Streptavidin-based containment systems for genetically engineered microorganisms

David L. Kaplan a,*, Charlene Mello b, Takeshi Sano c, Charles Cantor c, Cassandra Smith c

a Department of Chemical Engineering and Biotechnology Center, Tufts University, 4 Colby St., Medford, MA 02155, USA
b US Army Natick Research and Development Center, Biotechnology Division, Natick, MA 01760, USA
c Boston University, Center for Advanced Biotechnology, Boston, MA 02215, USA

Abstract

The use of genetically modified microorganisms for environmental remediation continues to be debated. Conditional lethal systems with tightly regulated gene expression can be used to contain released microorganisms and ameliorate some of the concerns about horizontal gene transfer. We have described streptavidin-based suicide systems to address these concerns and evaluated their function in Pseudomonas putida containing the TOL plasmid for aromatic hydrocarbon metabolism. Tight regulation of expression of a truncated streptavidin gene was required to avoid premature production of the toxic protein. Streptavidin expression was induced by the absence of 3-methyl benzoate (hydrocarbon substrate) which resulted in the elimination of 99.9% of the bacterial culture within eight hours. Low mutant escape rates at $10^{-7}$ per cell per generation were also realized. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Streptavidin; Containment systems; Microorganisms

1. Environmental remediation and genetically modified microorganisms

Environmental contamination due to the inappropriate storage and disposal of hazardous compounds is a world-wide problem. This contamination threatens water supplies, wildlife and human health. Potential solutions to this problem are varied, including combinations of chemical, physical and biological methods. Specifically, incineration, composting, solvent extraction, vitrification, and ozonation represent a partial list of technologies currently used or being explored. Key considerations with any environmental remediation option include transformation of the xenobiotic to less toxic or nontoxic byproducts, mineralization, or complexation of the compound into an intractable nontoxic matrix.

Many bioremediation methods are based on supplementation of natural microbial populations with appropriate nutrients to encourage inherent biodegradation capabilities, or pump-and-treat contaminated materials in a controlled bioreactor environment. In many instances, however, the concentration of contaminants is low, the addition of nutrients is undesirable, native populations of microorganisms do not harbor the requisite degradation capabilities, or the environmental conditions are not conducive to the expression of degradative genes. An alternative approach to this problem involves the use of genetically modified microorganisms. Engineered organisms have many potential applications to reduce environmental contamination in situ or in controlled bioreactors for the remediation of industrial waste streams. To develop a remediation system, genetic engineering can be used to address specific environmental conditions at a site or in a reactor, and suitable genetic control elements can be included to control survival in that environment.

A variety of genetically modified microorganisms have been constructed and evaluated for function, genetic stability and survivability [1,2]. Several systems have contained genes for suicidal function upon expression in Escherichia coli or Pseudomonas. Examples in-
clude genes encoding Gef or Hok peptides that destabilize cell membranes [3–7], lysis genes of bacteriophages [8], and RNases and DNases [9,10]. In addition to these direct controls, systems for monitoring gene flow within the environment have been described, to assess lateral gene transfer between bacteria [11]. A major problem with the above genetic control strategies has been the unacceptable mutant escape rates. This lack of adequate genetic control can lead to the release of these new genetic constructs into the environment, assumed to be an unacceptable risk.

To address the need to develop engineered organisms with stronger control over survival in the environment, we engineered Pseudomonas putida with catabolic plasmid capability and with tight regulation of survival. Decisions were required on the choice of host organism and the choice of catabolic activity. We engineered P. putida with a TOL plasmid and a streptavidin-based containment system. We found that a streptavidin-based containment system could be designed and used to control the survival of P. putida containing the native TOL plasmid, used to degrade toxic aromatic compounds [12]. Streptavidin is a naturally occurring protein with an extraordinarily high binding affinity for biotin. Biotin is an essential coenzyme for most microorganisms in biotin-dependent enzyme reactions involved in key metabolic pathways. The absence of biotin induces a lethal phenotype. Thus, we describe a new system for control of survival of genetically modified microorganisms based on the induction of a lethal genotype coupled to the activity of the TOL catabolic plasmid.

The choice of an organism for these studies was not simple. Many prior studies focused on engineering E. coli, which is less relevant to most scenarios for environmental remediation while nonetheless useful for fundamental insights into regulation and for facile cloning. We chose P. putida since the Pseudomonads are common in many environments, they are nutritionally versatile for the metabolism of low molecular weight organic compounds, they often harbor catabolic plasmids, they exhibit fast growth rates, and they could be used as industrially and environmentally relevant organisms. For the suicide control element, we chose streptavidin. Streptavidin, a tetrameric protein produced by Streptomyces avidinii, is a logical choice for containment for many reasons. The gene has been cloned and modified for many bioassays. Some mutant versions consisting of truncated genes still provide strong binding affinity to biotin while using a gene of reduced size to help limit possible mutation rates. Streptavidin is also known to irreversibly bind to d-biotin with a $K_d = 10^{-15} \text{ M}$. This type of containment system also could have some broader applications, such as in phytoremediation by engineering plants such as Arabidopsis thaliana, where lethal mutants defective in biotin biosynthesis have been described.

2. Construction of streptavidin-based genetic containment systems for bacteria

The experimental details involved in the construction of the genetically modified microorganism were described in Szafranski et al. [12] and will be summarized here. Recombinant methods were according to Sambrook et al. [13]. All subcloning was in E. coli K-12 strain XL1-Blue (Stratagene) and all suicide constructs were evaluated in P. putida mt-2 strain KT2440 [14]. The various plasmids utilized included pKK223-3, pCC102, pGEM-luc, pLysE, pRO1614, pTSA-13, pUC19 and pVLT33, and have been previously described ([15–20]; Promega, Pharmacia). Growth conditions and antibiotic concentrations were detailed previously [12]. Isopropyl-β-D-thiogalactopyranoside (IPTG) and m-methylbenzoate (3MB) were used at 1 mM and 0.2 mM, respectively.

A truncated streptavidin gene was used as the suicidal gene in the containment system. This expressed truncated protein, Stv-13, was described by Sano et al. [19] and consists of 119 residues and is 12.6 kDa. This truncated version of streptavidin still forms a tetramer but does not aggregate in the cell. Thus, it maintains high solubility and has a dissociation constant for biotin in the $10^{-14} \text{ M}$ range. These are important considerations to assure maximal biotin binding to the expressed streptavidin in the cells. The TOL plasmid, 117 kb, has been extensively characterized and encodes enzymes involved in the metabolism of xylenes, toluene and related hydrocarbons to tricarboxylic acid cycle intermediates. The plasmid is comprised of two operons, an upper and a meta-cleavage. The upper pathway oxidizes the aromatic hydrocarbons to the corresponding acids and is regulated by the xylR protein. The meta-cleavage pathway results in ring opening reactions for subsequent metabolism.

The genetic constructs were placed on two compatible broad host range plasmids, pCC-s05 (~15.3 kb) and pRO-ilp (~11.1 kb) as shown in Fig. 1. Copy numbers were 1–3 for pCC-s05 and 10–20 for pRO-ilp. pCC-s05 contains xylS2, lacI, and stv genes and pRO-ilp contains T7 RNA polymerase and lysozyme genes. The 1.3 kb fragment of the luc gene was inserted as a spacer to reduce expression of the T7 lysozyme gene. Controls containing only the N-terminal portion of the streptavidin protein, or a deletion of the $\Phi 10::stv$ fusion did not exhibit the response of the full constructs; they did not die in the absence of the 3-methylbenzoate inducer.

3. Genetic controls of streptavidin-based constructs

The general strategy used in these studies was to couple the expression of a lethal gene, in this case
Fig. 1. Physical maps of the two plasmids constructed for the genetic containment system.

**Version A:**

**Version**

3MB

(+)

(-)

3MB

X

Version B:

3MB

(+)

(-)

Fig. 2. Regulatory schemes developed. Version A represents the first design and Version B the final design. In both versions, the first parts illustrate the activity when the target xenobiotic (3-methylbenzoate) is present. In the second parts of each version, the activity shown represents conditions when the target xenobiotic has been consumed and streptavidin is expressed.
streptavidin, to the regulation of the catabolic (TOL) plasmid (Fig. 2). Placing the streptavidin gene directly under the control of a promoter that is negatively regulated by a repressor protein synthesized in response to an environmental signal would be a simple approach (Fig. 2, Version A). Our initial design for a streptavidin suicide system involved placement of the streptavidin gene directly under the control of the lac operon. In the presence of the toxic compound, 3-methylbenzoate, the xylS protein interacts with the Pm promoter to enhance expression. In the absence of 3-methylbenzoate, xylS does not bind to Pm, lacI is not expressed, Ptac is upregulated by placing the Pm promoter to activate expression of lacI. The cI protein binds to Ptac to block expression of T7 RNA polymerase. The polymerase is still expressed at low levels due to the leakiness of the Ptac, so T7 lysozyme, constitutively expressed under the regulation of a Ptet promoter, degrades any T7 RNA polymerase that is expressed. The result of this cascade is that stv expression is controlled by the T7 gene 10 promoter, φ10, and thus expression levels are minimized. When 3-methylbenzoate is consumed the xylS regulator protein can no longer bind to Pm, lacI expression is repressed, and Ptac is upregulated to express higher levels of T7 RNA polymerase. At this stage, the levels of RNA polymerase overwhelm the low constitutive levels of T7 lysozyme, sufficient T7 RNA polymerase is available to activate the φ10 promoter and the expression of streptavidin. Streptavidin expression results in the death of the cell due to binding of available biotin in the cell and shutting down all essential metabolic pathways. Alternatively, the addition of IPTG intervenes by activating the Ptac promoter directly, leading to higher levels of the T7 RNA polymerase and the same result as just described.

4. Efficacy of streptavidin containment systems

To assess efficacy of the constructs in P. putida to control survival, the appearance of clones resistant to controlled cell death was determined using published methods [21]. Efficiency of the construct was assessed by cell enumeration before and after depletion of the 3-methylbenzoate. Dilutions were incubated at least a week to assure recovery of weakened cells. Cells were recovered on media consisting of 3-methylbenzoate, biotin (50 μg/ml) and antibiotics corresponding to selectable markers on the plasmids. In Fig. 3, cell counts were reduced 99.9% within eight hours of removal of 3-methylbenzoate (curve s05, solid line). The combination of IPTG and the absence of 3-methylbenzoate accelerated the rate of cell death. Incubations at lower
Fluctuation tests were used to evaluate mutations allowing _P. putida_ to escape from the genetic containment system. Constructs with and without T7 lysozyme, with or without direct LacI–O _lac_ -dependent accessibility of the φ10 promoter, and with or without countertranscript protection against basal expression of the _stv_ gene were characterized. The dual plasmid system (pCC-8O5 and pRO-ilp) was about two orders of magnitude more stable than the same construct without the antisense expression (10⁻⁶–10⁻⁷ vs. 10⁻⁴–10⁻⁵ per cell per generation). The lowest frequency of resistant mutants, 10⁻⁷–10⁻⁸, was found for the _O lac_ next to the φ10.

5. Issues of genetic containment and stability

Biological containment using suicide genes should consider a number of issues:

1. Induction by environmentally relevant chemicals, such as a xenobiotic, as described by Contreras et al. [17]. Induction by IPTG, lactose or sucrose will not be appropriate for in situ remediation applications.

2. Use of environmentally relevant organisms such as _Pseudomonads_ and not _E. coli_ or other bacteria less capable of competing for survival in natural environments.

3. Lowering of mutation rates to approximate those experienced by natural populations. The mutation rates can be lowered with multiple independently regulated suicide genes within a single organism. In addition, mutation rates should be evaluated both during logarithmic growth phases and during stress conditions [24].

4. Plasmid-based containment systems will not suffice in field remediation scenarios; chromosomal-based systems will be required to enhance stability.

5. Monitoring of engineered organisms will be an important aspect of study with any engineered organism. Monitoring for short- and long-term impact of the introduced organisms in the field will be a key aspect of these studies.

To address environmental safety concerns with the use of genetically modified microorganisms, it is essential to consider efficacy, stability, and containment. Containment of engineered microorganisms is a significant issue because of the potential for horizontal gene transfer in the environment leading to concerns about impact on gene pools. This can be addressed with an appropriately designed suicide system. Efficacy can be addressed by utilizing suitable catabolic genes to target specific environmental contaminants. Stability can be addressed by lowering mutation rates which would otherwise lead to clones that escape the engineered regulatory control.

The rate of inactivation or the efficiency of the genetic constructs we have describe is 10⁻⁷–10⁻⁸ per cell per generation. This is a value that is significantly lower than other reports based on single toxic functions (range from 10⁻² to 10⁻⁶) which were reported in _E. coli_. These data suggest that suitable genetic controls can be designed based on this approach to lead to engineered microorganisms useful in environmental remediation. Post mutation analysis for the resistant mutants using restriction endonuclease analysis suggested that most changes in DNA involved the _stv_ and T7 RNA polymerase genes. The suicide system was evaluated under nutrient-rich conditions, and conditions promoting cell survival – presence of effectors. In natural conditions, such as environmental soils and waters where nutrient conditions are less favorable, the system may perform even more efficiently. Furthermore, the integration of other lethal genes and the incorporation of the plasmid-borne system into the chromosome could provide enhancements to the system.

The streptavidin-based control system offers an additional advantage of very sensitive monitoring of the presence of recombinant microorganisms when used in combination with biotinylated solid supports and biotinylated fluorescent probes. This is a unique advantage of this genetic containment strategy. Future studies with these systems should focus on incorporation of the genetic control functions into the chromosome, and the inclusion of additional suicide functions to address all of the key issues outlined at the beginning of this section.

Acknowledgements

We thank Przemyslaw Szafranksi for his efforts on this research.

References


