *P. putida* single-species biofilms. Similarly, combining the low eDNA-producing strains (*R. erythropolis* and *V. paradoxus*) produced a biofilm with an eDNA content statistically similar ($P > 0.01$) to the biofilms of the component strains. However, mixing the high and low eDNA-producing strains led to unpredictable results. Combining either *Pseudomonas* species with *R. erythropolis* produced a high eDNA biofilm (0.36 ± 0.13 g/g of cellular DNA and 0.39 ± 0.09 g/g of cellular DNA for *P. aeruginosa* and *P. putida*, re-

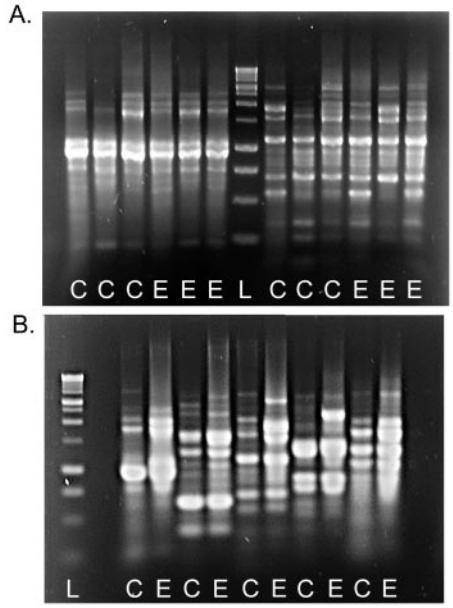

FIG. 2. Comparative RAPD analysis of *P. aeruginosa* cellular DNA and eDNA. (A) Three replicate cellular DNA and three replicate eDNA amplification products for primers 270 (left of the ladder) and 275 (right of the ladder) were compared to ascertain banding pattern reproducibility. Center lane, 1-kb ladder. (B) RAPDs of cellular DNA (left lane in each pair) and eDNA (right lane in each pair) for primers 270, 272, 275, 277, and 287, from left to right. The lanes are labeled as follows: C, cellular DNA; E, eDNA; and L, ladder.

spectively) whose eDNA content was not statistically different from that of either *Pseudomonas* species ($P > 0.1$). However, combining either *Pseudomonas* species with *V. paradoxus* produced a biofilm with relatively little eDNA (0.07 ± 0.02 g/g of cellular DNA and 0.14 ± 0.02 g/g of cellular DNA for *P. aeruginosa* and *P. putida*, respectively). The amount of eDNA in composite biofilms of *P. aeruginosa* and *V. paradoxus* was not significantly different from the single-species biofilms of either *R. erythropolis* or *V. paradoxus* ($P > 0.1$), while the eDNA of composition *P. putida* and *V. paradoxus* biofilms was significantly different from both ($P < 0.01$). It appears that while the bacterial species composing the biofilm do affect the amount of eDNA in a biofilm, they don't do so in a predictable manner.

RAPD comparison of *P. aeruginosa* cellular DNA and eDNA. The eDNA and cellular DNA pools of *P. aeruginosa* unsaturated biofilm were compared using the technique of RAPD analysis and eight different primers (Table 1). For all primers, the bright bands were common to the cellular DNA and eDNA (Fig. 2). Some of the fainter bands, particularly those with higher molecular weight, were present only in either cellular DNA or eDNA. These variations, however, were similar to the level of variations observed between replicate RAPD reactions (Fig. 2B). The correlation coefficients, based on band brightness values from cellular DNA and eDNA RAPDs, ranged from 0.790 to 0.989 (average, 0.913) and were equivalent to those of replicate RAPDs (0.823 to 0.992; average, 0.934). The eDNA appeared to produce brighter, more consistent bands of higher-molecular-weight fragments, while the cellular DNA produced more consistent bands of lower-molecular-weight fragments (Fig. 2).

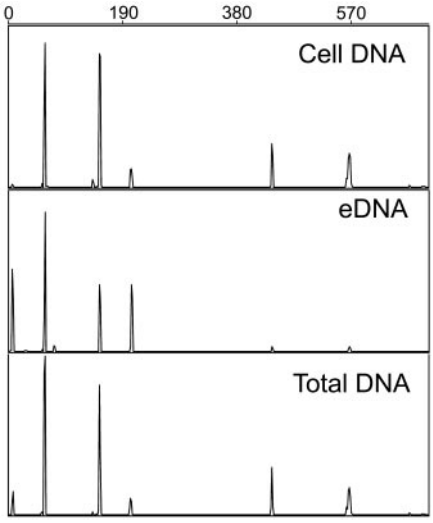

FIG. 3. TRFLP profiles generated from cellular DNA (top), eDNA (middle), and total DNA (bottom) from a multiple-species unsaturated biofilm comprised of *P. aeruginosa*, *P. putida*, *R. erythropolis*, and *V. paradoxus*.

TRFLP analyses of cellular DNA, eDNA, and total DNA. We compared the TRFLP profiles generated from cellular DNA, eDNA, and total DNA extracted from a biofilm containing all four species of bacteria. The resulting TRFLPs (Fig. 3) showed three major peaks and six minor peaks. The major peaks, located at 61 bp, 152 bp, and 566 bp, appeared in all the TRFLPs, as did the minor peaks located at 56 bp, 202 bp, 205 bp, and 438 bp. The remaining minor peaks, located at 140 bp and 562 bp, appeared only in the cellular DNA and the total DNA. Replication of TRFLP profiles for each DNA source ($n \geq 4$) showed excellent reproducibility, with the peak areas of each fragment length having variances ranging from 2.5% to 29.1% (average, 11.7%).

The TRFLP profile of the total DNA appeared very similar to that of the cellular DNA but slightly different from that of the eDNA (Fig. 3). Individual peak areas of the eDNA TRFLP fragments were significantly different ($P < 0.05$) from those of the cellular DNA profiles for all but the 56-bp fragment (Fig. 4). The peak areas of fragments in the total DNA TRFLP profiles were significantly different ($P < 0.05$) from those of the eDNA TRFLP profiles but were not significantly different ($P > 0.1$) from those of the cellular DNA TRFLP profiles (Fig. 4). The average Shannon-Weaver diversity indices for the total DNA, cellular DNA, and eDNA TRFLP profiles were 1.606 ± 0.041, 1.647 ± 0.021, and 1.244 ± 0.050, respectively. The average Shannon-Weaver index of the eDNA profile was significantly lower ($P < 0.001$) than either the cellular DNA or total DNA profiles. The average Shannon-Weaver diversity index from the profile of total DNA was statistically similar ($P > 0.1$) to that of the cellular DNA profile. The average evenness indices for the total DNA, cellular DNA, and eDNA TRFLP profiles were 0.732 ± 0.018, 0.750 ± 0.009, and 0.640 ± 0.026, respectively, and followed the same statistical pattern. The PCA of the TRFLP profiles was able to explain 66% and 24% of the variability between replicates on the first and second axes, respectively (Fig. 5). The replicates from the cellular

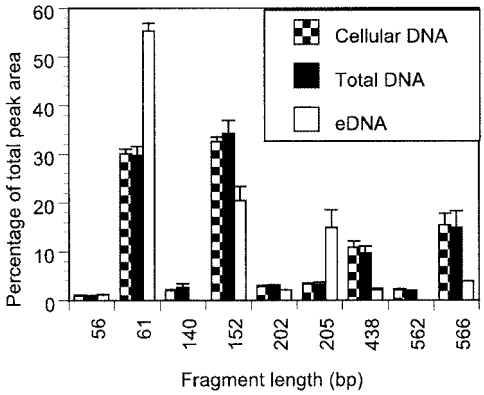

FIG. 4. Average percentage of total peak area contributed by individual fragments to the TRFLP profiles of cellular DNA ($n = 7$), total DNA ($n = 8$), and eDNA ($n = 4$). Error bars represent the standard error of each mean.

DNA and eDNA TRFLPs were grouped closely and clearly separated from each other. The total DNA TRFLP profiles also grouped, though less tightly, and overlapped the cellular DNA profiles. The ordination by correspondence analysis produced very similar distribution and groupings to those of the PCA ordination (data not shown).

DISCUSSION

Previously we reported that the EPS of unsaturated bacterial biofilms has a complex chemistry, comprised of eDNA, polysaccharides, and protein (51). Here, we investigated species composition as a factor governing eDNA and thus EPS complexity; we also investigated the potential for eDNA to influence culture-independent microbial community analyses. Our experimental results lead to several conclusions. First, some but not all bacteria produce large amounts of eDNA that persist when grown as either single- or dual-species unsaturated biofilms. Second, even if an isolated species is not a large producer of eDNA, the presence of a low eDNA producer can significantly influence the amount of eDNA in multiple-species biofilms. One explanation of this conclusion might be competitive interactions between strains that ultimately favor the growth of the lower eDNA producer (e.g., *V. paradoxus*). However, other explanations are also possible including ecological interactions due to differing growth rates (see Methods), different rates of cell death and lysis (26, 56), and the strain-dependent utilization of eDNA as a nutrient (14). Our observation that the genetic composition of eDNA and cellular DNA differed significantly in multispecies biofilms suggests that the mechanisms are likely to be complex and deserve further investigation.

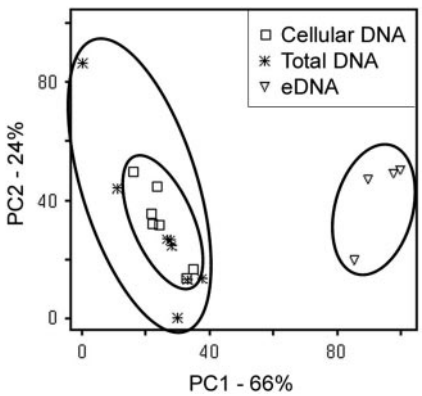

FIG. 5. Plot of principle components 1 (PC1) and 2 (PC2) representing the percent variability (axis labels) of the TRFLP profiles from cellular DNA, eDNA, and total DNA. PCA was performed from a similarity matrix based on comparing peak areas.

Another conclusion concerns the possible role of eDNA in confounding the analysis of natural microbial communities. Here, we report that the primary sequence of eDNA cannot be distinguished from that of cellular DNA for some species. Although eDNA did not contribute to the overall TRFLP profile in our low-diversity biofilms, eDNA and cellular DNA profiles differed significantly by TRFLP. Given that eDNA is typically recovered during DNA extraction (for examples, see references 2 and 38) and that it accumulates in biofilms disproportionately to the initial species distribution, eDNA could affect the culture-independent assessments of communities. Community diversity, ambient conditions, and mineralogy (e.g., clay content) are just a few of the variables in natural environments that could affect how eDNA affects the culture-independent assessments of whole communities. Previously, 16S rRNA gene amplicons in eDNA were shown to form a distinct denaturing gradient gel electrophoresis (DGGE) profile (2), but eDNA contributions to the total DGGE profile were not reported. Furthermore, our comparison of profiles was limited to that generated by amplifying genes for small-subunit rRNA. Given the amount of DNA outside of the cells, we cannot rule out that other sequences of interest, i.e., some encoding catabolic functions, could be amplified disproportionately from eDNA in comparison to biofilm species composition. Thus, because both the relative proportions of eDNA and cellular DNA in natural environments and the effect of eDNA on other nucleic acid-based assays (31) are currently unknown, it seems premature to dismiss any possible effect of eDNA on current culture-independent assessments.

Several questions still remain about the structure of eDNA. For example, why would more higher-molecular-weight RAPD products appear for *P. aeruginosa* eDNA relative to cellular DNA? One explanation could be disproportionate shearing of cellular DNA during the lysis of *P. aeruginosa* cells. However, the higher intensity could also be due to a difference in the average molecular weight of eDNA or to a difference in molecular conformation (i.e., secondary/tertiary structure.) While eDNA appears to have the same primary sequence as cellular DNA, whether eDNA is circular or linear and what proteins (if any) are associated with the secondary structure and maintenance of the eDNA are still unknown. eDNA of environmental biofilms may also be selectively enriched in specific sequences, particularly since coculturing microbes has been shown to stimulate plasmid DNA release (34). The resistance of biofilms to destruction by DNase after an initial growth period (58) may suggest that the DNA in the biofilm is somehow stabilized, which may inhibit the effectiveness of proposed biofilm treatment methods involving DNase (44). A better understanding of the structure of the eDNA may also elucidate why it con-

tributes so little to the total community fingerprint when analyzed by DGGE (2) or TRFLP.

In addition to the structure of eDNA, there are also many questions about the origins and functions of eDNA. It is unclear whether eDNA is excreted, as suggested by Hara and Ueda (19), or is a product of cell lysis, a natural part of biofilm development (26, 56). The variable levels of eDNA produced among bacterial species suggests that eDNA may be an environmental adaptation, although the ecological importance remains unknown. Strong cases have been made for the roles of eDNA in exchanging genetic information (14, 34, 36), in stabilizing biofilms (36, 58), and as a nutrient during starvation (14). It is probable that eDNA also fulfills many roles frequently ascribed to exopolysaccharides (59). Continued study of eDNA is warranted to eliminate uncertainty about the effects on apparent microbial community composition by culture-independent techniques and to discern its roles in the microbial ecology of diverse environments. More broadly, a better understanding of eDNA may have consequences beyond its effect on analyses of community composition. For example, more knowledge of eDNA structure could allow researchers to increase the effectiveness of DNase treatments for cystic fibrosis and persistent infections (5, 6). Further, because the amount of eDNA in EPS can substantially impact the physiochemical properties of industrial biofilms (49), knowing the origin and function of eDNA in saturated biofilms could be of economic importance. Finally, the cryptic sequences in eDNA could potentially be used to trace the growth and transport of bacteria in natural environments (2).

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