Quantitative PCR for Tracking the Megaplasmid-Borne Biodegradation Potential of a Model Sphingomonad

Erica M. Hartmann,a Jonathan P. Badalamenti,a Rosa Krajmalnik-Brown,a,b and Rolf U. Haldenc,b,c

The Swette Center for Environmental Biotechnology, The Biodesign Institute, Arizona State University, Tempe, Arizona, USA; School of Sustainability and the Built Environment, Ira A. Fulton Schools of Engineering, Arizona State University, Tempe, Arizona, USA; and Department of Environmental Health Sciences, Johns Hopkins University, Bloomberg School of Public Health, Baltimore, Maryland, USA

We developed a quantitative PCR method for tracking the dxnA1 gene, the initial, megaplasmid-borne gene in Sphingomonas wittichii RW1’s dibenzo-p-dioxin degradation pathway. We used this method on complex environmental samples and report on growth of S. wittichii RW1 in landfill leachate, thus furnishing a novel tool for monitoring megaplasmid-borne, dioxygenase-encoding genes.

The genus Sphingomonas is an unusual group of alphaproteobacteria known for their extraordinary ability to degrade recalcitrant environmental pollutants (24). A survey of 18 sphingomonads showed that almost all strains carried 2 to 5 different, large (50- to 500-kb) megaplasmids (2). The biodegradative genes of these sphingomonads are often contained on extrachromosomes (2), but they may not be organized in traditional operons or located on the same plasmid (21). The frequency with which biodegradative sphingomonads are discovered, coupled with their unique genetic structure and reluctance to share plasmids among other genera, suggests that these megaplasmid-harboring organisms have an adaptive advantage to metabolize anthropogenic environmental pollutants (20).

Sphingomonas wittichii RW1 is the first identified aerobic bacterium that can degrade mono- through hexachlorinated dioxins (5, 7, 14) and use dibenzo-p-dioxin or dibenzofuran as the sole source for carbon and energy (18). Polychlorinated dibenzo-p-dioxins are ubiquitous environmental pollutants from various sources, including waste incineration (6). S. wittichii RW1 has been investigated as a bioremediation agent and shows promise (13, 14). It is also a model sphingomonad, thanks to its high degree of characterization and the complete sequencing of its genome and two megaplasmids (12).

Dibenzo-p-dioxin degradation proceeds through the dioxin dioxygenase, which is encoded by the dxnA1 and dxnA2 genes, together denoted dxnA1A2, on the 220-kb plasmid, pSWIT02 (GI: 148550845) (12). This enzyme catalyzes the initial dihydroxylation of the aromatic ring (18), leading to its spontaneous cleavage into 2,2′,3-trihydroxybiphenyl, which is dihydroxylated by a second enzyme into 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate and then hydrolyzed to form salicylic acid (19). These downstream metabolites can then be funneled into the citric acid cycle and used for carbon and energy. The dioxin dioxygenase also transforms chlorinated congeners of dibenzo-p-dioxin, resulting in the formation of chlorinated salicylates (19). This initial transformation destroys and detoxifies the planar structure of the molecule (15), but the resultant chlorinated intermediates typically do not support growth.

Due to its crucial role in dioxin degradation, the dioxin dioxygenase is therefore a promising candidate biomarker (4). A protein-based detection method for dioxin dioxygenase exists (4), and standard PCR primers have been published for the study of the dxnA1A2 cistron (1, 2) and for ring-hydroxylating dioxygenases in general (8). However, due to the amplicon length or lack of specificity, these primers are not appropriate for quantitation, which is important in linking microbial activity with chemical transformations to track bioremediation (22).

In the present study, we developed quantitative PCR (qPCR) primers to detect the dxnA1 gene with sufficient sensitivity and specificity for use in environmental samples. We then used this method to determine the copy number of the pSWIT02 megaplasmid and monitor S. wittichii RW1 in bioaugmented landfill leachate.

S. wittichii RW1 was routinely grown in pure culture in minimal medium supplemented with dibenzofuran as previously described (4). Plasmid DNA was extracted using a BACMAX DNA purification kit (Epitect, Madison, WI). The dxnA1A2 gene cluster was amplified as described previously (2). To generate a positive control, the product was treated using components of the QiaQuick PCR purification kit (Qiagen, Valencia, CA), cloned into the pCR4-TOPO plasmid using the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA), and sequenced using an Applied Biosystems 3730 capillary sequencer. A Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/) search of the sequence of the cloned product showed 99.95% identity to dxnA1 (GI:4007779) with 0% gaps and complete coverage.

The qPCR primers for dxnA1 were designed using the Primer3 software v1.1.4 (17) and evaluated for secondary structures using NetPrimer (Premier Biosoft International, Palo Alto, CA). The specificity of the primers was checked in silico using Primer-BLAST, which found no nonspecific amplification for the selected primer pair (product size between 60 and 300 bp; minimum of 6 mismatches to ignore targets).

Optimized qPCR conditions were as follows: (i) 95°C for 2 min; (ii) 40 cycles, with 1 cycle consisting of 95°C for 10 s, 58°C for 20 s, and 68°C for 30 s. A melting curve was generated at the end of
the last cycle. Primers were supplied at a concentration of 300 nM. The qPCR procedure was performed using 5 PRIME RealMasterMix for SYBR green (Fisher Scientific, Pittsburgh, PA) on a Mastercycler ep realplex instrument (Eppendorf, Hamburg, Germany). qPCR mixtures contained 4 µl of template DNA in a final reaction mixture volume of 10 µl.

The primer pair dxnA1fwd321-dxnA1rev451 (Table 1) produced a standard curve using 10-fold serial dilutions of our dxnA1 control plasmids with a slope of 3.33, indicating a near-ideal amplification efficiency of 1.01, linearity over 8 orders of magnitude, and a limit of quantification of 62 copies (Fig. 1). The corresponding melting curve showed only one peak, indicating a unique product.

To evaluate primer specificity, DNA extracted from *S. wittichii* RW1 was introduced at concentrations ranging from 1 ng/µl to 0.001 ng/µl (10^2 to 10^6 copies), into a background of DNA extracted from an activated sludge sample from the aeration tank of the Mesa Northwest Wastewater Reclamation Project in Mesa, AZ, or agricultural soil samples from Baltimore, MD (23) (Fig. 1). The corresponding melting curve showed only one peak, indicating a unique product.

Amplification of target DNA was linear over 4 orders of magnitude, from 10^2 to 10^6 copies (R^2 = 0.99); the fluorescence signal from the background samples was similar to that of the nontemplate controls. This performance is similar to that of other methods used for 16S rRNA genes (linearity from 10^2 to 10^7 target cells, with each cell containing between 1 and 15 copies of the 16S rRNA gene, against a background of DNA from 7 nontarget bacteria) (11) and the benzylsuccinate synthase gene *bssA* (linearity from 10^2 to 10^6 genome copies against a background of 3 nontarget bacteria) (3). Probe-based, as opposed to SYBR green-based, qPCR methods can achieve detection limits an order of magnitude lower (16).

To determine the copy number of the pSWIT02 megaplasmid, qPCR was also performed targeting the 16S rRNA genes (11). There are two copies of this gene on the chromosome and none on either megaplasmid (12). A comparison of *dxnA1* to 16S rRNA gene copy number in *S. wittichii* RW1 grown on dibenzofuran showed a ratio of 1.0 ± 0.1 (average ± standard error; n = 19), implying a 2.0 ± 0.2 ratio of pSWIT02 to chromosome.

Microcosms were created to test the survival of *S. wittichii* RW1 in landfill leachate. The leachate was known to contain acetone, benzene, 2-butane, 1,4-dichlorobenzene, dichloroethane, dichloroethene, dichloropropane, ethylbenzene, methylene chloride, 4-methyl-2-pentanone, tetrachloroethene, toluene, trichloroethene, vinyl chloride, and xylenes in the µg/liter range. Dibenzo-furan from a methanol stock solution was added to sterile test tubes to a final concentration of 0.5 mg/ml. Landfill leachate was pasteurized to inactivate endogenous microorganisms by heating to 65°C for 1 h in a water bath in sealed containers to prevent evaporative compound losses and added to the test tubes after cooling to room temperature. Microcosms were inoculated with 50 µl of a pure culture of *S. wittichii* RW1, resulting in an approximate density of 10^7 CFU/ml, which is comparable to previous studies (5). This high inoculum is necessary because the population declines as the contaminant is transformed (5, 13),

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’ Base</th>
<th>3’ Base</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dxnA1fwd321</td>
<td>321</td>
<td>341</td>
<td>TCATG GGCTG GTTGT CAATA</td>
</tr>
<tr>
<td>dxnA1rev451</td>
<td>431</td>
<td>451</td>
<td>CGAAA ATCAG CCCCT TGTA</td>
</tr>
</tbody>
</table>

*In the primer names, fwd stand for forward and rev stands for reverse.*

*The positions in *S. wittichii* RW1 are shown.*

**FIG 1** Correlation of observed copy number and concentration of template DNA from a pure culture of *Sphingomonas wittichii* RW1 and samples spiked with DNA background. Genomic DNA from pure culture was diluted in water (RW1) or in DNA extracted from sludge (RW1 + sludge) or soil (RW1 + soil). Copy numbers calculated from the unspiked sludge and soil samples were less than the limit of quantitation. Data are shown on a log-log plot for ease of visualization. Each point is an average of three replicates; error bars representing the standard error may be smaller than the marker. The two insets show the slopes of the respective dilutions (top right graph) and the qPCR standard curve for the designed primer set (dxnA1fwd321-dxnA1rev451) (bottom left graph). Standard curves were generated using a recombinant plasmid containing the gene encoding dioxin dioxygenase. Results shown here are typical of several qPCR runs.
FIG 2 Measurement of total DNA, dxnA1 copy number, and colony counts from *Sphingomonas wittichii* RW1-bioaugmented landfill leachate microcosms. The points at days 3, 6, 9, 12, and 14 show the average values ± standard errors (error bars) of three biological replicate samples. All other measurements are single data points. Measurements are normalized per milliliter of culture.

likely because the chlorinated metabolites, especially chlorocatechols, can inhibit enzymes in this and other catabolic pathways (5, 19). The tubes were incubated horizontally at room temperature without agitation and sampled daily with three replicate samples taken 3, 6, 9, 12, and 14 days postinoculation. Colony counts were performed by spreading dilutions on Luria broth (LB) agar plates. DNA was extracted with the NucleoSpin soil kit and diluted 1:10 in water for qPCR. Fluorescence signal from uninoculated leachate was not significantly different from that of the nontemplate control.

Total DNA, *dxnA1* copy numbers, and colony counts all showed a similar trend (Fig. 2), indicating that the population of *S. wittichii* RW1 increased initially and then was stable. Results from the qPCR assay showed less variation than colony counts because of the greater specificity of the non-culture-based assay that was insensitive to a background of leachate-endogenous microorganisms that survived pasteurization and persisted at levels at 1 or more orders of magnitude less than *S. wittichii* RW1.

In the leachate microcosms, there were 0.5 ± 0.1 (average ± standard error; n = 13) *dxnA1* copies per CFU, which is lower than the pure culture copy number, suggesting that a part of the RW1 community is making use of carbon sources in leachate other than dibenzofuran. The correlation between colony count and *dxnA1* was poor (R² = 0.26). Other studies have also noted discrepancies between colony counts and qPCR assays (10). That *dxnA1* is on a megaplasmid may contribute to the lack of correlation, as not every cell contains the plasmid. While the percentage of the population carrying the plasmid varies, it is unlikely to be transferred to other organisms, even in the presence of a selective advantage (2).

The *dxnA1* qPCR results were consistently lower than the colony counts, which is what would be expected if plasmid copy number were less than one. This observation suggests that biodegradative genes can be lost, so culture-based assays or methods targeting chromosomal genes, e.g., 16S rRNA genes, may overestimate biodegradative capability when the necessary genes are carried on plasmids, as is often the case with *sphingomonads* (2). Similar observations have been made in the anaerobic world concerning vinyl chloride reductase genes in *Dehalococcoides* (22), which are flanked by insertion sequences (9) and therefore also subject to loss in the absence of positive pressure (3).

Previous studies looking at *S. wittichii* RW1 activity in contaminated environments have been hampered by a lack of molecular tools to adequately evaluate the bacterial population. Instead, they rely on dioxin removal rates as a proxy for bacterial activity and survival (5, 13). The actual population levels are therefore unknown. We believe this qPCR method will help answer questions about population levels in the face of inhibitory metabolites and the retention of biodegradative capability despite the availability of other carbon sources. We have already provided evidence that biodegradative capability can be lost in a complex medium containing multiple carbon sources by comparing *dxnA1* copy number to colony counts in landfill leachate microcosms. For *in situ* studies where it is impractical to perform colony counts due to the presence of other microorganisms, quantification of *dxnA1* could be combined with general or *Sphingomonas*-specific 16S rRNA gene assays to compare population levels and biodegradative capability.

We created a qPCR method for *dxnA1*, the gene encoding dioxin dioxygenase, an important enzyme in the dioxin degradation pathway of *S. wittichii* RW1. This qPCR method is accurate over 8 orders of magnitude and has a limit of quantitation of 62 copies per reaction mixture. We have demonstrated the specificity of the primers over 4 orders of magnitude, down to 10² copies/reaction mixture against environmental DNA backgrounds. Finally, we have used this method to quantify *dxnA1* in environmental samples and, for the first time, showed growth of *S. wittichii* RW1 in landfill leachate, suggesting that the organism could be used to remediate dioxins in contaminated leachates. Our findings indicate that loss of megaplasmids occurs in *S. wittichii* RW1 populations under the conditions tested. This underscores the value of the present method for tracking the biodegradation potential of *S. wittichii* RW1 cells released into contaminated environments for remediation purposes. The method illustrated here may be adapted for targeting biodegradative, plasmid-based genes extant in other *sphingomonads* to accurately gauge their bioremediation potential.

ACKNOWLEDGMENTS

We acknowledge Nicole Hansmeier, Benny Pycke, and He-Ping Zhao for their assistance.

This project was supported in part by award number R01ES015445 from the National Institute of Environmental Health Sciences (NIEHS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIEHS or the National Institutes of Health.

REFERENCES


5. Halden RU, Halden BG, Dwyer DF. 1999. Removal of dibenzofuran, dibenzo-p-dioxin, and 2-chlorodibenzo-p-dioxin from soils inoculated...


