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Microbial Inactivation of *Pseudomonas putida* and *Pichia pastoris* Using Gene Silencing

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Antisense deoxyoligonucleotide (ASO) gene silencing was investigated as a potential disinfection tool for industrial and drinking water treatment application. ASOs bind with their reverse complementary mRNA transcripts thereby blocking protein translation. While ASO silencing has mainly been studied in medicine, it may be useful for modulating gene expression and inactivating microorganisms in environmental applications. In this proof of concept work, gene targets were *sh ble* (zeocin resistance) and *todE* (catechol-2,3-dioxygenase) in *Pichia pastoris* and *npt* (kanamycin resistance) in *Pseudomonas putida*. A maximum 0.5-fold decrease in *P. pastoris* cell numbers was obtained following a 120 min incubation with single-stranded DNA (ssDNA) concentrations ranging from 0.2 to 200 nM as compared to the no ssDNA control. In *P. putida*, a maximum 5.2-fold decrease was obtained after 90 min with 400 nM ssDNA. While the silencing efficiencies varied for the 25 targets tested, these results suggest that protein activity as well as microbial growth can be altered using ASO gene silencing-based tools. If successful, this technology has the potential to eliminate some of the environmental and health issues associated with the use of strong chemical biocides. However, prior to its dissemination, more research is needed to increase silencing efficiency and develop effective delivery methods.

Introduction

Many industrial applications as well as water treatment technologies rely heavily on the use of chemical disinfectants or ultraviolet irradiation to control undesirable microbial growth in water and on surfaces (*1, 2*). While effective at controlling the propagation of microorganisms, there are several unwanted side effects stemming from such methods. For instance, in industrial applications, bacteria such as *Mycobacterium avium* have developed resistance to chlorination (*3*), and there are well-documented cases of *Legionella* spp. resisting treatment by means of hiding and replicating within a host including amoeba or protozoa (*4*). In the context of drinking water applications, the use of chlorine has been shown to lead to the formation of carcinogenic disinfection byproducts (*5, 6*). Furthermore, there have been several reports of pathogens such as *Cryptosporidium parvum* and *Giardia lamblia* developing resistance to conventional chemical disinfectants (*7*). Finally, studies show that viruses can show resistance to UV treatment by a process where the viruses rearrange their DNA after treatment (*8*). All of these reports point to the fact that there is a need to develop new disinfection technologies which address these concerns. The current study was undertaken to test the potential for gene silencing to be used as a disinfection technology.

Two main gene silencing mechanisms exist. The first method is antisense oligodeoxynucleotide (ASO) silencing and relies on single strands of oligodeoxynucleotides generally 12−22 nucleotides in length, which are reverse and complementary to the target mRNA of interest (*9*). When binding occurs between the ASO and target mRNA, steric translational inhibition occurs at the ribosomal complex, and RNase H is induced, which cleaves the 3′−O−P bond of the RNA molecule (*10*). ASO silencing has been used in the biomedical field and was recently demonstrated to be a naturally occurring phenomenon (*11*). The second mechanism is RNA interference (RNAi), which has been demonstrated to be common among all eukaryotes (*12*). RNAi gene silencing is initiated via a double-stranded RNA molecule, which is cleaved into discrete fragments 21−23 nucleotide in length. These short interfering RNAs (siRNAs) form a ribonucleoprotein−endonuclease complex known as the RNA Induced Silencing Complex (RISC). The siRNAs proceed to unwind, and the antisense strand directs the complex to the targeted mRNA, which is cleaved by the endonuclease activity of the RISC (*12*). A similar mechanism known as "clustered regularly interspaced short palindrome repeats" (CRISPR) is known to occur in prokaryotes (*13*). In ASO silencing, RNAi or CRISPR, silencing is ultimately achieved via molecular interactions between mRNA and short single-stranded nucleotide strands through reverse complementary (ASO) or short-interfering RNA (siRNA) (*10*).

Gene silencing technology has mostly focused on medical applications, including cancer and viral infectious treatments (*14, 15*), with little research in other fields. Theoretically, gene silencing could be used to control microbial growth in a variety of fluids by silencing genes essential for cell survival. The targets could be designed specifically for applications where a single class of microorganisms need to be removed (i.e., *Legionella* spp. in cooling towers) or more broadly for other applications where multiple microorganisms must be inactivated (e.g., water disinfection). In this study, we provide proof of concept data demonstrating that gene silencing technology can be used for inactivation as well as gene expression modulation of prokaryotic and eukaryotic microorganisms in water.

Materials and Methods

Strains and Materials. *Pichia pastoris* DO2-1 was engineered using the pPICZ expression system to express the *todE* gene (Y18245) and produce catechol-2,3-dioxygenase. *P. pastoris* DO2-1 was a gift from Dr. Paul Szaniszlo at the University of Texas at Austin. *P. pastoris* was grown on a yeast peptone dextrose (YPD) medium at 30 °C on a VWR DS2-500 × 10^{-1} orbital shaker table (West Chester, PA) at 150 rpm in a Fisher Scientific Incubator (Hampton, New Hampshire). Zeocin was purchased from Invitrogen (Carlsbad, CA) and added at a final concentration of 100 μg/mL in all experiments. *Pseudomonas putida* BBC443 was grown in a Luria−Bertani (LB) media at ambient temperature (22−23 °C) in the presence of 50 μg/mL kanamycin purchased from Invitrogen.

ssDNA Target Sequences and Delivery. DNA sequences were designed to block expression of genes coding for zeocin resistance (*sh ble*, NCBI Accession L36850) and catechol-

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2,3-dioxygenase (*todE*, NCBI Accession Y18245) in *P. pastoris* and neomycin phosphotransferase, which conveys kanamycin resistance (*npt*, NCBI Accession AB158755 *16, 17*) in *P. putida*. The ssDNA sequences, which were used are shown in Tables S1 and S2 of the Supporting Information for *P. pastoris* and *P. putida*, respectively. The relative location within their respective genes as well as GC content is also provided for each target. For the purpose of this study, high and low GC content were defined as GC content 50% and higher and 49% or lower, respectively. All ssDNA was purchased from Integrated DNA Technologies (Coralville, IA). One of the biggest obstacles to gene silencing technology is the identification of a delivery method compatible with the application of choice. Much research has been performed on delivery systems some of which include microinjection, nanogels, liposomes, and viral delivery (*18, 19*). However, in this study, no engineering was performed to increase cellular uptake. Rather, cells were incubated in the presence of ssDNA and uptake was limited to that naturally passing through the cellular membrane through passive diffusion or transformation. The cells were not made competent prior to any incubation. While this method is much less efficient than engineered delivery technologies, the entry of any oligonucleotide into the cell that yielded a measurable response was deemed to be sufficient for this proof of concept work.

Zeocin Resistance (*sh ble*) Gene Silencing. Because zeocin resistance is required for fungal survival, fungal growth was used as an indicator of zeocin resistance gene silencing. *P. pastoris* was grown in a 100 mL YPD medium in 250 mL Erlenmeyer flasks for 48 h starting from an individual colony obtained from an agar plate. Following growth, 4 mL of log-phase cells were removed and placed in fresh 25 mL Erlenmeyer flasks containing 10 mL of YPD. Average starting cell concentration was $(1.1 \pm 0.1) \times 10^8$ cells/mL. Zero, 0.2, 2, 20, or 200 nM of ssDNA (zeo1−5) was added to each flask. For mixture experiments, 100 nM of each target (zeo3 and zeo4) was used for a combined concentration of 200 nM. Two negative controls were also prepared, consisting of no ssDNA and 200 nM scrambled ssDNA conditions. The cultures were then incubated at 30 °C for 3 h on an orbital shaker table at 150 rpm in a Fisher Scientific temperature-controlled incubator (Hampton, NH). Samples were taken hourly for cell count determination. For each treatment condition, experiments were performed in triplicate, starting from three independent *P. pastoris* colonies. All cell count measurements were performed at least in duplicate for all replicates.

Fungal Growth Conditions for *TodE* Silencing Experiments. *P. pastoris* was grown as described above. The culture was exposed to 200 nM of TodE1−5 ssDNA. Two negative controls were also prepared consisting of no ssDNA and 200 nM scrambled ssDNA conditions. The cultures were incubated at 30 °C on a VWR DS2-500 $\times 10^{-1}$ orbital shaker table (Hampton, NH) at 150 rpm in a Fisher Scientific temperature-controlled incubator for 2 h. Following the incubation, 10 mL samples were taken for the catechol assay. For each treatment condition, experiments were performed in triplicate, starting from three independent *P. pastoris* colonies.

Catechol Assay. The catechol assay was performed as described by Edghill et. al (*20*). Briefly, after a 4 h incubation period, the fungal cells were washed with 1× phosphate buffer saline (PBS). The solution was centrifuged for 10 min at 4200 rpm using an Eppendorf 5804 centrifuge (Eppendorf, Inc., Woodbridge, NJ) at room temperature, and the supernatant was removed by aspiration. The cells were then resuspended in 1× PBS and centrifuged for 10 min at 4200 rpm at room temperature. The supernatant was again removed by aspiration, and the cells were resuspended in PBS. The suspension was finally diluted to a final OD_{620} of 0.5. OD measurements were taken using a using a Hach RR/4000 U spectrophotometer (Loveland, CO). Five millilters of the dilution was subsequently exposed to 0.1% (m/v) catechol and incubated at 28 °C for 10 min. Finally, the samples were centrifuged, and the supernatant OD was measured at 375 nm. The product originating from catechol-2,3-dioxygenase enzymatic activity is *cis,cis*-muconate and is yellow in color. Thus, high OD_{375} indicates high levels of enzymatic activity, while low OD_{375} indicates catechol-2,3-dioxygenase silencing. All activity measurements were performed at least in duplicate for all replicates.

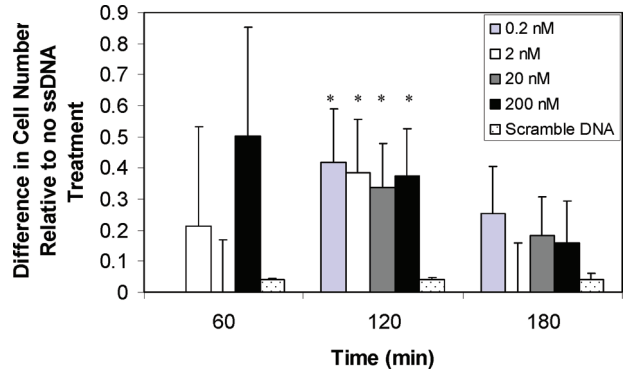

FIGURE 1. Effect of ssDNA concentration on *P. pastoris* growth. *P. pastoris* was cultured in the presence of either 0, 0.2, 2, 20, or 200 nM zeo4 or 200 nM scrambled ssDNA. *P. pastoris* cell numbers were measured hourly for 3 h. Data are expressed as one minus the ratio of the difference in cell numbers at each time point relative to time zero over the same difference for the no ssDNA control. Error bars represent ± one standard deviation. Star (*) indicates significant difference in cell number relative to no ssDNA at a 95% confidence interval.

Kanamycin Resistance (*npt*) Gene Silencing. *P. putida* was grown in 100 mL of Luria−Bertani (LB) medium at ambient temperature (∼22−23 °C) in 250 mL Erlenmeyer flasks on an orbital shaker table at 150 rpm. At the beginning of each experiment, 8 mL of log-phase culture was transferred into 25 mL Erlenmeyer flasks containing 6 mL of LB medium. Average starting cell concentration was $(4.4 \pm 0.3) \times 10^8$ cells/mL. The cultures were then exposed to 0, 0.4, 4, 40, and 400 nM of ssDNA (npt1−14) and incubated at room temperature for 2 h. A concentration of 0.4 nM is approximately equivalent to 4.8×10^{14} ssDNA copies/L for a DNA fragment 21 nucleotides in length. For mixture experiments, 200 nM of each target was used for a combined concentration of 400 nM. Two negative controls were also prepared consisting of no ssDNA and 400 nM scrambled ssDNA conditions. Cell numbers for each treatment were monitored every 30 min. Cell numbers were linearly correlated to the optical density measured at 620 nm (OD_{620}, $R^2 = 0.97$). OD_{620} was measured on a Thermo Electron Corporation Multiskan Ex spectrophotometer (Vantaa, Finland). Independent triplicate experiments were carried out starting from plated *P. putida*.

Statistical Analysis. All the data are expressed as either the difference in cell number or enzyme activity relative to the no ssDNA treatment ± one standard deviation. The statistical difference between the treatments and the no ssDNA control as well as between groups were examined using an unpaired Student *t* test.

Results and Discussion

Effects of ssDNA Concentration on Cell Growth. Concentration effects were tested using zeo4 in *P. pastoris* and npt5 in *P. putida* over a period of 180 and 120 min, respectively. The difference in cell numbers in samples treated with zeo4 relative to the no ssDNA control ranged from 0 to a 0.5-fold decrease (Figure 1). The strongest effect was observed at the 120 min time point for all concentrations. No significant

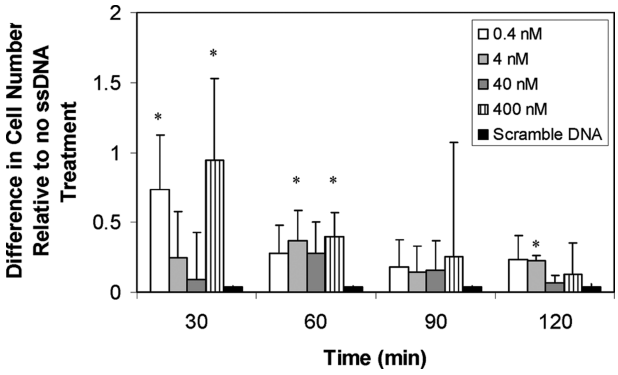

FIGURE 2. Effect of ssDNA concentration on *P. putida* growth. *P. putida* was cultured in the presence of either 0, 0.4, 4, 40, or 400 nM npt5 or 400 nM scrambled ssDNA. *P. putida* cell numbers were measured every 30 min for 2 h. Data are expressed as one minus the ratio of the difference in cell numbers at each time point relative to time zero over the same difference for the no ssDNA control. Error bars represent ± one standard deviation. Star (*) indicates significant difference in cell number relative to no ssDNA at a 95% confidence interval.

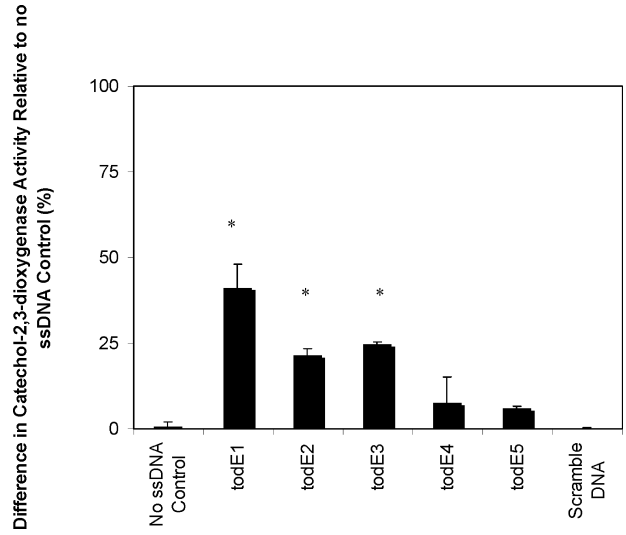

FIGURE 3. Effect of *todE* targets on catechol-2.3-dioxygenase activity. *P. pastoris* was cultured in the presence of 200 nM target ssDNA. After 2 h, optical density (OD) measurements were taken to determine the level of C23DO activity. Data are expressed as one minus the ratio of treatment OD mean values ± SD to the no ssDNA control. Star (*) indicates significant difference in activity relative to no ssDNA at a 95% confidence interval.

difference was observed at either the 60 or 180 min time points at any loading. This result was unexpected as oligonucleotides are known to degrade rapidly, and for this reason, a decreased impact with time was expected. Crooke and Bennett (*21*) reported a half-life on the order of 30−60 min for unmodified oligonucleotides in serum, whereas our data suggest that stability might be a little longer in the growth medium used in this study. However, because a direct measure of DNA was not performed, care should be taken when extrapolating these data to oligonucleotide stability observations. It is possible that the effect is correlated to oligonucleotide stability in *P. pastoris* rather than in the growth medium itself. Furthermore, because the observed impact is relatively small (i.e., less than 50%) and the variability is quite large (i.e., standard deviations up to 30% in some cases) at the 60 min time point, it is possible that any significant impact at the shorter time point was missed entirely. Finally, the ssDNA concentration did not impact total cell numbers for concentrations ranging from 0.2 to 200 nM. These data suggest that over saturation of ssDNA within the cell may be occurring even at concentrations as low as 200 pM. Because the ssDNA concentration in the cell is likely higher than the corresponding mRNA transcript concentration to which the ssDNA is binding, the addition of more ssDNA would not have an effect as all mRNA transcripts are already in the bound conformation. Others have reported similar trends (*22*).

In *P. putida*, varying effects were observed over the 120 min sampling period in the presence of 0.4−400 nM npt5 (Figure 2). The largest inhibition (0.9-fold decrease) was observed in the 400 nM treatment at the 30 min sampling point. This decrease was approximately 2-fold higher than the maximum level in *P. pastoris* and occurred much earlier in *P. putida*. It is possible that this may be attributed to the stability of the ssDNA within each class of microorganism. Studies show that the half-life or measure of ssDNA stability in *P. pastoris* is higher than the half-life of free genetic material in a bacterial cell (*23, 24*). Thus, it is possible that overtime some of the target ssDNA degraded, which may account for some of the observed differences.

For *P. putida* and *P. pastoris*, the data indicate that the effect on microbial growth is transient with maximum effects observed at 30 and 120 min for npt5 and zeo4, respectively. This result is not unexpected as ssDNA's half-life is known to be short, especially in a more complex medium (*10*). While this may be a drawback in traditional drinking water treatment applications where some residual disinfectant is desired, there are many applications where this degradation might be useful. For instance, microelectronics manufacturing requires ultraclean water that is free of chemicals and biologicals. In those situations, a disinfectant that degrades rapidly but provides targeted inactivation may be desirable.

TABLE 1. Effect of *npt* Targets on *P. putida* Growth[a]

	cell number decrease relative to no ssDNA treatment			
	30 min	60 min	90 min	120 min
npt1	2.3 ± 0.2	1.8 ± 0.2	0.4 ± 0.2	ns
npt2	ns	ns	0.3 ± 0.1	0.4 ± 0.1
npt3	ns	1.0 ± 0.2	ns	0.5 ± 0.2
npt4	ns	1.0 ± 0.2	5.2 ± 0.4	ns
npt5	0.4 ± 0.1	0.9 ± 0.2	ns	ns
npt6−14	ns	ns	ns	ns

[a] *P. putida* was cultured in the presence of either 400 nM target ssDNA or 400 nM scrambled ssDNA. *P. putida* cell numbers were measured every 30 min for 2 h. Data are expressed as one minus the ratio of the difference in cell numbers at each time point relative to time zero over the same difference for the no ssDNA control. Treatments with no significant decrease in cell number ($p < 0.05$) relative to the no ssDNA control are indicated as ns.

Effect of ssDNA Target Location and GC Content. In *P. pastoris and P. putida*, significant silencing was observed using targets with different GC contents at the start and middle of the gene (Figure 3 and Table 1). However, there were no consistent trends suggesting that other factors also contribute to silencing efficacy and that the design of targets is very specific to the microorganism and gene themselves.

In *P. pastoris,* the highest decrease of catechol-2,3-dioxygenase activity was observed on the target in the beginning of *todE* (Figure 3). Sequence todE1 decreased activity 41% relative to the no ssDNA control while 21% and 24% reduction was observed for todE2 and todE3, respectively. The targets in the last part of the gene had the lowest effect with todE4 and todE5 not significantly decreasing activity relative to the no ssDNA controls. No significant difference was observed for targets zeo1−4 (data not shown). No noticeable differences was observed with respect to GC

content. This finding was rather surprising as previous research in eukaryotes shows that the efficacy of an RNAi sequence is related to the relative and absolute stability of the two basepairs at the 5′ end of the sequence (*25*). On the basis of this report, expectations were that sequences with higher GC content contents near the 3′ end of the target strand and lower GC content at the 5′ strand have higher silencing rates. However, in this study, the highest silencing level was observed with todE1 which was designed to have high GC content throughout the target and is located at the 5′ end of the gene. Two out of the three targets with high 5′-GC and low 3′-GC (i.e., todE4 and todE5) did not significant silence catechol-2,3-dioxygenase activity relative to the no ssDNA control. It is possible that these targets hybridized to an off-target in *P. pastoris*, resulting in nonspecific silencing. However, additional research is needed to verify that this is in fact why no effect was observed.

Similar results to those found in *P. pastoris* were obtained in *P. putida* (Table 1 and Tables S3 and S4 of the Supporting Information). Several targets located at the beginning and middle of the *npt* gene had a significant effect on bacterial growth ($P < 0.05$); however, the results varied greatly from one target to another. Therefore, no generalizations can be made on the basis of location or GC content related to efficiency. The maximum effect was a 5.2-fold decrease between treatment and no ssDNA control for npt4 at the 90 min time point. An important difference for prokaryotic as compared to eukaryotic silencing applications is that target location should be partially based on either blocking ribosomal attachment (i.e., blocking the ribosome binding sites) or displacement of the 16S rRNA that is used during translation. As a result, targets closer to the start of the gene should be more efficient at displacing or blocking the ribosome (*26, 27*). In this study, all targets with significant effects on cell numbers relative to no ssDNA control were located at the beginning and middle of the *npt* gene. However, the relative decrease in cell number as well as the optimal treatment time varied greatly with target suggesting that factors other than location and GC content are important to consider in such design.

Effect of Using Sequence Mixtures on Silencing Efficacy. To determine whether a mixture of ssDNA targets within a specific gene is more effective at gene silencing than an individual ssDNA, the silencing efficiency of a mixture of ssDNA sequences was compared to the mixture decomposed into individual sequences in *P. putida* and *P. pastoris*. In *P. putida*, the combination of ssDNA sequences did not enhance microbial inhibition as compared to the individual components (Figure 4). Microbial growth was significantly different in the presence of the individual ssDNA as compared to their combinations at various time points; however, when microbial growth inhibition was significant, the decrease was always larger in the presence of one or both of the individual components (Figure 4). No significant difference was observed when comparing the inhibition by ssDNA mixture and the scramble control at any time point ($p > 0.05$). In *P. pastoris*, growth was not statistically significantly different in the presence of the individual targets as compared to their combination (Figure 5). It should be noted that these experiments were conducted at the higher target concentration (200 nM), and therefore, cells might be oversaturated as previously discussed. In the case of RNAi, as the concentration of siRNA saturates the RISC, off targeting can occur where the sense strand attaches to the RISC and decreased silencing efficiency as well (*22, 28*). Similarly, if all target mRNA transcripts are bound, the additional ssDNA strands might bound nonspecifically to other transcripts. Therefore, additional experiments should be performed where multiple genes are targeted in a single microorganism as well as in mixed microbial communities. In those cases, the addition

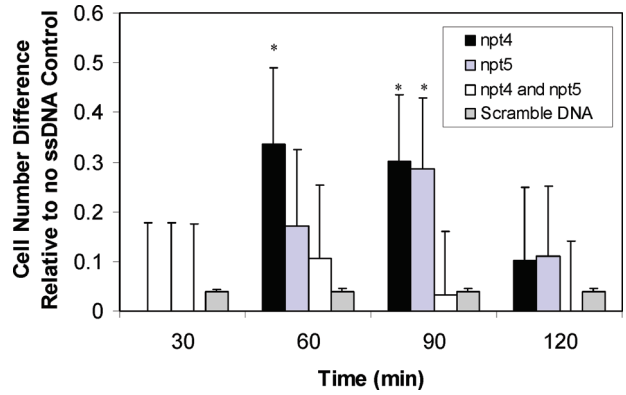

FIGURE 4. Effect of target mixtures on *P. putida* growth. *P. putida* was cultured in the presence of 400 nM total target ssDNA or 400 nM scrambled ssDNA. *P. putida* cell numbers were measured every 30 min for 2 h. Data are expressed as one minus the ratio of the difference in cell numbers at each time point relative to time zero over the same difference for the no ssDNA control. Error bars represent ± one standard error. Star (*) indicates significant difference in cell number relative to no ssDNA at a 95% confidence interval.

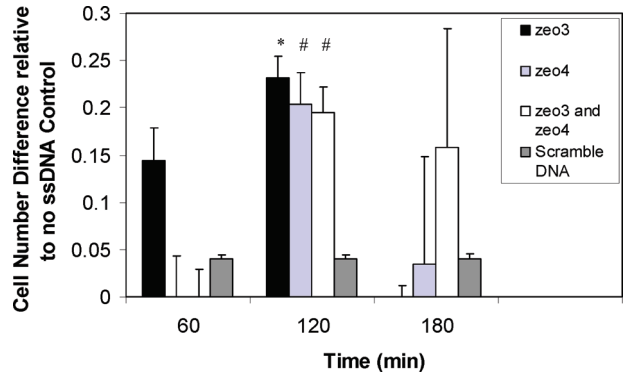

FIGURE 5. Effect of target mixtures on *P. pastoris* growth. *P. pastoris* was cultured in the presence of 200 nM total target ssDNA or 200 nM scrambled ssDNA. *P. pastoris* cell numbers were measured every hour for 3 h. Data are expressed as one minus the ratio of the difference in cell numbers at each time point relative to time zero over the same difference for the no ssDNA control. Error bars represent ± one standard deviation. Star (*) and pound (#) indicate significant difference in cell number relative to no ssDNA at a 95 and 90% confidence interval, respectively.

of ssDNA combinations might have a different effect than that observed herein.

Use of Gene Silencing-Based Technologies for Microbial Inactivation. While there is still much research needed in order to identify the optimal conditions for gene silencing-based pathogen inactivation, as demonstrated in this study, this technology appears promising. This proof of concept work indicates that ssDNA can be used to silence specific microbial functions in prokaryotic and eukaryotic microorganisms. However, before this technology can be brought to market, several areas of research should be targeted. First, the microbial inactivation efficiency needs to be increased. While efficiencies less than 99% might be acceptable for applications aimed at reducing microbial corrosion or membrane biofouling, significant improvements are needed before this method could be used for other applications such as water purification, where high efficiency as well as diverse pathogens must be targeted concurrently. Second, delivery methods compatible with environmental applications must be developed. Several existing technologies including viral vectors (*29*) or the use of coated molecules, which increase

intracellular stability (*30*) show significant promise and could be designed to be cost-effective through the use of self-sustaining vectors encapsulated in a wide range of recycled plastic materials. Proper engineering should be performed to ensure that appropriate oligonucleotide concentrations can be delivered to achieve the desired inactivation level. Third, pathogen and gene portfolios should be expanded to determine the optimal gene targets that have the ability to deactivate a wide range of microorganisms. Finally, a comprehensive toxicity study must be done to determine the possible health effects of using antisense gene silencing in environmental applications to ensure the development of a safe and green biocidal technology.

Acknowledgments

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Supporting Information Available

Detailed maps showing the location of each target, tables summarizing the target sequences in *P. putida* and *P. pastoris*, and tables depicting the effect of target location and GC Content on *P. putida* growth. This information is available free of charge via the Internet at http://pubs.acs.org.

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