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Development of Quantitative Real-Time PCR Assays for Detection and Quantification of Surrogate Biological Warfare Agents in Building Debris and Leachate[▽]

Pascal E. Saikaly,* Morton A. Barlaz, and Francis L. de los Reyes III

Department of Civil, Construction, and Environmental Engineering, North Carolina State University, Raleigh, North Carolina 27695

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Evaluation of the fate and transport of biological warfare (BW) agents in landfills requires the development of specific and sensitive detection assays. The objective of the current study was to develop and validate SYBR green quantitative real-time PCR (Q-PCR) assays for the specific detection and quantification of surrogate BW agents in synthetic building debris (SBD) and leachate. *Bacillus atrophaeus* (vegetative cells and spores) and *Serratia marcescens* were used as surrogates for *Bacillus anthracis* (anthrax) and *Yersinia pestis* (plague), respectively. The targets for SYBR green Q-PCR assays were the 16S-23S rRNA intergenic transcribed spacer (ITS) region and *recA* gene for *B. atrophaeus* and the *gyrB*, *wzm*, and *recA* genes for *S. marcescens*. All assays showed high specificity when tested against 5 ng of closely related *Bacillus* and *Serratia* nontarget DNA from 21 organisms. Several spore lysis methods that include a combination of one or more of freeze-thaw cycles, chemical lysis, hot detergent treatment, bead beat homogenization, and sonication were evaluated. All methods tested showed similar threshold cycle values. The limit of detection of the developed Q-PCR assays was determined using DNA extracted from a pure bacterial culture and DNA extracted from sterile water, leachate, and SBD samples spiked with increasing quantities of surrogates. The limit of detection for *B. atrophaeus* genomic DNA using the ITS and *B. atrophaeus recA* Q-PCR assays was 7.5 fg per PCR. The limits of detection of *S. marcescens* genomic DNA using the *gyrB*, *wzm*, and *S. marcescens recA* Q-PCR assays were 7.5 fg, 75 fg, and 7.5 fg per PCR, respectively. Quantification of *B. atrophaeus* vegetative cells and spores was linear ($R^2 > 0.98$) over a 7-log-unit dynamic range down to 10^1 *B. atrophaeus* cells or spores. Quantification of *S. marcescens* ($R^2 > 0.98$) was linear over a 6-log-unit dynamic range down to 10^2 *S. marcescens* cells. The developed Q-PCR assays are highly specific and sensitive and can be used for monitoring the fate and transport of the BW surrogates *B. atrophaeus* and *S. marcescens* in building debris and leachate.

The first recorded attempt to use pathogens as biological warfare (BW) agents was in the 14th century when the Mongols catapulted plague-infected victims into the city of Kaffa (Feodosiya, Ukraine) to spread the disease (33, 53). During and after World War II, the development and use of pathogens, such as *Yersinia pestis* (plague), *Bacillus anthracis* (anthrax), and *Francisella tularensis* (tularemia), as BW agents intensified (41, 53). Recently, there has been concern about the potential "weaponization" of pathogens for bioterrorism use, with the October 2001 bioterrorist attack with *B. anthracis* in the United States as the most prominent example (17, 41).

The 2001 event has sparked renewed interest in the development of detection platforms for BW agents (8, 17, 24, 33, 48, 57), methods for inactivation (16, 39, 61) and decontamination of *Bacillus* spores (4, 44), sampling protocols for recovery of *Bacillus* spores from surfaces (5, 20), and methods for viability assessment (31, 47). The decontamination of a building following a terrorist attack with BW agents will generate a significant amount of building decontamination residue that is likely to remain contaminated with BW agents. One disposal alternative is burial in a landfill. Despite the significance of the aforementioned studies in improving bioterrorism preparedness, information on the fate and transport of microorganisms in general and specifically of BW agents in landfills is lacking. This knowledge will assist in bioterrorism preparedness and in the assessment of alternatives for the safe disposal of building decontamination residue.

Evaluation of the fate and transport of BW agents in landfills requires the development of specific and sensitive detection assays. However, surrogates are required, as it is sometimes not feasible to use actual BW agents (40). Several surrogate organisms of BW agents have been used in previous research (40). Specifically, *Bacillus atrophaeus* has been used as a surrogate for *B. anthracis* in studies to develop methods to detect *B. atrophaeus* spores (8, 52, 55), to determine the effects of electric charge and field on the viability of airborne bacteria (34), to develop methods for viability assessment of *Bacillus* spores (31), to evaluate the effect of electric beam irradiation for inactivation of *Bacillus* spores in envelopes (16), and to investigate the effectiveness of decontamination methods against *B. atrophaeus* spores present on furniture (4, 44). *Serratia marcescens* has been used as a surrogate for *Yersinia pestis* for examining the fate of pathogens in indoor air (56).

Traditional monitoring of biocontaminants relies on culture-based techniques that are time-consuming and can detect only culturable cells (5). However, recent developments in nucleic acid-based detection systems, in particular quantitative real-time PCR (Q-PCR), offer significant advantages over culture-based methods for the detection and quantification of BW

* Corresponding author. Mailing address: Department of Civil, Construction, and Environmental Engineering, North Carolina State University, Raleigh, NC 27695. Phone: (919) 515-6275. Fax: (919) 515-7908. E-mail: pesaikal@ncsu.edu.

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TABLE 1. Bacterial strains used for assessment of primer specificity and the C_T values obtained in Q-PCR

Species (strain)[a]	C_T value obtained in Q-PCR with[b]:					
	ITS	$recA_{Ba}$	*gyrB*	$recA_{Sm}$	*wzm*	16S rRNA genes
Bacillus atrophaeus (ATCC 9372)	16 ± 0.17	18 ± 0.23	–	–	37 ± 0.12	–
Bacillus brevis (CBSC 15-4865)	–	–	–	–	–	–
Bacillus cereus (CBSC 15-4870)	36 ± 0.25	–	–	–	–	33 ± 0.12
Bacillus mycoides (CBSC 15-4871)	–	–	–	–	–	34 ± 0.23
Bacillus megaterium (CBSC 15-4900)	–	34 ± 0.20	–	–	–	–
Bacillus sphaericus (CBSC 15-4908)	–	–	–	–	36 ± 0.17	36 ± 0.45
Bacillus subtilis (CBSC 15-4921)	–	–	–	–	37 ± 0.28	36 ± 0.61
Bacillus thuringiensis (CBSC 15-4926)	–	–	–	–	–	35 ± 0.12
Bacillus badius (DSM 23)	–	–	–	–	36 ± 0.53	35 ± 0.32
Bacillus flexus (DSM 1320)	–	–	–	–	–	34 ± 0.42
Bacillus amyloliquifaciens (DSM 7)	–	–	–	–	–	35 ± 0.29
Bacillus mojavensis (DSM 9205)	–	–	–	–	–	–
Bacillus fusiformis (DSM 2898)	–	–	–	–	37 ± 0.30	–
Paenibacillus validus (DSM 3037)	–	38 ± 0.40	–	–	–	–
Paenibacillus chondroitinus (DSM 5051)	–	–	–	–	–	34 ± 0.10
Serratia marcescens (ATCC 13880)	–	–	17 ± 0.15	18 ± 0.43	16 ± 0.22	14 ± 0.11
Serratia ficaria (DSM 4569)	36 ± 0.33	–	–	38 ± 0.33	–	38 ± 0.22
Serratia fonticola (DSM 4576)	–	–	–	–	37 ± 0.13	35 ± 0.58
Serratia odorifora (DSM 4582)	–	–	–	–	–	–
Serratia plymuthica (DSM 4540)	–	–	–	–	36 ± 0.45	35 ± 0.34
Serratia quinivorans (DSM 4597)	–	–	–	–	–	36 ± 0.22
Serratia entomophila (DSM 12358)	–	–	–	–	–	–
Pseudomonas fluorescens (ATCC 13525)	–	–	–	–	36 ± 0.19	33 ± 0.39

[a] ATCC, American Type Culture Collection, Manassas, VA; CBSC, Carolina Biological Supply Company, Burlington, NC; DSM, German Collection of Microorganisms and Cell Cultures.
[b] C_T values are mean values ± standard deviations of duplicates. –, no detection above threshold before cycle 40.

agents. Q-PCR provides high specificity, sensitivity, and speed (38, 49). In addition, it allows the detection of cells irrespective of their culturability. Several Q-PCR assays have been developed and validated for the detection and quantification of BW agents (e.g., *Y. pestis*, *B. anthracis*, *F. tularensis*, and smallpox virus) (17, 18, 21, 23, 24, 33, 43, 48, 57). However, to date, only one Q-PCR assay for the BW surrogate *B. atrophaeus* and one Q-PCR assay for the BW surrogate *S. marcescens* have been reported (4, 5, 25). While Buttner et al. (4, 5) reported the detection of *B. atrophaeus* using Q-PCR targeting the *B. atrophaeus recA* gene, the specificity of the assay was not reported. In addition, the sequence of the target gene could not be identified in published sequences in the GenBank, DNA Data Bank of Japan (DDBJ), and European Molecular Biology Laboratory (EMBL) databases. Iwaya et al. (25) used the 16S rRNA gene for designing a Q-PCR assay for detecting *S. marcescens* in blood. Although the 16S rRNA gene has commonly been used for modern species classification, it has limitations in discriminating species of closely related taxa because of high 16S rRNA sequence similarities (13). Instead, protein-coding genes and the 16S-23S rRNA intergenic transcribed spacer (ITS) region, which both exhibit higher sequence variation than the more conserved 16S rRNA gene, have been used for identifying species of closely related taxa (6, 9, 10, 27, 42, 43, 50). Therefore, to facilitate the use of *B. atrophaeus* and *S. marcescens* as surrogates for pathogens that could potentially be used as BW agents, nucleic acid target sequences that provide the most discrimination between the surrogate organism and its nearest evolutionary neighbor are needed for the design of specific Q-PCR assays for detection and quantification of *B. atrophaeus* and *S. marcescens*.

The objective of this study was to develop and validate a set of SYBR green Q-PCR assays for the specific detection and quantification of BW surrogates *S. marcescens* and *B. atrophaeus*. The targets for SYBR green Q-PCR assays were the 16S-23S rRNA ITS region and *recA* gene for *B. atrophaeus* and the 16S rRNA and *gyrB*, *wzm*, and *recA* genes for *S. marcescens*. The Q-PCR assays were validated using DNA extracted from a pure bacterial culture and DNA extracted from sterile water, synthetic building debris (SBD), and leachate spiked with surrogates.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. All strains were grown on Difco nutrient agar (Becton, Dickinson, and Co., Sparks, MD) at 30°C for 24 h with the exception of *S. marcescens* ATCC 13880, *Serratia plymuthica* DSM 4540, and *Pseudomonas fluorescens* ATCC 13525 that were grown at 26°C. In addition to obtaining *B. atrophaeus* ATCC 9372 vegetative cells (formerly *Bacillus subtilis* var. *niger*) from the American Type Culture Collection (Manassas, VA), purified spore suspensions of *B. atrophaeus* ATCC 9372 were obtained from the North American Science Associates, Inc., (NAMSA) Ohio laboratory (Northwood, OH).

Primer design for SYBR green Q-PCR. The primers utilized in this study are summarized in Table 2.

***B. atrophaeus*-specific Q-PCR primers.** The sequences of the primers (ITS_F and ITS_R) targeting the 16S-23S rRNA ITS region specific for *B. atrophaeus* were designed on the basis of the 16S-23S rRNA ITS region of *B. atrophaeus* ATCC 49337 (GenBank accession number AF478080 [60]). The 16S-23S rRNA ITS sequences of 23 *Bacillus* spp., 9 *Paenibacillus* spp., 6 *Brevibacillus* spp., 2 *Geobacillus* spp., 1 *Marinibacillus* species, and 1 *Virgibacillus* species were retrieved from the GenBank, EMBL, and DDBJ databases (accession numbers AF478062 to AF478111 [60], AB05068, AJ544538, AY157575, and AY149473). The retrieved sequences were aligned via the multiple alignment tool ClustalW (54) using BioEdit v7.0.5 (14). Sequence regions suitable for the design of *B. atrophaeus* ITS-specific Q-PCR primers (ITS_F and ITS_R) were selected by visual inspection of ClustalW multiple alignments. For the amplification of *B. atrophaeus recA* gene, previously designed Q-PCR primers were used, although the specificity of this previously developed Q-PCR assay was not reported (4, 5).

TABLE 2. Primers used for SYBR green Q-PCR assays

Target gene	Primer[a]	Primer sequence (5′–3′)	T_m (°C)[b]	% GC	Amplicon length (bp)	Accession no. (position on gene)[c]
B. atrophaeus spacer region	ITS_F	CATTCGATTCTTCGAGATG	48	42	75	AF478080 (259–333)
	ITS_R	GGTCTTACTTTTGAATGTGATGTC	52	38		
B. atrophaeus recA gene	$recA_{Ba}$_F	ACCAGACAATGCTCGACGT	57	53	131	NA
	$recA_{Ba}$_R	CCCTCTTGAAATTCCCGAAT	53	45		
S. marcescens gyrB gene	*gyrB*_F	AGTGCACGAACAAACTTACAG	53	43	138	AJ300536 (113–251)
	*gyrB*_R	GTCGTACTCGAAATCGGTCACA	57	50		
S. marcescens recA gene	$recA_{Sm}$_F	CAAGGCGAATGCCTGTAACT	56	50	202	M22935 (1526–1727)
	$recA_{Sm}$_R	GAGGATAGGCGCCACATAAA	55	50		
S. marcescens integral membrane protein gene	*wzm*_F	GGTCATGCGGGTTCAAATAC	54	50	153	L34166 (547–699)
	*wzm*_R	ATGACCGAGCGTGGAAATAC	55	50		
S. marcescens 16S rRNA gene	16S_F	GGTGAGCTTAATACGTTCATCAATTG	55	39	179	AJ233431 (435–613)
	16S_R	GCAGTTCCCAGGTTGAGCC	59	63		

[a] The primer is named after the target gene, and F and R at the end of the primer name indicate forward and reverse orientations, respectively.
[b] Theoretical melting temperature (T_m) calculated using the OligoAnalyzer 3.0 from Integrated DNA Technologies (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx; Integrated DNA Technologies, Coralville, IA).
[c] Positions of genes are given according to the accession numbers. NA, not applicable.

The specificity of the *recA* Q-PCR assay was tested in this study. To differentiate the *B. atrophaeus recA* Q-PCR assay from the *S. marcescens recA* Q-PCR assay, *B. atrophaeus recA* is hereafter referred to as $recA_{Ba}$ and *S. marcescens recA* is hereafter referred to as $recA_{Sm}$.

***S. marcescens*-specific Q-PCR primers.** The sequences of the primers (*gyrB*_F and *gyrB*_R) targeting the *gyrB* gene specific for *S. marcescens* were designed on the basis of the *gyrB* sequence of *S. marcescens* ATCC 13880 (EMBL accession number AJ300536) (9). The *gyrB* sequences of 10 *Serratia* spp., 1 *Citrobacter* species, 2 *Enterobacter* spp., 1 *Hafnia* species, 2 *Klebsiella* spp., 1 *Morganella* species, 1 *Pectobacterium* species, 2 *Proteus* spp., 1 *Providencia* species, and 1 *Salmonella* species were retrieved from the EMBL database (accession numbers AJ300528 to AJ300554 [9]). The retrieved sequences were aligned as described above.

The sequences of the primers ($recA_{Sm}$ _F and $recA_{Sm}$_R) targeting the *recA* gene specific for *S. marcescens* were designed on the basis of the *recA* sequence of *S. marcescens* (GenBank accession number M22935) (2). The *recA* sequences of 2 *Serratia* spp., 11 *Erwinia* spp., 4 *Yersinia* spp., 2 *Pantoea* spp., 1 *Hafnia* species, 1 *Rahnella* species, 7 *Pectobacterium* spp., 5 *Brenneria* spp., 1 *Samsonia* species, 1 *Salmonella* species, 3 *Enterobacter* spp., 1 *Klebsiella* species, 2 *Shigella* spp., 2 *Escherichia* spp., 1 *Citrobacter* species, 2 *Bacillus* spp., 2 *Pseudomonas* spp., 1 *Photobacterium* species, and 2 *Vibrio* spp. were retrieved from the GenBank, EMBL, and DDBJ databases (accession numbers M22935 [2], DQ859854 to DQ859890, DQ196414, AF301039, AY332960, AY332972, AY219007, AY727899, AY208918, AJ580873, AY686537, DQ995254, AJ511368, AJ515542, AY707924, AF301120, AY217064, AY332993, DQ458613, AJ223882, and AJ316152). The retrieved sequences were aligned as described above.

The sequences of the primers (*wzm*_F and *wzm*_R) targeting the *wzm* gene specific for *S. marcescens* were designed on the basis of the *wzm* sequence of *S. marcescens* (GenBank accession number L34166) (45). All available *wzm* gene sequences were retrieved from the GenBank, EMBL, and DDBJ databases (accession numbers L34166 [45], DQ907230, AY528413, BK000051, AJ605741, AY659979, Z18920, AJ007311, AJ007747, AY558875, AY337617, AY319940, AF337647, AY442352, AY653208, AF503594, AF328862, AY376146, AY253301, AF097519, AF285636, AE012010, AE012158, AF18284, AY028370, AF047478, AF189151, AF064070, L41518, L31775, D14156, AB010296, AB010293, and U63722). The retrieved sequences were aligned as described above.

The Q-PCR primers (16S_F and 16S_R) used for the 16S rRNA gene of *S. marcescens* have been described (25). The specificity of the primers (16S_F and 16S_R) has been tested previously (25) and in the present study.

All primers were tested for in silico specificity using the Basic Local Alignment Search Tool (BLAST v.2.2.15) (1). The specificity of the primers was also tested against 5 ng of genomic DNA isolated from pure cultures of target organisms (in this study, surrogate organisms) and nontarget organisms (closely related organisms) (Table 1). All primers used in this study were purchased from Integrated DNA Technologies (Coralville, IA).

DNA extraction from microbial cells and spores. Five methods of DNA extraction were evaluated for spore lysis and DNA release. Spores of *B. atrophaeus* ATCC 9372 were placed in sterile phosphate-buffered saline to 10^4 spores/ml, and then 100 μl of the suspension (equivalent to 10^3 spores) was subjected to different DNA extraction treatments as described below.

(i) Treatment 1 (freeze-thaw cycles followed by chemical lysis and bead beat homogenization). Samples were frozen at −80°C for 5 min and then immediately placed in a water bath at 65°C for 1 min to rapidly thaw. This process was repeated four times. Samples were then added into a 2-ml mini-bead beater tube containing a mixture of PowerSoil beads and 300 mg of 106-μm glass beads (Sigma-Aldrich, St. Louis, MO). Then, 60 μl of solution C1 (a lysis buffer containing sodium dodecyl sulfate) provided in the PowerSoil kit was added to the mixture, and the tube was placed in a mini-bead beater (Biospec Inc., Bartlesville, OK) and homogenized for 3 min at maximum speed. After homogenization, the genomic DNA was extracted using the PowerSoil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA) according to the manufacturer's instructions.

(ii) Treatment 2 (hot detergent treatment followed by bead beat homogenization). Samples were added to a 2-ml mini-bead beater tube containing a mixture of PowerSoil beads and 300 mg of 106-μm glass beads (Sigma-Aldrich, St. Louis, MO). Then, 60 μl of solution C1 was added to the mixture, and the mixture was incubated at 70°C for 20 min. After incubation, the tube was homogenized for 3 min at maximum speed. Genomic DNA was extracted using the PowerSoil DNA extraction kit protocol.

(iii) Treatment 3 (bead beat homogenization and chemical lysis). The genomic DNA was extracted following the PowerSoil DNA extraction kit protocol with one modification: 300 mg of 106-μm glass beads were added.

(iv) Treatment 4 (sonication at different times followed by chemical lysis. Samples were sonicated in 2-ml centrifuge tubes containing 100 mg of 106-μm glass beads using a Fisher Scientific model 550 Sonic Dismembrator (Pittsburgh, PA) at 20 kHz for 1, 2, or 3 min. After sonication, mixtures were transferred into 2-ml mini-bead beater tubes, and 60 μl of solution C1 was added to each mixture. The mixtures were vortexed for 5 seconds. After the mixtures were vortexed, the genomic DNA was extracted by following the PowerSoil DNA extraction kit protocol but without the bead beating step.

(v) Treatment 5 (sonication followed by chemical lysis and bead beat homogenization). Treatment 5 is the same as treatment 4, but after the mixtures were vortexed, the tubes were subjected to bead beat homogenization for 3 min at maximum speed.

The quality (A_{260}/A_{280}) and quantity (A_{260}) of extracted genomic DNA was determined with a Nanodrop (NanoDrop Technologies, Wilmington, DE) spec-

trophotometer. The extracted genomic DNA from each treatment was amplified in duplicate using Q-PCR, and the threshold cycles (C_T) were recorded (see below).

Q-PCR conditions. Real-time PCR was performed in a 25-μl reaction mixture volume containing 12.5 μl of 2× iQ SYBR green supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM [each] deoxynucleoside triphosphate, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR green I, 20 nM fluorescein, stabilizers) (Bio-Rad Laboratories, Hercules, CA), 0.5 μM of each primer, sample template (1 μl for the specificity experiment, 5 μl for the spore lysis experiment, 2 μl for standard curves using DNA extracted from a pure bacterial culture, and 5 μl for the spiking experiment), and RNase-free sterile water to a final volume of 25 μl. Amplification was performed using the iQ5 real-time detection system (Bio-Rad Laboratories, Hercules, CA) using the following program: (i) an initial denaturing step at 95°C for 5 min; (ii) 45 cycles, with 1 cycle consisting of denaturation at 95°C for 50 s, annealing at 63°C for 50 s, and extension at 72°C for 45 s; and (iii) a final extension step at 72°C for 7 min. All assays were identical in primer concentration and annealing temperature. Q-PCR assays with C_T values over 40 were considered negative. For each PCR run, a negative (no-template) control was used to test for false-positive results or contamination. The presence of nonspecific products or primer dimers was confirmed by observation of a single melting peak in a melting curve analysis using the iCycler iQ5 optical system software v1.0. In addition, the PCR products were subjected to gel electrophoresis on 1.5% agarose gels stained with ethidium bromide to confirm the absence of nonspecific products or primer dimers.

Q-PCR standard curves. SBD contained the following components (dry weight shown as a percentage in parentheses) (based on information in reference 32): ceiling tile (12.79), carpet (4.47), vinyl (0.78), electronics (5.56), furniture (33.68), white office paper (34.29), folders/cardboard (4.22), and mixed office paper (4.21). The SBD materials were shredded and mixed in the proportions given above. After the material was shredded, it was ground in a Wiley mill to pass a 1-mm screen for use in the spiking experiment. Leachate for the spiking experiment was obtained from a 208-liter drum containing well-decomposed residential waste incubated at 37°C.

To determine the limits of detection, linear ranges, and amplification efficiencies of the Q-PCR assays, two types of standard curves were constructed: (i) standard curves based on DNA extracted from a pure bacterial culture; and (ii) standard curves constructed using DNA extracted from spiked sterile water, SBD, and leachate samples.

Standard curves with DNA extracted from a pure bacterial culture were constructed using serial dilutions of genomic DNA (75 ng, 7.5 ng, 0.75 ng, 75 pg, 7.5 pg, 0.75 pg, 75 fg, 7.5 fg, and 0.75 fg per amplification reaction mixture) from *B. atrophaeus* ATCC 9372 and *S. marcescens* ATCC 13880. For each Q-PCR assay, the experiment was repeated twice on separate plates with triplicate Q-PCRs per experiment ($n = 6$) to determine intra-assay reproducibility (within a plate) and interassay reproducibility (between plates). No-template controls were included in all PCR runs.

To construct standard curves based on spiked environmental samples, overnight cultures of *B. atrophaeus* ATCC 9372 vegetative cells and *S. marcescens* ATCC 13880 cells grown in nutrient broth were harvested by centrifugation at $4,000 \times g$ for 10 min. The resulting pellets were resuspended in sterile water, and total bacterial counts were determined using a Cellometer (Nexcelom Bioscience, Lawrence, MA) and phase-contrast microscope at a magnification of ×40. In addition, four replicate *B. atrophaeus* ATCC 9372 spore suspensions were counted using a Cellometer. The serial dilutions for spiking experiments were based on those counts. *B. atrophaeus* ATCC 9372 vegetative cells or spores (100 μl) and *S. marcescens* ATCC 13880 cell suspensions (100 μl) were simultaneously added in increasing amounts (ranging from 10^0 to 10^7 cells or spores) to leachate (0.3 ml), SBD (0.5 g), and sterile water (0.3 ml) samples. Immediately after spiking, genomic DNA from each sample was extracted using the hot detergent and bead beat homogenization method (treatment 2). The spiking experiment was repeated twice on the same day with triplicate Q-PCRs per spiking experiment/assay ($n = 6$). Duplicate spiking experiments/assays were run on the same plate. No-template controls and nonspiked samples were included in all Q-PCR runs.

Data analysis. Data collection and analysis were performed using iQ5 optical system software v1.0 (Bio-Rad Laboratories, Hercules, CA). All statistical analyses were performed using Microsoft Excel 2003. The amplification efficiency, E, was calculated from the slope of the standard curve using the formula $E = 10^{-1/\text{slope}} - 1$ (19). The coefficients of variation (CV) for the slope, y intercept, R^2, and E values were used to determine intra-assay reproducibility (within a plate) and interassay reproducibility (between plates). The slopes and y intercepts among replicate standard curves obtained from two DNA extractions (corresponding to duplicate spiking experiments), each quantified in triplicate on the same plate ($n = 6$) were compared using paired sample t tests at $P < 0.05$.

RESULTS AND DISCUSSION

Specificity of the assays. The annealing temperatures and primer concentrations of all six assays (Table 2) were optimized to determine the temperature and primer concentration that gave the best specificity without reduction in yield. The optimal annealing temperature and primer concentration for all Q-PCR assays were determined to be 63°C and 500 nM, respectively.

The BLAST results did not show any significant homology to other published sequences in the GenBank, DDBJ, and EMBL databases. The specificity of the primers in the present study was also verified empirically by running Q-PCR using 5 ng of genomic DNA isolated from 21 closely related organisms (Table 1). When testing the specificity of the newly developed Q-PCR assays (ITS, *gyrB*, $recA_{Sm}$, and *wzm*), the majority of non-*S. marcescens* and non-*B. atrophaeus* species showed negative C_T values (C_T values over 40 were considered negative) with the exception of a few species that were detected but only at higher C_T values ($C_T \geq 36$) compared to the C_T values ($16 \leq C_T \leq 18$) of the target organisms (Table 1). These findings indicate that the Q-PCR assays developed in this study are specific and that a minimum of 5 ng of DNA is required to generate a positive signal for some nontarget organisms. The $recA_{Ba}$ primers developed by Buttner et al. (4, 5) as tested here showed high specificity (Table 1). However, this is the first report of their specificity.

Although the 16S rRNA primers (16S_F and 16S_R) have been used successfully for the specific identification of *S. marcescens* in blood samples (25), they resulted in poor specificity in this study, as most of the tested organisms showed slight, but detectable, amplification at C_T values of ≥33 (Table 1). The difference in specificity results between the two studies may be attributed to the fact that a different group of species was tested for specificity in the current study. In addition, SYBR green chemistry was used in this study and TaqMan probe chemistry was used in the previous study (25), which may have contributed to higher specificity. Despite the higher specificity of TaqMan probe chemistry compared to SYBR green chemistry, it is not always possible to design assays for TaqMan probe chemistry. These assays require the design of primers and probes that must satisfy rigid constraints that cannot always be easily applied, especially with the gene sequences selected in this study. For subsequent experiments, we decided to use only *gyrB*, $recA_{Ba}$, and *wzm* Q-PCR assays for the detection and quantification of *S. marcescens*.

The absence of nonspecific products or primer dimers was confirmed by performing a melting curve analysis. The melting curve analysis showed a single clear melting peak for all Q-PCR assays and no formation of nonspecific products (Fig. 1). In addition, agarose gel electrophoresis on 1.5% agarose gels stained with ethidium bromide confirmed the absence of nonspecific products or primer dimers (data not shown). All no-template controls tested negative for the six Q-PCR assays used in this study.

The rationale behind the choice of the 16S-23S rRNA ITS region and protein-coding genes for the design of specific Q-

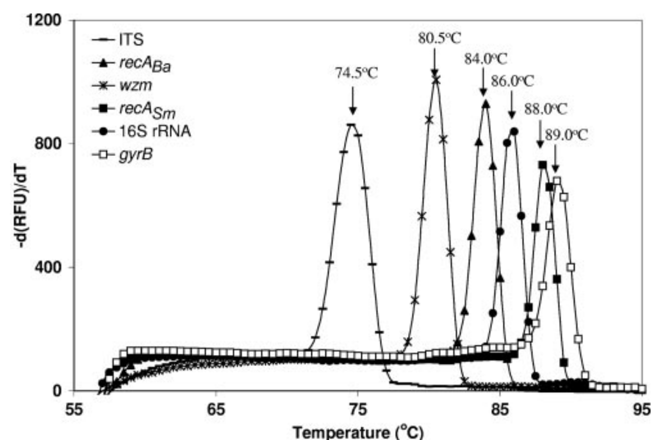

FIG. 1. Melting curve analysis with the corresponding melting temperatures of the six Q-PCR assays. RFU, relative fluorescence units.

PCR assays for the detection of *B. atrophaeus* and *S. marcescens* is that these target sequences exhibit higher genetic variation than the more conserved 16S rRNA gene and therefore can be utilized for differentiating species of closely related taxa (6, 9, 10, 27, 42, 43, 50). For example, because of its high nucleotide sequence variability, the *gyrB* gene has been shown to be a more reliable phylogenetic marker than the 16S rRNA gene for classifying *Serratia* species (9). Qi et al. (43) utilized the *rpoB* gene to differentiate *B. anthracis* from closely related bacilli. Similarly, Palmisano et al. (42) reported the use of *rpoB* gene to differentiate *Bacillus licheniformis* from the closely related species *Bacillus sonorensis*. Chun and Bae (6) demonstrated the usefulness of the *gyrA* gene for the rapid identification of *B. subtilis* and related taxa. Johnson et al. (27) and Shaver et al. (50) reported the use of 16S-23S rRNA ITS region to differentiate *B. subtilis* from the closely related species *B. atrophaeus*.

Extraction methods tested for maximum recovery of DNA from lysed spores. Spore suspensions were tested for purity by phase-contrast microscopy at a magnification of ×100 to check for the presence of vegetative cells. In addition, to check for the presence of extracellular DNA that could be carried over from vegetative cells during spore preparation, 1 ml of spore suspension (10^8 spores) was subjected to centrifugation at 14,000 × *g* for 15 min to pellet the spores. The supernatant was then subjected to DNA extraction followed by Q-PCR. The results from both phase-contract microscopy and Q-PCR revealed the high purity of the spore suspensions obtained from the NAMSA Ohio laboratory (Northwood, OH). The C_T values from an evaluation of several spore lysis methods were all between 31.5 and 33.5 (data not shown), suggesting that the methods tested were equally efficient in lysing the spores. In addition, spores were sonicated for different times (1, 2, or 3 min), with or without a bead beating step, using a sonicator probe in the presence of 106-μm glass beads and the calculated C_T values were again similar (data not shown).

Several studies have reported that bead beat homogenization and sonication are effective in lysing spores (3, 15, 27, 28, 37, 59). Kuske et al. (30) reported that hot detergent and freeze-thaw (40 cycles) treatments were not effective in lysing *B. atrophaeus* and *Fusarium moniliforme* spores and that bead beat homogenization was the most effective method for extracting DNA from spores. It was shown using microscopic counts that *Bacillus* spores are resistant to freeze-thaw treatment (37). Similarly, Keswani et al. (28), Williams et al. (59), and Haugland et al. (15) have demonstrated that the bead beating method was most effective in recovering DNA from fungal spores. Belgrader et al. (3) improved the sonication time for spore lysis (30 seconds using a sonicator probe and 2 min using a sonicating water bath) by using 106-μm glass beads in the suspension. They reported that using a sonicator probe improved the limit of detection of spores by 3 log units.

Based on the comparisons of spore lysis methods presented here, the hot detergent treatment followed by bead beat homogenization (treatment 2) was selected to extract genomic DNA from bacterial cells and spores because it was shown to be equally effective in lysing spores, easy to conduct, and fast and has high sample processing capacity compared to treatments with sonication.

Limits of detection, linear ranges, and reproducibility of the Q-PCR assays using pure culture DNA. Standard curves for the five Q-PCR assays developed in this study were constructed (not shown). The limit of detection, corresponding to the smallest amount of template DNA resulting in positive amplification in all replicates (35, 40, 46), for *B. atrophaeus* genomic DNA using the ITS and $recA_{Ba}$ Q-PCR assays was 7.5 fg per PCR (2 genome equivalents). The limits of detection of *S. marcescens* genomic DNA using *gyrB*, *wzm*, and $recA_{Sm}$ Q-PCR assays were 7.5 fg (1 genome equivalent), 75 fg (13 genome equivalents), and 7.5 fg per PCR (1 genome equivalent), respectively. Genome equivalents were calculated assuming that one molecule of *B. atrophaeus* and *S. marcescens* DNA corresponds to 4.45 and 5.4 fg of DNA, respectively, according to equation 1 below (46) and considering a genome size of 4.2 and 5.1 Mb as determined for *Bacillus subtilis* subsp. *subtilis* strain 168 (29) and *Serratia marcescens* strain Db11 (information found at Sanger Institute [http://www.sanger.ac.uk/Projects/S_marcescens/]), respectively.

$$\text{DNA (fg)} = [\text{genome size (bp)}]\left(\frac{600\ \text{Da}}{\text{bp}}\right) \times \left(\frac{1.6 \times 10^{-27}\text{kg}}{\text{Da}}\right)\left(\frac{10^{18}\ \text{fg}}{\text{kg}}\right) \quad (1)$$

Quantitative amplification parameters for *B. atrophaeus* were optimal with linearity over an 8-log-unit dynamic range ($R^2 >$ 0.99 for both ITS and $recA_{Ba}$ Q-PCR assays), and the overall PCR amplification efficiencies for the ITS and $recA_{Ba}$ Q-PCR assays were 90.39% and 97.98%, respectively. Similarly, quantification of *S. marcescens* was linear over a 7-log-unit (*gyrB*) to 8-log-unit ($recA_{Sm}$ and *wzm*) dynamic range ($R^2 > 0.99$), and the overall PCR amplification efficiencies for the *gyrB*, $recA_{Sm}$, and *wzm* Q-PCR assays were 95.08%, 95.21%, and 96.04%, respectively. From the amplification efficiency values, it can be determined that the slopes of the standard curves were very close to the theoretical optimum of −3.32. A lower *y* intercept indicates greater sensitivity at a given cycle number. The *y* intercept values from the standard curves showed that the ITS Q-PCR assay (*y* intercept = 15.503) has greater sensitivity than

TABLE 3. Intra- and interassay CVs for reproducibility of the Q-PCR assays

Q-PCR assay	CV											
	R^2			E (%)			y intercept			Slope		
	Expt 1[a]	Expt 2[a]	Expt 1 + 2[b]	Expt 1	Expt 2	Expt 1 + 2	Expt 1	Expt 2	Expt 1 + 2	Expt 1	Expt 2	Expt 1 + 2
ITS	0.20	0.41	0.44	3.24	1.92	2.43	0.02	0.61	5.36	2.35	1.40	1.78
$recA_{Ba}$	0.74	0.12	0.82	1.86	2.28	2.08	0.93	0.16	5.00	1.34	1.66	1.49
gyrB	0.55	0.26	0.44	2.94	3.14	2.93	0.75	1.01	0.74	2.16	3.18	2.15
wzm	0.02	0.17	0.42	3.71	5.12	3.81	0.12	0.20	1.94	2.71	3.85	2.75
$recA_{Sm}$	0.08	0.18	0.24	1.80	1.64	2.21	0.02	0.59	3.17	1.30	1.24	1.60

[a] Intra-assay CV. Each experiment consists of triplicate Q-PCRs/assay/plate. Experiments 1 and 2 were run on separate plates.
[b] Interassay CV for a total of six replicate Q-PCRs/assay in two separate experiments (experiments 1 and 2, with triplicate Q-PCRs/experiment).

the $recA_{Ba}$ Q-PCR assay (y intercept = 17.238), and the *gyrB* (y intercept = 17.354) and *wzm* (y intercept = 17.46) Q-PCR assays have greater sensitivity than the $recA_{Sm}$ Q-PCR assay (y intercept = 20.067).

The presence of multiple copies of genes of interest on the genome of the target organism is important because it provides a means to increase the sensitivity of the assay and allows detection of a low number of target organisms in environmental samples (36). The fact that the ITS Q-PCR assay was more sensitive than the $recA_{Ba}$ Q-PCR assay is likely due to the presence of seven copies of the 16S rRNA and 23S rRNA genes per genome in *B. atrophaeus* (information found at The Ribosomal RNA Operon Copy Number Database [http://rrndb.cme.msu.edu]). Data on the number of $recA_{Ba}$ gene copies in *B. atrophaeus* and $recA_{Sm}$ and *wzm* gene copies in *S. marcescens* could not be identified. The *gyrB* gene is present as a single-copy gene in all bacteria (22). The results show that *gyrB*, *wzm*, and $recA_{Sm}$ Q-PCR assays have similar amplification efficiencies that are very close to the optimal value of E = 1 (i.e., the template is doubled in each amplification cycle), and under these conditions, it is possible to calculate the number of gene copies of *wzm* and $recA_{Sm}$, according to equation 2 (46):

$$\text{Number of } wzm \text{ or } recA_{Sm} \text{ genes} = 2^{-dC_T} \quad (2)$$

where $dC_T = C_T$ (*wzm* or $recA_{Sm}$) $- C_T$(*gyrB*). From this equation, the calculated detectable average numbers of *wzm* and $recA_{Sm}$ gene copies were 1 and 0.17, respectively. The fractional copy number of $recA_{Sm}$ does not imply an actual gene copy number but a detectable copy number relative to that of *gyrB*. Thus, for the same mass of target DNA, *gyrB* and *wzm* Q-PCR assays will detect sixfold-more genes (1/0.17) than the $recA_{Sm}$ Q-PCR assay.

To evaluate the reproducibility of the Q-PCR assays, two experiments (experiments 1 and 2) were conducted. Each experiment consisted of triplicate standard curves generated by plotting the C_T values versus log ng DNA/PCR. Experiments 1 and 2 were run on separate plates. A total of six standard curves were constructed (three standard curves/experiment). The slope, y intercept, percent efficiency, and R^2 values from the three standard curves in each experiment were pooled, and the averages and standard deviations were used to calculate the intra-assay CVs (within an experiment) and interassay reproducibility (between experiments) (Table 3). The low CVs for both the inter- and intra-assay variability (0.02% to 5.36%) indicate good reproducibility of the Q-PCR standard curves. In the literature, most of the variability in Q-PCR assays has been described based on C_T values and gene copy numbers (11, 51). Smith et al. (51) reported intra-assay CVs for the numbers of 16S rRNA gene copies between 3.16% and 9.09%. They also reported interassay CVs between 0.27% and 1.50% for the C_T values and between 11.24% and 26.02% for the gene copy number. A similar range of CVs for the C_T and gene copy number was reported by Dionisi et al. (11). In the present study, the CVs for the C_T values ranged between 0.09% and 5.22% for intra-assay variability and between 0.27% and 7.37% for interassay variability.

Limits of detection and linear ranges of the Q-PCR assays in sterile water, SBD, and leachate samples spiked with surrogate organisms. Quantitative amplification parameters for the Q-PCR assays are presented in Table 4. Amplification parameters for *B. atrophaeus* using the ITS Q-PCR assay were optimal, with linearity ($R^2 > 0.98$) over a 7-log-unit dynamic range and a detection limit of 10^1 *B. atrophaeus* vegetative cells or spores (or 2×10^1 cells or spores per g of SBD or ml of leachate or sterile water), with amplification efficiencies ranging between 0.93 and 1.04 (Table 4). For comparison, the $recA_{Ba}$ Q-PCR assay was used to determine the detection limit of *B. atrophaeus* spores in SBD where linearity ($R^2 > 0.99$) was over a 6-log-unit dynamic range, and the detection limit was 20×10^1 spores/g of SBD (Table 4). The fact that the amplification parameters for *B. atrophaeus* vegetative cells and spores in leachate samples were very similar (Table 4) suggests that the hot detergent treatment followed by bead beat homogenization was effective in spore lysis. In contrast, amplification parameters for *B. atrophaeus* vegetative cells and spores in SBD were different (Table 4). This difference may be because *B. atrophaeus* spores are hydrophobic (12) and they adhere to different solid matrices (e.g., soil and building debris), thus requiring treatment for spore recovery before lysis. Dragon and Rennie (12) reported that solutions containing both a nonionic detergent (Triton X-100 or Nonidet P-40) and buoyant concentrations of sucrose, which helps to lift spores after disruption of hydrophobic forces using nonionic detergents, improved the recovery of *B. anthracis* spores from soil. Similarly, Ryu et al. (48) showed that more *B. anthracis* spores in soil samples were detected when a solution of sucrose and Triton X-100 was used than when a solution of phosphate-buffered saline and Triton X-100 was used. In the present study, no pre-PCR treatment for spore recovery from SBD samples was made.

Quantitative amplification parameters for *S. marcescens* using the *gyrB*, *wzm*, and $recA_{Sm}$ Q-PCR assays were optimal, with

TABLE 4. Quantitative amplification parameters for the Q-PCR assays in leachate and SBD

Q-PCR assay	Sample	Target	Amplification parameter[a]				Linear range (no. of cells or spores)
			E	Slope	y intercept	R^2	
ITS	Leachate	*B. atrophaeus* vegetative cells	0.99 ± 0.07	−3.34 ± 0.18	37.7 ± 0.8	>0.98	10^1–10^7
		B. atrophaeus spores	0.97 ± 0.04	−3.39 ± 0.12	38.7 ± 0.6	>0.98	10^1–10^7
	SBD	*B. atrophaeus* vegetative cells	0.96 ± 0.05	−3.42 ± 0.13	36.7 ± 0.6	>0.98	10^1–10^7
		B. atrophaeus spores	1.04 ± 0.22	−3.24 ± 0.52	39.6 ± 2.7	>0.98	10^1–10^7
	Sterile water	*B. atrophaeus* vegetative cells	0.93 ± 0.08	−3.51 ± 0.21	38.6 ± 1.0	>0.98	10^1–10^7
recA$_{Ba}$	SBD	*B. atrophaeus* spores	0.87 ± 0.01	−3.66 ± 0.04	39.9 ± 0.3	>0.99	10^2–10^7
gyrB	Leachate	*S. marcescens*	1.04 ± 0.04	−3.24 ± 0.09	39.4 ± 0.6	>0.98	10^2–10^7
	SBD	*S. marcescens*	0.82 ± 0.07	−3.85 ± 0.28	43.9 ± 1.4	>0.99	10^2–10^7
recA$_{Sm}$	Leachate	*S. marcescens*	1.03 ± 0.05	−3.26 ± 0.11	43.1 ± 0.7	>0.97	10^2–10^7
	SBD	*S. marcescens*	0.78 ± 0.03	−3.98 ± 0.11	46.7 ± 0.4	>0.99	10^2–10^7
wzm	Leachate	*S. marcescens*	0.94 ± 0.05	−3.47 ± 0.13	38.9 ± 0.5	>0.98	10^2–10^7
	SBD	*S. marcescens*	0.82 ± 0.02	−3.86 ± 0.07	40.6 ± 0.4	>0.99	10^2–10^7
	Sterile water	*S. marcescens*	0.81 ± 0.03	−3.87 ± 0.12	41.2 ± 0.9	>0.99	10^2–10^7

[a] The value for each parameter except R^2 is a mean ± standard deviation of two DNA extractions (corresponding to two spiking experiments), each quantified in triplicate on the same plate ($n = 6$).

linearity ($R^2 > 0.98$) over a 6-log-unit dynamic range and a detection limit of 10^2 *S. marcescens* cells (or 20×10^1 cells per g of SBD or ml of leachate or sterile water) (Table 4). The amplification efficiencies for all three assays were higher in leachate (ranging between 0.94 and 1.04) than in SBD (ranging between 0.78 and 0.82). In addition, higher sensitivities (lower y intercept) were observed for leachate than for SBD. Although *wzm* and *gyrB* Q-PCR assays resulted in similar sensitivities when using DNA extracted from a pure bacterial culture, their sensitivities in environmental samples were different, with *wzm* Q-PCR assay showing higher sensitivity (Table 4). These findings suggest that the sensitivity of an assay using DNA extracted from a pure bacterial culture does not always reflect the performance of the assay in environmental samples. All five Q-PCR assays tested negative for the no-template and nonspiked samples.

To assess the variability of the Q-PCR assays between duplicate DNA extractions corresponding to duplicate spiking experiments, paired sample t tests were used, and the results showed that the difference in slopes and y intercepts among replicate standard curves obtained from two DNA extractions (corresponding to two spiking experiments), each quantified in triplicate on the same plate ($n = 6$), were not statistically significant ($P > 0.05$). These results illustrate the low variability in DNA extraction efficiencies between duplicate spiking experiments.

A major limitation of Q-PCR is the occurrence of false-negative results due to the presence of PCR inhibitors in environmental samples. Internal controls have been used by several investigators (35, 46) for the identification of false-negative PCR using TaqMan chemistry. The C_T values obtained for leachate and SBD were similar to the C_T values obtained for sterile water samples using the ITS and *wzm* Q-PCR assays (data not shown), suggesting that PCR inhibition due to the presence of nonbiological contaminants in leachate and SBD was insignificant.

Another limitation to the application of Q-PCR-based tests is the potential variability in cell lysis and DNA extraction efficiency. Therefore, this variation must be taken into consideration for reliable quantification. Several investigators (7, 26, 58) have used internal controls to quantify losses due to cell lysis and nucleic acid extraction. Although the actual target organism is the best control for quantifying losses in nucleic acid extraction, it is rarely used because of the potential natural occurrence of the target organism in the same environmental sample. To evaluate extraction efficiency, standard curves for quantification were constructed by spiking known amounts of *B. atrophaeus* ATCC 9372 (vegetative cells or spores) and *S. marcescens* ATCC 13880 cell suspensions into leachate, SBD, and sterile water. Quantification by this method corrects for losses in cell lysis and target DNA during extraction. In addition, all nonspiked samples tested in this study resulted in negative PCR, suggesting that *B. atrophaeus* and *S. marcescens* were not present in the SBD and leachate samples used in this study.

In summary, the developed Q-PCR assays are highly specific and sensitive and can be used for monitoring the fate and transport of the BW surrogates *B. atrophaeus* and *S. marcescens* in building debris and leachate samples which is our intended application. Although the assays used in this study were designed primarily for biodefense research, they could be applied to detect and quantify *B. atrophaeus* and *S. marcescens* in any sample, provided that PCR inhibition is minimal and nucleic acid extraction losses can be quantified.

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