ORIGINAL ARTICLE

Influence of industrial contamination on mobile genetic elements: class 1 integron abundance and gene cassette structure in aquatic bacterial communities

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The acquisition of new genetic material via horizontal gene transfer allows bacteria to rapidly evolve. One key to estimating the contribution of horizontal gene transfer to bacterial evolution is to quantify the abundance of mobile genetic elements (MGEs) in bacterial communities under varying degrees of selective pressure. We quantified class 1 integrase (*int1*) gene abundance in total community DNA extracted from contaminated and reference riverine and estuarine microhabitats, and in metal- or antibiotic-amended freshwater microcosms. The *int1* gene was more abundant in all contaminant-exposed communities indicating that relative gene transfer potential is higher in these communities. A second key to assessing the contributions of MGEs to bacterial evolution is to examine the structure and function of the MGE-associated gene pool. We determined that the gene cassette pool is a novel and diverse resource available for bacterial acquisition, but that contamination has no discernible effect on cassette richness. Gene cassette profiles were more similar within sites than among sites, yet bacterial community profiles were not, suggesting that selective pressures can shape the structure of the gene cassette pool. Of the 46 sequenced gene cassette products, 37 were novel sequences, while the 9 gene cassettes with similarity to database sequences were primarily to hypothetical proteins. That class 1 integrons are ubiquitous and abundant in environmental bacterial communities indicates that this group of MGEs can play a substantial role in the acquisition of a diverse array of gene cassettes beyond their demonstrated impact in mediating multidrug resistance in clinical bacteria.

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Introduction

Mobile genetic elements (MGEs) involved in bacterial acquisition and recombination of foreign DNA contribute to genetic diversity within bacterial assemblages and enable bacteria to rapidly evolve (Ochman *et al.*, 2000; O'Brien, 2002; Dobrindt *et al.*, 2004; Gogarten and Townsend, 2005; Boucher *et al.*, 2007). Previous approaches to examine the role of horizontal gene transfer in microbial evolution have

involved the use of a comparative approach to reconstruct historical gene transfer events (for example, Boucher *et al.*, 2003; Kunin *et al.*, 2005; Comas *et al.*, 2006; Doolittle and Bapteste, 2007), the use of *in vivo* or *in vitro* gene transfer experiments where gene transfer rates are estimated under controlled conditions (for example, Dahlberg *et al.*, 1998; van Elsas and Bailey, 2002) or molecular investigations of cellular regulation and machinery involved in the process (for example, Thorsted *et al.*, 1998; Lambertsen *et al.*, 2004). While these methods have merit for revealing evolutionary relationships among organisms and the mechanisms that lead to transfer, they rarely reflect *in situ* gene transfer potential in microbial assemblages.

One key to estimating gene transfer potential and its role in bacterial evolution is to first estimate MGE

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abundance in bacterial communities under varying degrees of selective pressure. Several studies have detected MGEs including plasmids, transposons and integrons across a wide variety of habitats (for example, Sobecky, 1999; Smalla and Sobecky, 2002; van Elsas and Bailey, 2002; Frost *et al.*, 2005), but rarely in a quantitative manner due to previous methodological constraints. In those studies that did quantify MGE abundance in bacterial communities, class 1 integrase (*intI1*) genes were shown to be abundant in Gram-positive and Gram-negative bacteria in poultry litter obtained from farms with varying antibiotic usage regimes regardless of antibiotic usage (Nandi *et al.*, 2004), while mercury exposure was recently demonstrated to increase the abundance of IncP-1 plasmids (Smalla *et al.*, 2006). Yet, how MGE abundance is affected by environmental conditions and selective pressures is poorly understood and remains to be tested systematically.

In this study, we examined the potential contribution of integrons to bacterial evolution under varying degrees of selective pressure from metal exposure. Integrons are genetic elements that incorporate exogenous sources of mobile DNA called gene cassettes into the recipient genome via site-specific recombination. Various classes of integrons have been identified (Nield *et al.*, 2001; Nemergut *et al.*, 2004; Boucher *et al.*, 2007), but each class shares a common structure including an integrase gene (*intI*) that encodes for the enzyme catalyzing the site-specific recombination of a gene cassette at an integron-associated site designated *attI*. Where examined, integrons also possess a promoter, P_c that allow the transcription of the typically promoterless open reading frames (ORFs) contained within adjacent gene cassettes (Figure 1). The units of insertion into integrons are gene cassettes. These are independently mobilizable genetic units that normally consist of a single ORF and a cassette-associated recombination site designated a 59-base element or *attC*. Class 1 integrons were first identified due to their role in the

dissemination of antibiotic resistance gene cassettes in clinical bacteria (Stokes and Hall, 1989), but have since been found in a phylogenetically broad range of clinical and environmental bacteria including Gram-negative and Gram-positive bacteria (Nandi *et al.*, 2004; Stokes *et al.*, 2006; Boucher *et al.*, 2007). Environmental surveys of integron-associated gene cassettes indicate that the majority of cassettes include novel sequences unrelated to known antibiotic resistance genes (Stokes *et al.*, 2001; Holmes *et al.*, 2003), with one conservative estimate of cassette richness being at least 2343 different gene cassettes in a 50-m² sediment plot (Michael *et al.*, 2004). Integrons therefore represent a potential genetic resource by which bacteria in a variety of environments can adapt through the acquisition of new genetic material.

To estimate the potential contribution of integrons to the evolution of aquatic bacteria, we compared the relative abundance of class 1 integrons and examined the structure and function of the gene cassette pool in aquatic microbial communities under varying degrees of contaminant exposure. We compared the abundance of *intI1* genes in bacterial communities from industrially contaminated and reference riverine and estuarine systems, and in freshwater microcosms where bacterial exposure to metals and antibiotics was directly manipulated. To further investigate how environmental conditions influence the structure and function of genes contained within integrons, we compared gene cassette diversity and similarity metrics using gene size fragment analysis, and assessed their predicted function through sequence analysis.

Materials and methods

Sampling

Savannah River Site. We collected samples from two streams located on the United States Department of Energy's Savannah River Site (SRS) near Aiken, SC, USA on 9 November 2005. Effluent from ash-settling basins associated with the SRS D-400 coal-fired power plant enters into Beaver Dam Creek at a site designated Site A, the first sampling point. Sediment here consists of cobble, sand and coal ash particles with elevated concentrations of a variety of metals associated with coal ash effluent including selenium, arsenic and strontium (Wright *et al.*, 2006). Beaver Dam Creek Site D is located 3.2 km downstream from Site A, where sediment is primarily sandy, and dissolved metal concentrations are typically lower than at Site A. Meyers Branch is a historically unimpacted blackwater stream with sandy sediment, which has previously been used as a reference stream on the SRS (McArthur and Tuckfield, 2000; Wright *et al.*, 2006). Neither stream has a point source of anthropogenic antibiotics.

We collected bacteria from four common stream microhabitats to examine the abundance of MGEs

Figure 1 Structure and orientation of integrons. The integrase enzyme, *intI*, catalyzes the site-specific recombination of the gene cassette (GC) *attC* site at the *attI* location within the integron downstream of the promoter, P_c .

in a variety of microbial communities including epiphytic biofilm, *Corbicula fluminea* (Asiatic Clam) digestive tracts, surface sediments and surface water. Epiphytic biofilm was collected from oak wooden dowels (2×45 cm) deployed at each site on 3 June 2005. On the sampling date, we transported three dowels from each site back to the laboratory in sterile whirlpacks on ice for further processing. To represent microbial communities harbored in the digestive tract of invertebrates, we collected nine *C. fluminea* individuals. Grab samples of surface sediments were collected in triplicate in sterile whirlpacks to analyze sediment bacterial communities, and we collected 1 l of stream water in triplicate in autoclaved and UV-sterilized polycarbonate bottles to sample bacterial communities in the water column.

Estuarine sites. We sampled three estuaries located along the Georgia and South Carolina coasts that vary in degree and type of industrial contamination in October 2005. Both Shipyard Creek (Charleston, SC, USA) and the LCP Site (Brunswick, GA, USA) are designated on the US Environmental Protection Agency National Priorities List for their history of hazardous industrial contamination. Sediments contain elevated concentrations of chromium and cadmium (Shipyard Creek), and mercury (LCP Chemical) among other contaminants. The Ashepoo-Combahee-Edisto (ACE) Basin estuary (Beaufort, SC, USA), a historically unimpacted US National Wildlife Refuge, served as a reference site. We collected samples in triplicate as above for surface sediments and water samples during both ebb and flood tides.

Savannah River microcosms. Bacterial exposure to metals and antibiotics was experimentally manipulated using a series of freshwater microcosms conducted by (Stepanauskas *et al.*, 2006). We collected surface water from the Savannah River near the intake for the SRS D-400 coal-fired power plant on 29 September 2003, which was then aliquoted into triplicate tetracycline (30 mg l^{-1}), cadmium (0.1 mM) or control microcosms. Selected concentrations represent treatment microcosms from Stepanauskas *et al.* (2006) that generated an increase in metal and antibiotic resistance phenotypes relative to controls in the microcosm series. Microcosms were incubated for 7 days at 23°C with slow rotation (100 r.p.m. on an orbital shaker) after which 250 ml of water were collected for DNA extraction.

Metal analysis

Sediment samples were collected from each site to confirm that a gradient of metal concentrations existed among sites. Sediment samples were frozen until processing as in Wright *et al.* (2006) at the Savannah River Ecology Laboratory analytical

facility and analyzed using ICP-MS with the appropriate calibration standards and blanks during digestion and analysis.

Sample processing

Samples were processed immediately in the laboratory. For biofilm samples, we aseptically scraped biofilm material from the wooden dowels using a sterilized razor blade and extracted DNA from 0.25 g of this material using the MoBio PowerSoil DNA Extraction kit according to the manufacturer's instructions. To extract DNA from *Corbicula* digestive tracts, we aseptically removed and combined the hindguts of three individuals per replicate to obtain enough material to process using the MoBio UltraClean DNA Extraction kit. We extracted sediment DNA from 0.4 g of sediment using the MoBio PowerSoil DNA Extraction kit. For water samples, we filtered 1 l of water (or 250 ml for the microcosms), onto $0.2 \mu\text{m}$ filters. Because of suspended particles, multiple filters had to be used that were combined in 50 ml Eppendorf tubes containing 10 ml of autoclaved, UV-sterilized nanopure water. Tubes were vortexed until all visible particles were detached and centrifuged at $4700 g$ for 15 min to pellet bacteria. We extracted DNA from the pellets using MoBio UltraClean DNA Extraction kit. DNA was quantified using gel standards with AlphaEaseFC Software version 3.1.2 (Alpha Innotech Corp., San Leandro, CA, USA).

Quantitative PCR protocol

Primers. Primers targeting the *intI1* gene were designed to estimate the abundance of class 1 integrons (Figure 1). To account for potential differences among samples in bacterial abundance and PCR inhibitors, universal primers were also used to quantify 16S rRNA genes as used in Fierer *et al.* (2005). Primers used and their annealing temperatures are detailed in Table 1.

Calibration standards. Calibration standards for use in quantitative PCR (qPCR) assays were generated from conventional PCR products using the appropriate primer pair with DNA extracted from each sample type as the template. We purified PCR products using the Qiagen PCR Purification kit according to the manufacturer's instructions, and purified amplicons were then quantified using AlphaEaseFC software. Six 10-fold dilutions were subsequently prepared with each purified PCR product for use as standards in qPCR assays.

Quantitative PCR conditions. Quantitative PCR for *intI1* and 16S rRNA genes consisted of $1 \mu\text{l}$ template, $1 \mu\text{l}$ of each $1 \mu\text{M}$ *intI1* primer ($10 \mu\text{M}$ 16S rRNA primers), $12.5 \mu\text{l}$ of SuperArray SYBR Green Master Mix (SuperArray, Frederick, MD, USA), and PCR-grade water to a reaction volume of $25 \mu\text{l}$. Triplicate

Table 1 Primers used in this study used conventional and quantitative PCR (qPCR) reactions, expected amplicon size and annealing temperature

Target	PCR type	Primer	Sequence (5' → 3')	Amplicon size (bp)	Annealing temperature (°C)	Reference
<i>intI1</i>	qPCR	qIntI1F	ACCAACCGAACAGGCTTATG	~ 286	62	Nemergut <i>et al.</i> (2004)
		qIntI1R	GAGGATGCGAACCACTTCCAT			
<i>attC</i>	Conventional	HS286	(FAM)-ATCCTCSGCTKGARCGA	Variable	55	Stokes <i>et al.</i> (2001)
		HS287	MTTGTTAGVC GGGATCCGCSGCTKANCTCVRRCGTTAGSC			
16S rRNA	qPCR	Eub338	ACTCCTACGGGAGGCAGCAG	~ 200	63	Fierer <i>et al.</i> (2005)
		Eub518	ATTACCGCGGCTGCTGG			
16S rRNA	Conventional	E334F	(FAM)-CCAGACTCCTACGGGAGGCAGC	~ 605	55	Baker <i>et al.</i> (2003)
		E939R	CTTGTGCGGGCCCCCGTCAATTC			

reactions for each sample were performed using the Bio-Rad iQCyler Real-Time Detection System in Bio-Rad iCyler 96-well plates, where triplicate no-template controls were also used. The thermocycler protocol for *intI1* and 16S rRNA reactions consisted of 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 62 °C for 30 s (53 °C for 16S rRNA), 72 °C for 30 s and a final 10 min at 72 °C for extension. To ensure specificity for each reaction, multiple analyses were conducted. A melt curve was analyzed after each qPCR run, while randomly selected samples were run on a 1% agarose gel in 10 × TBE solution for 30 min at 80 V to check for the presence of a single band of the appropriate size, and an additional subset of *intI1* PCR products were selected for sequencing (see below). Copy number of each target gene was calculated using the average molecular mass of 660 g mol⁻¹ for a double-stranded DNA molecule. Estimates of 16S rRNA copy number were corrected for amplification in no-template control reactions.

Gene cassette profiling

To assess the structure of the gene cassette community, fluorescently labeled primers targeting the *attC* site (HS286 and HS287 in Figure 1) were used to amplify gene cassettes from freshwater and estuarine sediment samples and microcosms as in Michael *et al.* (2004). PCRs were run in triplicate for each sample consisting of 400 nM of each primer, 10 × PCR buffer, 1.5 mM MgCl₂, 200 μM dNTPs, 0.02 U JumpStart Taq (Sigma-Aldrich, St Louis, MD, USA) and 5 ng of template DNA in a 25-μl reaction volume. PCR conditions were as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 30 s, 72 °C for 2 min 30 s and a final extension at 72 °C for 10 min. PCR amplicons were subsequently analyzed using fragment analysis on an ABI 3130xl sequencer to separate gene cassettes by size, combining 1 μl of PCR product with 1.5 μl

internal NAUOX size standard (to 1002 bp) (DeWoody *et al.*, 2004) and 7.5 μl of HiDi/formamide. We limited analysis of cassette sizes to those within 250–999 bp, as the false-positive rate for PCR fragments smaller than 250 bp increases significantly (as determined from our sequencing results and as in Michael *et al.*, 2004), and size class calling accuracy decreases with increasing amplicon size. To account for variability among PCR experiments, three triplicate PCRs were analyzed separately for each sample using GeneMapper (Applied Biosystems, Foster City, CA, USA) with the peak detection algorithm set to detect peaks greater than 20 fluorescence units above the threshold. We compiled the data from the three runs by retaining peaks that were present in at least two out of the three PCR runs and calculated mean peak area as a measure of relative cassette abundance.

tRFLP analysis

We assessed bacterial community structure by amplifying 16S rRNA fragments for terminal restriction length polymorphism (tRFLP) analysis. PCR mixture consisted of 400 nM of the universal primers 5'-FAM labeled E334F and unlabeled E939R (Table 1), 10 × PCR buffer, 1.5 mM MgCl₂, 200 μM dNTPs, 0.02 U JumpStart Taq (Sigma) and 5 ng of template DNA. The thermocycler program consisted of 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. Duplicate PCRs were run for each sample, combined and purified using QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. PCR products were subsequently quantified using DNA standards and the AlphaImager system and software. To generate restriction fragments, we incubated 30 ng of PCR product with 1 μl of *RsaI* enzyme (New England Biolabs, Ipswich, MA, USA) (*RsaI* was demonstrated to generate the most peaks

in initial testing of samples), 2 µl of NEBuffer 1, and water to a reaction volume of 20 µl at 37 °C for 1 h, followed by 20 min at 65 °C to inactivate the enzyme. Ten microliters of the products were then purified, precipitated and resuspended in TE following the methods of Sambrook and Russell (2000). For fragment analysis on the ABI 3130xl sequencer, we denatured 1 µl of the purified digest product with 0.7 µl of NAUOX internal size standard and 13 µl of 1:1 HiDi/formamide at 95 °C for 5 min. Peaks above 20 fluorescence units were detected and sized using a global Southern algorithm in GeneMapper software (Applied Biosystems).

Sequencing

We assessed potential gene cassette function by transforming gene cassette PCR products from one randomly selected sample of each sample type (for example, Savannah River Site sediment, Shipyard Creek ebb tide, tetracycline microcosm and so on) into sequencing vectors using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Clones were screened for the presence of the insert using conventional PCR with M13 primers. We then analyzed PCR products on a 1% agarose gel for 30 min at 80 mA and selected clones for sequencing representing a range of sizes. Sequencing reactions using M13 forward and reverse primers and BigDye 3.1 Terminators (Applied Biosystems) were resolved on an ABI 3130xl sequencer. We joined contigs and manually edited sequences using Sequencher software (GeneCodes, Ann Arbor, MI, USA) to ensure accuracy of the readings. After confirming the presence of both HS286 and HS287 primer sequences in the obtained sequences, we both manually identified predicted ORFs and used NCBI's ORF Finder (www.ncbi.nlm.nih.gov/gorf/gorf.html) to locate predicted proteins within gene cassettes. To assess potential function of predicted proteins, we conducted a BLASTX search to identify similar amino-acid sequences (Altschul *et al.*, 1990).

The *intI1* qPCR products were also transformed with the TOPO TA Cloning Kit and sequenced using M13 primers as above to verify amplification of the correct target.

Statistical analyses and gene cassette diversity and similarity metrics

Differences among sites in log-transformed integrase relative abundance (*intI1* copy number/16S rRNA copy number) were analyzed by analysis of variance in SAS (SAS Institute, Cary, NC, USA), where site was the main effect. Site means were compared using the LSMEANS option with $\alpha = 0.05$. Each sample set (freshwater, estuarine and microcosms), and each microhabitat were analyzed separately because of potential differences in PCR efficiencies and inhibitors among different bacterial community types.

Gene cassette richness, Shannon diversity and community similarity values (Chao's abundance-based Jaccard similarity) were calculated using EstimateS software version 7.5 (Colwell, 2005). Richness (the number of detected gene cassette sizes) and diversity values were calculated for each site using compiled data from the three replicate PCRs for each sample. A distance matrix was constructed for gene cassette profiles from each sample set based on similarity values, and a multi-dimensional scaling plot was subsequently generated using these data in SAS. The same procedure was followed for analyzing tRFLP profiles for similarity among samples at each site.

Results

Metal analysis

Multiple metals were elevated in sediments from Beaver Dam Creek Sites A and D relative to Meyers Branch (Table 2). Shipyard Creek sediments had higher concentrations of several metals compared with ACE Basin and LCP Chemical sediments, but LCP Chemical sediments were higher in mercury (Table 2).

16S rRNA abundance

For freshwater samples, 16S rRNA gene abundance did not differ between sites for biofilm samples, but there were significantly fewer 16S rRNA copies at Site D in *Corbicula*, sediment and water column microhabitats (Figure 2a). 16S rRNA copies were significantly more abundant in sediment and water

Table 2 Sediment metal concentrations (mg kg⁻¹) from reference and contaminated freshwater and estuarine samples

Site	Type	Cr	Co	Ni	Cu	Zn	As	Se	Sr	Cd	Hg	Be	V	Mo
Meyers Branch	Freshwater	BDL	1.86	1.47	0.74	13.80	BDL	1.43	BDL	0.02	BDL	BDL	1.36	BDL
Beaver Dam Site D	Freshwater	3.85	1.89	2.88	3.16	15.44	1.02	3.40	BDL	0.04	BDL	0.25	8.33	BDL
Beaver Dam Site A	Freshwater	BDL	1.76	4.72	3.87	17.08	2.59	1.75	0.53	0.22	0.07	0.09	3.03	BDL
ACE basin	Estuarine	53.68	7.39	16.38	9.03	51.35	10.81	2.31	66.00	0.24	BDL	1.60	56.04	0.82
LCP Chemical	Estuarine	33.52	4.26	10.12	8.19	40.65	5.79	BDL	39.79	0.09	1.92	0.88	34.61	0.46
Shipyard Creek	Estuarine	596.66	6.21	33.36	59.09	198.84	10.65	BDL	114.40	1.22	BDL	0.87	45.66	8.61

Abbreviations: As, arsenic; BDL, below detection limit; Be, beryllium; Cd, cadmium; Co, cobalt; Cr, chromium; Cu, copper; Hg, mercury; Mo, molybdenum; Ni, nickel; Se, selenium; Sr, strontium; V, vanadium; Zn, zinc.

Estuarine values represent mean concentrations of five replicate samples collected during October 2005.

Figure 2 16S rRNA copy number (●), class 1 integrase (*int11*) copy number (△) and the 16S rRNA: *int11* copy number ratio for the various sites and microhabitats as assessed by quantitative PCR. (a) Savannah River Site freshwater microhabitats; (b) estuarine microhabitats; and (c) experimental microcosm.

column samples at Meyers Branch than at Site A, but this pattern was reversed for *Corbicula* samples. For estuarine sites, in both ebb and flood samples, there were significantly fewer copies of 16S rRNA genes at the Shipyard Creek site compared with ACE Basin and LCP Chemical sites, but there were no site-specific differences among sediment samples (Figure 2b). 16S rRNA copy number was greatest in

the initial samples but declined over the 7 days of incubation for all microcosms (Figure 2c). 16S rRNA qPCR efficiencies ranged between 100.2% and 105.3% among the sample types, with correlation coefficients between 0.90 and 0.96 for the standard calibration curves. Melt curve analysis displayed a single peak confirming the specificity of the reactions.

intI1 relative abundance

The relative *intI1* abundance was significantly greater in bacteria from contaminated sites compared with the reference site across the tested bacterial communities. The *intI1*/16S rRNA ratio was significantly lower for biofilm, sediment and water microhabitats at the reference riverine site, Meyers Branch, relative to the two contaminated sites; however, there was no statistical difference in the ratio for *Corbicula* samples among the three sites (Figure 2a). Copies of *intI1* genes were significantly more abundant at Site D than Site A for sediment samples, while there was no statistical difference between the two sites for the other sampled microhabitats. Compared with the two contaminated estuarine sites, significantly fewer copies of *intI1* were detected in ACE Basin for ebb, flood and sediment samples, while the ratio of *intI1*/16S rRNA was significantly greater in Shipyard Creek than LCP Chemical samples for ebb samples (Figure 2b). In microcosm samples, *intI1* relative abundance was greatest in the cadmium treatment, followed by the tetracycline treatment, while there was no statistical difference between initial and control microcosms (Figure 2c). qPCR efficiencies ranged from 85.5% to 86.8%, while standard curve correlation coefficients ranged from 0.910 to 0.982. All retrieved *intI1* sequences were highly similar to previously characterized *intI1* genes and have been deposited in GenBank under the following accession numbers (EU43506–EU43511).

Gene cassette structure

Gene cassette richness (the number of unique gene cassette sizes) and Shannon diversity varied significantly among sites, but with no consistent pattern between contaminant-exposed and reference communities (Table 3). Richness and diversity were significantly greater in contaminant-exposed communities from the freshwater and microcosm samples. However, cassette richness and diversity were significantly greater in the reference site, ACE

Basin and LCP Chemical site than in Shipyard Creek. The composition of the gene cassette pool varied among sites and microcosms, as gene cassette profiles were more similar within rather than among each site or microcosm treatment (Table 3). Furthermore, replicates from each sample clustered together in comparison with replicates from other sites or treatments in a multidimensional scaling plot (Figure 3a).

Gene cassette function

Of the 46 sequenced gene cassettes obtained from total community DNA extracted from each sample type, 43 had predicted ORFs with start and stop codons in typical gene cassette orientation (Stokes *et al.*, 2001), while the three with no obvious ORF were all less than 209 bp in length. Gene cassette sequences have been deposited in GenBank under the following accession numbers: EU435312–EU435354. Several ORFs had putative stop codons out of frame ($n=15$) or stop codons in the middle of the predicted coding sequence ($n=8$). The majority of gene cassette sequences contained ORFs that had no similar matches in GenBank at either the nucleotide or amino-acid level ($n=34$). Only nine gene cassettes were similar to GenBank sequences at the amino-acid level (Table 4). Two gene cassettes were similar to previously detected gene cassettes, and four gene cassettes encoded functions that were similar to hypothetical proteins. No known antibiotic or metal resistance genes were recovered, but gene cassettes encoding proteins with similarity to proteins of known function included partial matches to magnesium chelatases, *N*-acetyltransferases and pantothenate synthetases. However, the CdMicrocosm_01 cassette likely underwent a frameshift as there was an inframe stop codon in the middle of the coding sequence, and no inframe stop at the end. The gene cassette encoding a product similar to pantothenate synthetases involved in vitamin B₅ synthesis had an inframe stop codon as did the InitMicrocosm_06 cassette.

Table 3 Gene cassette richness, diversity (Shannon), gene cassette and tRFLP similarity (Chao's abundance-based Jaccard similarity) both within a site and among sites for sediment samples as calculated in EstimateS for each of the three sample groups (Colwell, 2005)

Sample	Cassette richness	Cassette diversity	Cassette similarity within group	Cassette similarity among groups	tRFLP similarity within group	tRFLP similarity among groups
Meyers branch	202 (4.2)	4.0	0.77 (0.05)	0.30 (0.02)	0.25 (0.04)	0.28 (0.08)
Beaver dam site D	259 (5.9)	4.9	0.48 (0.12)	0.31 (0.03)	0.29 (0.01)	0.25 (0.07)
Beaver dam site A	268 (5.5)	4.6	0.77 (0.02)	0.36 (0.02)	0.27 (0.04)	0.26 (0.09)
ACE basin	255 (6.6)	4.2	0.91 (0.03)	0.66 (0.05)	0.88 (0.03)	0.67 (0.17)
LCP chemical	255 (6.8)	4.5	0.86 (0.04)	0.69 (0.06)	0.75 (0.07)	0.71 (0.17)
Shipyard creek	234 (5.0)	4.3	0.90 (0.01)	0.59 (0.07)	0.63 (0.30)	0.60 (0.19)
Initial	21 (NA)	2.2	NA	0.052 (0.02)	NA	0.48 (0.16)
Control	30 (5.2)	3.1	0.07 (0.04)	0.027 (0.01)	0.48 (0.15)	0.45 (0.13)
Cadmium	91 (8.3)	4.1	0.04 (0.04)	0.041 (0.01)	0.77 (0.09)	0.52 (0.13)
Tetracycline	95 (7.6)	3.6	0.11 (0.09)	0.025 (0.01)	0.54 (0.31)	0.53 (0.14)

Abbreviations: NA, not applicable because there is only one initial sample; tRFLP, terminal restriction length polymorphism. Values in parentheses represent 1 s.d. ($n=3$).

Figure 3 Multidimensional scaling plots of sediment gene cassette pool as assessed by amplifying gene cassettes with HS286 and HS287 primers (Figure 1), and bacterial community structure as assessed by 16S rRNA terminal restriction fragment length polymorphism (tRFLP) analysis, based on a distance matrix calculated from Chao's abundance-based Jaccard similarity (see Table 2 for similarity values). (a) Savannah River Site freshwater microhabitats; (b) estuarine microhabitats; and (c) experimental microcosm.

tRFLP community similarity

Bacterial community composition was not identifiably distinct between sites as assessed by the similarity of 16S rRNA tRFLP profiles, where Chao's

abundance-based Jaccard similarity values indicate that similarity was not greater within a site compared with that among the sites (Table 3) as analyzed separately for each of the three sample sets.

Table 4 The nine gene cassettes out of 46 sequenced gene cassettes with GenBank matches based on amino-acid similarity (BLASTX).

Cassette name	Cassette size (bp)	E-value	Predicted protein	Accession number	Stop codon in middle of predicted ORF	No inframe stop codon	Organism and accession number
Initial Microcosm_01 (EU435351)	359	5.00e ⁻²²	Panthothenate synthetase		Yes		<i>Acidothermus cellulolyticus</i> 11B (YP_871971)
Initial Microcosm_02 (EU435327)	494	1.00e ⁻¹⁰	Gene cassette		Yes		<i>Vibrio cholerae</i> (AAL68646)
Initial Microcosm_03 (EU435347)	533	3.00e ⁻³⁵	ORF-SIK7-1341 GNAT family				<i>Pseudodalteromonas tunicata</i> D2 (ZP_01131714)
Initial Microcosm_04 (EU435350)	555	1.00e ⁻³⁰	N-acetyltransferase Hypothetical protein				<i>Xylella fastidiosa</i> Temecula 1 (NP_779100)
Initial Microcosm_05 (EU435326)	596	2.00e ⁻⁵²	Hypothetical protein				<i>Saccharophagus degradans</i> str 2-40 (YP_527802)
Initial Microcosm_06 (EU435345)	621	5.00e ⁻³⁵	Hypothetical protein		Yes		<i>Delftia acidovorans</i> (ZP_01577442)
Control Microcosm_01 (EU435343)	620	7.00e ⁻²⁰	Hypothetical protein				<i>Hahella chejuensis</i> KCTC 2396 (YP_436158)
Cadmium Microcosm_01 (EU435333)	401	1.00e ⁻²⁴	Mg-chelatase ATPase subunit D		Yes	Yes	<i>Rhodopseudomonas palustris</i> (YP_487620)
SYC Sed_01 (EU435314)	448	8.00e ⁻¹⁶	Gene cassette			Yes	Environment (AAL24528)

Additionally, replicates from each site did not cluster together in a multidimensional scaling plot (Figure 3b).

Discussion

Class 1 integron abundance

Genetic elements involved in the acquisition of exogenous sources of DNA were more abundant in contaminant-exposed bacterial communities across a variety of freshwater and estuarine microbial habitats and in experimental microcosms where metal and antibiotic exposure was manipulated. That this effect of industrial contamination on *intI1* gene abundance was observed across multiple bacterial communities in multiple types of ecosystems indicates its generality. In this study, the *intI1* gene was relatively more abundant in Site D than in Site A in the contaminated freshwater stream counter to the prediction that *intI1* would be most abundant in the most contaminated site. Metal concentrations were elevated at Site D on the sampling date likely due to both a period of increased stream discharge associated with the coal-fired plant operations (data not shown) and the long-term cumulative effects of sediment transport from more contaminated sites upstream. Regardless of variance within Beaver Dam Creek, *intI1* was consistently more abundant across multiple microhabitats in comparison with the reference site, Meyers Branch.

Culture-based and molecular estimates of *intI1* abundance in bacteria of environmental origin indicate that between 1% and 10% of bacteria possess these genes (C Baker-Austin, unpublished data; Nandi *et al.*, 2004; Boucher *et al.*, 2007), although Biyela *et al.* (2004) found class 1 integrons in over 50% of isolates with multiple drug resistances from an urban South African river. Variation in the proportion of a bacterial community possessing class 1 integrons outside of clinical settings is likely to be the result of several factors including (1) distance to an enriched source of class 1 integrons (for example, sewage treatment plant); (2) integron dispersal ability (for example, mobilization of the integron via physical linkage to a plasmid or transposon); (3) the taxonomic composition of the community; (4) horizontal gene transfer potential in a given habitat; and (5) intensity of selective pressure favoring the maintenance of genes contained within the integron.

To date, class 1 integrons have primarily been characterized from γ -proteobacteria of clinical origin, but have more recently been detected in environmental bacteria from diverse habitats and taxonomic relationship. For example, based on a BLASTN search of 759 bacterial genomes (completed and whole-genome shotgun projects) in the Microbial Genome Resources database (<http://www.ncbi.nlm.nih.gov/genomes/MICROBES/>

microbial_taxtree.html), eight genomes (1.1%) yielded sequences similar to *intI1* (*E*-value cutoff: $1e^{-50}$). Seven of these genomes were from enteric γ -proteobacterial pathogens of clinical origin, while the exception to this was the Gram-positive human pathogen *Corynebacterium diphtheriae* (Actinobacteria) (Supplementary Table 1). In a BLASTN search of all GenBank bacterial sequences, more than 300 unique nucleotide sequences produced similar alignments to *intI1* (*E*-value cutoff: $1e^{-50}$). Again, the majority of these were from plasmids and transposons in clinical isolates of Gram-negative proteobacteria pathogens reflecting the bias in the database toward pathogenic bacteria and antibiotic resistance studies. However, a broader group of bacteria possess these elements as evidenced by Nandi *et al.* (2004) detecting *intI1* in Gram-positive bacteria and the recent description by Stokes *et al.* (2006) of four nonpathogenic soil bacteria (all β -proteobacteria) possessing class 1 integrons. A BLASTN search of archaeal sequences found no matches to *intI1* indicating that to date no class 1 integrons have been detected in Archaea (date of analyses: 27 March 2007). Thus sequence database evidence to date suggest that class 1 integrons are concentrated in Gram-negative γ -proteobacteria, but this picture will likely change with sequence analysis of bacteria from terrestrial and aquatic systems.

Elevated class 1 integron abundance in industrially contaminated sites can either be the result of selection for the integron itself (the ability to acquire and insert new genetic material in a site-specific location), adaptive gene cassettes contained within integrons (direct selection) or other genetic elements to which the integron is physically linked (coselection). In support of the last hypothesis, class 1 integrons are frequently detected on larger MGEs including insertion sequence common regions, transposons and plasmids (Tennstedt *et al.*, 2003, 2005; Stokes *et al.*, 2006; Toleman *et al.*, 2006a,b; Walsh, 2006) that contain genes encoding other functions. A good example of this is Tn21, a transposon containing a mercury resistance transposon in which a class 1 integron is embedded (Liebert *et al.*, 1999). Selective pressures favoring the maintenance of genes within these genetic elements (for example, metal contamination maintaining metal resistance genes) may therefore maintain other genes including integrons that are physically linked. However, what regulates integron-mediated gene cassette recombination, and the expression of cassette-associated genes is yet to be fully understood, thereby limiting a rigorous assessment of the mechanisms contributing to the increased abundance of *intI1* genes observed in metal-contaminated sites.

Gene cassette structure and function

The effects of industrial contamination on gene cassette pool richness and diversity are equivocal, as

the riverine and microcosm data suggest that this selective pressure increases the richness and diversity of the gene cassette pool, whereas this pattern was not observed in the estuarine sites where cassette richness and diversity was lower at Shipyard Creek. We predicted that contamination would have a homogenizing effect on the gene cassette pool, as selective pressures would favor the propagation of cassettes conferring a selective advantage (Turner *et al.*, 2002), but alternatively it may be that the sampled sites are too heterogeneous to favor the dominance of a few cassettes.

The analysis of cassette richness using these methods is likely to underestimate gene cassette richness. This is in large part because the primers used do not recover all integron-associated gene cassettes (Michael *et al.*, 2004). Other potential biases relate to the use of gene cassette size as a proxy for distinguishing between different cassettes. Previously, Michael *et al.* (2004) detailed this potential bias, where the same cassette size class can contain multiple cassettes. However, this problem is not unique to this method, as the same limitation is present in techniques such as tRFLP and DGGE. These biases result in richness values that underestimate the true number of unique gene cassettes in a bacterial community, but what is still apparent is that gene cassettes are a diverse genetic resource for bacterial evolution in the environment.

Similarity patterns indicate that the gene cassette pool at each site or microcosm treatment was more similar in gene cassette size composition and relative abundance compared with the other sites for each sample set. This finding likely reflects differences in selective pressures at each site maintaining a different pool of cassettes. Alternatively, gene cassette profiles could reflect taxonomic or biogeographic artifacts, but 16S rRNA bacterial community fingerprints as assessed by tRFLP analysis indicate that overall community structure was not distinct among sites within each of the three sample sets. This strengthens the conclusion that gene cassette pools were distinct at each site because of differential selective pressure. However, tRFLP fingerprinting analysis is a relatively coarse metric of community similarity, which can mask finer scale taxonomic variation in bacterial community composition, and biogeography may still have an effect through dispersal limitation of gene cassettes between sites or through variations in gene cassette composition entering each site due to differences in watershed drainages.

One key to assess whether gene cassettes are selected for at the various sites is to identify the potential function of their genes. An assessment of whether any of the most abundant gene cassettes is of potential adaptive significance is hampered by the lack of recovery of the most abundant cassette sizes, and by the fact that most sequenced cassettes were novel. Of interest was the fact that several gene cassettes likely encode nonfunctional proteins due

to frameshifts, and inframe stop codons, suggesting that they are not encoding a protein under selection. Those cassettes that are similar to GenBank sequences are of mostly unknown function, whereas the three sequences were similar to proteins with identified function, although were not associated with known metal- or antibiotic resistance genes. Two cassettes (Initial Microcosm_02 and SRS Sed_01) encoded proteins that had low-level similarity at the amino-acid level to products from gene cassettes previously recovered, further demonstrating that gene cassettes themselves can evolve. The majority of sequenced gene cassettes are novel, as recovered using gene cassette PCR, but this method does not allow for a characterization of the integron context in which they are associated. Furthermore, this finding is consistent with previous surveys of environmental gene cassettes supporting the hypothesis that this pool is a diverse and distinct genetic resource from that found statically in bacterial chromosomes (Boucher *et al.*, 2007).

Summary

Integrations are ubiquitous in bacterial communities regardless of whether industrial contamination is present, demonstrating that these MGEs play a substantial role in the acquisition of new genetic material in environmental bacteria. That they were more abundant in sites with contamination across a variety of habitats and ecosystems suggests that bacteria at these sites have a higher potential for gene exchange, whether it is for the acquisition of antibiotic resistance gene cassettes or cassettes of unknown function. The gene cassette pool is a diverse resource available for bacterial acquisition via horizontal gene transfer, whereas the composition of the pool reflects selective pressures at each site. Whether these findings are applicable to other MGEs besides class 1 integrons remains to be tested, and where further study would enhance our understanding of the effect of stress on evolutionary processes in bacteria.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990). Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

- Baker GC, Smith JJ, Cowan DA. (2003). Review and re-analysis of domain-specific 16S primers. *J Microbiol Methods* **55**: 541–555.
- Biyela PT, Lin J, Bezuidenhout CC. (2004). The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Sci Technol* **50**: 45–50.
- Boucher Y, Douady CJ, Papke RT, Walsh DA, Boudreau ME, Nesbø CL *et al.* (2003). Lateral gene transfer and the origins of prokaryotic groups. *Annu Rev Genet* **37**: 283–328.
- Boucher Y, Labbate M, Koenig JE, Stokes HW. (2007). Integrons: mobilizable platforms that promote genetic diversity in bacteria. *Trends Microbiol* **15**: 301–309.
- Colwell RK. (2005). EstimateS: statistical estimation of species richness and shared species from samples. Version 7.5. User's Guide and Application; published at: <http://purl.oclc.org/estimates>.
- Comas I, Moya A, Azad RK, Lawrence JG, Gonzalez-Candelas F. (2006). The evolutionary origin of Xanthomonadales genomes and the nature of the horizontal gene transfer process. *Mol Biol Evol* **23**: 2049–2057.
- Dahlberg C, Bergstrom M, Hermansson M. (1998). *In situ* detection of high levels of horizontal plasmid transfer in marine bacterial communities. *Appl Environ Microbiol* **64**: 2670–2675.
- DeWoody JA, Schupp J, Kenefic L, Busch J, Murfitt L, Keim P. (2004). Universal method for producing ROX-labeled size standards suitable for automated genotyping. *Biotechniques* **37**: 348–352.
- Dobrindt U, Hochhut B, Hentschel U, Hacker J. (2004). Genomic islands in pathogenic and environmental microorganisms. *Nat Rev Microbiol* **2**: 414–424.
- Doolittle WF, Baptiste E. (2007). Pattern pluralism and the Tree of Life hypothesis. *Proc Natl Acad Sci USA* **104**: 2043–2049.
- Fierer N, Jackson JA, Vilgalys R, Jackson RB. (2005). Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl Environ Microbiol* **71**: 4117–4120.
- Frost LS, Leplae R, Summers AO, Toussaint A. (2005). Mobile genetic elements: the agents of open source evolution. *Nat Rev Microbiol* **3**: 722–732.
- Gogarten JP, Townsend JP. (2005). Horizontal gene transfer, genome innovation and evolution. *Nat Rev Microbiol* **3**: 679–687.
- Holmes AJ, Gillings MR, Nield BS, Mabbitt BC, Nevalainen KM, Stokes HW. (2003). The gene cassette metagenome is a basic resource for bacterial genome evolution. *Environ Microbiol* **5**: 383–394.
- Kunin V, Goldovsky L, Darzentas N, Ouzounis CA. (2005). The net of life: reconstructing the microbial phylogenetic network. *Genome Res* **15**: 954–959.
- Lambertsen LM, Molin S, Kroer N, Thomas CM. (2004). Transcriptional regulation of pWW0 transfer genes in *Pseudomonas putida* KT2440. *Plasmid* **52**: 169–181.
- Liebert CA, Hall RM, Summers AO. (1999). Transposon Tn21, flagship of the floating genome. *Microbiol Mol Biol Rev* **63**: 507–522.
- McArthur J, Tuckfield RC. (2000). Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. *Appl Environ Microbiol* **66**: 3722–3726.
- Michael CA, Gillings MR, Holmes AJ, Hughes L, Andrew NR, Holley MP *et al.* (2004). Mobile gene cassettes: a fundamental resource for bacterial evolution. *Am Nat* **164**: 1–12.

- Nandi S, Maurer JJ, Hofacre C, Summers AO. (2004). Gram-positive bacteria are a major reservoir of Class 1 antibiotic resistance integrons in poultry litter. *Proc Natl Acad Sci USA* **101**: 7118–7122.
- Nemergut DR, Martin AP, Schmidt SK. (2004). Integron diversity in heavy-metal-contaminated mine tailings and inferences about integron evolution. *Appl Environ Microbiol* **70**: 1160–1168.
- Nield BS, Holmes AJ, Gillings MR, Recchia GD, Mabbutt BC, Nevalainen KM *et al.* (2001). Recovery of new integron classes from environmental DNA. *FEMS Microbiol Lett* **195**: 59–65.
- O'Brien TF. (2002). Emergence, spread, and environmental effect of antimicrobial resistance: how use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. *Clin Infect Dis* **34**: S78–S84.
- Ochman H, Lawrence JG, Groisman EA. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**: 299–304.
- Sambrook J, Russell DW. (2000). *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press: Cold Spring Harbor.
- Smalla K, Haines AS, Jones K, Krögerrecklenfort E, Heuer H, Schlöter M *et al.* (2006). Increased abundance of IncP-1 beta plasmids and mercury resistance genes in mercury-polluted river sediments: first discovery of IncP-1 beta plasmids with a complex mer transposon as the sole accessory element. *Appl Environ Microbiol* **72**: 7253–7259.
- Smalla K, Sobecky PA. (2002). The prevalence and diversity of mobile genetic elements in bacterial communities of different environmental habitats: insights gained from different methodological approaches. *FEMS Microbiol Ecol* **42**: 165–175.
- Sobecky PA. (1999). Plasmid ecology of marine sediment microbial communities. *Hydrobiologia* **401**: 9–18.
- Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, King CJ *et al.* (2006). Coselection for microbial resistance to metals and antibiotics in freshwater microcosms. *Environ Microbiol* **8**: 1510–1514.
- Stokes HW, Hall RM. (1989). A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol Microbiol* **3**: 1669–1683.
- Stokes HW, Holmes AJ, Nield BS, Holley MP, Nevalainen KM, Mabbutt BC *et al.* (2001). Gene cassette PCR: sequence-independent recovery of entire genes from environmental DNA. *Appl Environ Microbiol* **67**: 5240–5246.
- Stokes HW, Nesbo CL, Holley M, Bahl MI, Gillings MR, Boucher Y. (2006). Class 1 integrons potentially predating the association with Tn402-like transposition genes are present in a sediment microbial community. *J Bacteriol* **188**: 5722–5730.
- Tennstedt T, Szczepanowski R, Braun S, Puhler A, Schlüter A. (2003). Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. *FEMS Microbiol Ecol* **45**: 239–252.
- Tennstedt T, Szczepanowski R, Krahm I, Puhler A, Schlüter A. (2005). Sequence of the 68,869 bp IncP-1 alpha plasmid pTB11 from a waste-water treatment plant reveals a highly conserved backbone, a Tn402-like integron and other transposable elements. *Plasmid* **53**: 218–238.
- Thorsted PA, Macartney DP, Akhtar P, Haines AS, Ali N, Davidson P *et al.* (1998). Complete sequence of the IncP beta plasmid R751: implications for evolution and organisation of the IncP backbone. *J Mol Biol* **282**: 969–990.
- Toleman MA, Bennett PM, Walsh TR. (2006a). Common regions e.g. orf513 and antibiotic resistance: IS91-like elements evolving complex class 1 integrons. *J Antimicrob Chemother* **58**: 1–6.
- Toleman MA, Bennett PM, Walsh TR. (2006b). ISCR elements: novel gene-capturing systems of the 21st century? *Microbiol Mol Biol Rev* **70**: 296–316.
- Turner SL, Bailey MJ, Lilley AK, Thomas CM. (2002). Ecological and molecular maintenance strategies of mobile genetic elements. *FEMS Microbiol Ecol* **42**: 177–185.
- van Elsas JD, Bailey MJ. (2002). The ecology of transfer of mobile genetic elements. *FEMS Microbiol Ecol* **42**: 187–197.
- Walsh TR. (2006). Combinatorial genetic evolution of multiresistance. *Curr Opin Microbiol* **9**: 476–482.
- Wright MS, Peltier GL, Stepanauskas R, McArthur JV. (2006). Bacterial tolerances to metals and antibiotics in metal-contaminated and reference streams. *FEMS Microbiol Ecol* **58**: 293–302.

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