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Short communication

Evolution of an alkane-inducible biosensor for increased responsiveness to short-chain alkanes

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ABSTRACT

Synthetic alkane-inducible biosensors have applications as detectors for environmental hydrocarbon contamination and as novel inducible expression systems with low-cost inducers. Here, we have assembled and evolved an alkane-responsive biosensor with a fluorescence output signal in *Escherichia coli* by utilizing regulatory machinery from *Pseudomonas putida's* alkane metabolism. Within our system, the transcriptional regulator, AlkSp, is activated by the presence of alkanes and binds to the P_{alkB} promoter, stimulating transcription of a Green Fluorescent Protein reporter. Through two successive rounds of directed evolution via error prone PCR and fluorescence activated cell sorting, we isolated *alkS* mutants enabling up to a 5 fold increase in fluorescence output signal in response to short-chain alkanes such as hexane and pentane. Further characterization of selected mutants demonstrated altered responsiveness to a wide range of linear alkanes (pentane to dodecane). Sequence analysis highlighted the S470T mutation as a likely candidate responsible for increased effectiveness of the AlkS protein for short-chain alkanes. This work represents the first evolution of a synthetic biosensor system for alkanes.

1. Introduction

Alkane-responsive biosensors are a useful synthetic biology part. In particular, these components can be used to sense hydrocarbon contamination in ground water and water ways, as hydrocarbon pollution represents a widespread problem to native organisms in a wide range of environments (Atlas, 1991; Jaspers et al., 2001; Louati et al., 2001). Additionally, the low-cost of alkanes warrants their study for applications as inducible gene expression systems. Combining these two aspects, it is possible to create a sentinel organism that can both sense and remediate environmental alkane contaminants using an alkane biodegradation pathway (Eggink et al., 1987; Paton et al., 2004; Tecon and van der Meer, 2008). Notably, the n-alkane hexane (C_6) has well-known long-term toxicity towards humans, and potential carcinogenic properties (Hathaway, 1991; Sabatini et al., 2005). While previous studies have developed biosensors which exhibit noticeable response at octane concentrations as low as 10 nM (Jaspers et al., 2001), the current range and level of responsiveness of biosensors towards short-chain alkanes such as pentane or hexane is significantly lower (5 μM for a noticeable response) (Sticher et al., 1997). Improving these biosensors for increased responsiveness towards hexane through protein engineering expands the promise of synthetic biology by creating novel and controllable two-component inducible systems (Young and Alper, 2010).

The native metabolism of *Pseudomonas pudita* GPo1 is regulated by a two component genetic circuit activated by linear alkanes (van Beilen et al., 2001). The *alkS* ORF encodes a regulatory protein that binds to and activates the P_{alkB} (Yuste et al., 1998). Transcription from this P_{alkB} promoter is completely dependent on an alkane-activated AlkSp protein (Fig. 1a). Here, we sought to replicate this inducible system in *Escherichia coli* and evolve it for alternative short-chain alkane inputs. Natively, the alkSp protein is transcriptionally controlled through both catabolite repression and feedback inhibition (Canosa et al., 2000, 1999). To avoid these expression issues (Dinamarca et al., 2003; Menart et al., 2003), the *alkS* gene was expressed using a heterologous constitutive promoter. Here, we use the green fluorescent protein, GFP, as a reporter gene to avoid screening issues present in an alternative, hexanal-dependent luciferase system (Sticher et al., 1997). P_{alkB}-GFP fusions function well in *E. coli* (Jaspers et al., 2001), and luciferase has a rapidly dynamic hexanal-dependent response, disallowing flow cytometry screening or sorting. A GFP-based biosensor displays more constant fluorescence and thus is a suitable reporter for fluorescence activated cell sorting and further flow cytometry analysis.

Through evolving this synthetic biosensor, we demonstrate that responsiveness of the system can be improved for both native and non-native alkane inducers. In doing so, we create a broad-range

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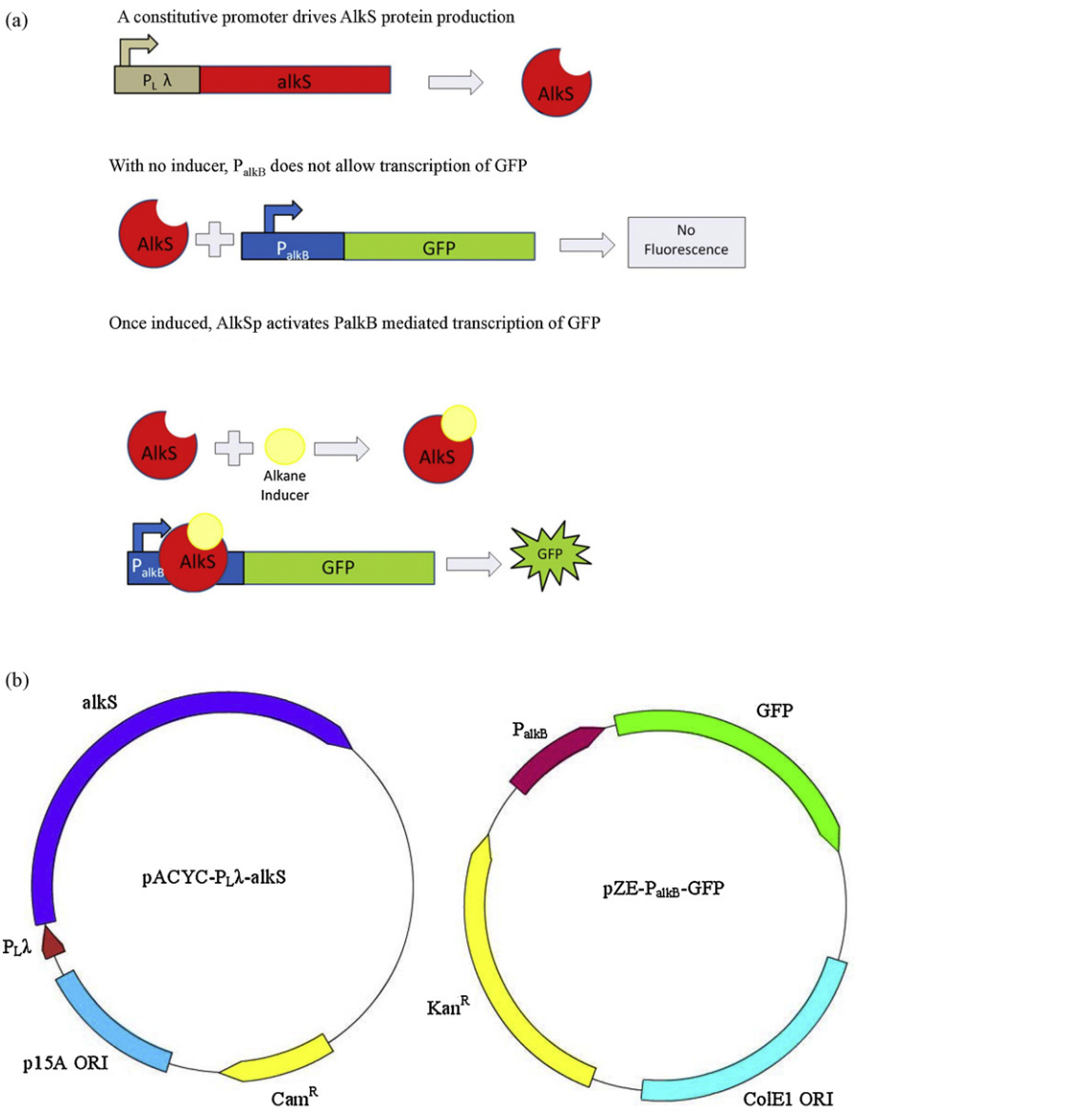

Fig. 1. *Overview of the alkS-P_{alkB} genetic circuit and plasmid map schematics for pACYC-$P_L\lambda$-alkS and pZE-P_{alkB}-GFP.* (a) A constitutive promoter drives the expression of AlkSp. AlkSp only binds to the P_{alkB} promoter to allow expression of the GFP reporter gene when activated by an alkane inducer. (b) A two-plasmid system was created to separate the production of the AlkSp regulator and the promoter-GFP reporter system.

alkane biosensor with particularly novel responsiveness to the short-chain alkanes pentane and hexane.

2. Materials and methods

2.1. Strains and media

E. coli strain DH10β was used for all experiments. DH10β was grown at 37 °C (unless stated otherwise) with constant shaking. LB media (Teknova) was supplemented with 10 μg/ml kanamycin and 34 μg/ml chloramphenicol as necessary to make media LB-kan-cam. M9 medium was made as described previously (Wood, 1983) and was supplemented with 0.1% casamino acids, 10 μg/ml kanamycin, and 34 μg/ml chloramphenicol as stated. Solid media was prepared by adding 15 g/L agar (Teknova).

2.2. Cloning procedures

All restriction enzymes were purchased from New England Biolabs, and all digestions were performed according to standard protocols. PCR reactions were performed with recommended conditions using Phusion DNA polymerase (Finnzymes). Ligations were performed overnight at 16 °C using T4 DNA Ligase (Fermentas). Gel extractions were performed using the GeneClean gel extraction kit (MP Biomedicals). *E. coli* minipreps were performed using the Zyppy Plasmid Miniprep Kit (Zymo Research Corporation). Transformation of *E. coli* strains was performed using standard electroporation protocols (Sambrook and Russell, 2001).

2.3. Construction of a dual plasmid biosensor reporter system

Two plasmids, pACYC-$P_L\lambda$-alkS and pZE-P_{alkB}-GFP (Fig. 1b), were constructed separately and transformed into *E. coli* to create a functional alkane responsive reporter system. Plasmid pACYC-$P_L\lambda$-alkS was constructed by the insertion of $P_L\lambda$ promoter to plasmid pACYC184 (Rose, 1988) using XbaI/HindIII, followed by the addition of the *alkS* ORF using KpnI/BamHI. Plasmid pZE-P_{alkB}-GFP was constructed by replacing the $P_L\lambda$ promoter from plasmid pZE-GFP with the P_{alkB} promoter using AatII/EcoRI. *AlkS* and P_{alkB} sequences were amplified from *Pseudomonas putida* GPo1 (ATCC. 29347) gDNA, and

Table 1
List of primers used in this study. The primers used in this study are listed with their names, sequences, amplification products, and restriction enzymes attached.

Primer Name	Sequence	PCR product	Restriction sites
PL-F	Caattccgacgtctaagaaaccattattatcatgacattaac	$P_L\lambda$	XbaI
PL + gap-R	CCCAAGCTTCCTTAATTAA cccTTGGCGCGCCgcggtacctttctcctctttaatgaattc		HindIII/PacI/KpnI
P_{alkB} -F	TCCGACGTCttacccggaagcccggg	P_{alkB}	AatII
P_{alkB}-R	CCGGAATTCtttggtgttctccaatttttattaaattagtcg		EcoRI
alkS-F	CGGGGTACCgcatgaaaataataataaataatgatttcccggt	*alkS*	KpnI
alkS-R	CGCGGATCCttagataattccttgacgctcag		BamHI
alkSmut-F	cattaaagaggagaaaggtaccgcATG	*alkS*	KpnI
alkSmut-R	ccggccacgatgcgtccggcgtagaggatccTTA		BamHI

$P_L\lambda$ was amplified from pZE-GFP (Alper et al., 2005). Primers are listed in Table 1.

2.4. Biosensor induction

Each biosensor, with mutated or wildtype AlkSp, was grown overnight in 50 ml LB-kan-cam. Biosensors were reinoculated at an OD_{600} of 0.01 into 5 mLs M9 medium supplemented with casamino acids, kanamycin, and chloramphenicol and grown overnight. 500 μl of this culture and 500 μl of alkane inducer were added to 5 ml fresh M9 medium supplemented with casamino acids (8.33% v/v inducer). Hexane induction refers to induction of the biosensor with hexane as an inducer and likewise for all alkane inductions. All biosensors and inducer combinations were tested simultaneously under these described conditions. Alkane inducers included pentane, hexane, heptane, octane, nonane, decane, undecane, and dodecane (henceforth C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, and C_{12}, respectively). Induction with water was employed as a negative control. Cultures were incubated for one hour at 30 °C with agitation in sealed 14 ml polypropylene culture tubes (BD Falcon) to attain full alkane-mediated biosensor induction. One hour was shown to be an optimal time for maximal fluorescence with no loss of cell viability (data not shown). Fully induced cells were pelleted and then resuspended in chilled, sterile water for further evaluation of fluorescence response.

2.5. Directed evolution of AlkSp

2.5.1. Error-prone PCR of alkS and alkSm4

Error-prone PCR (epPCR) of the *alkS* gene was performed using the GeneMorph II Kit (Stratagene) using plasmid pACYC-$P_L\lambda$-alkS as template with primers alkSmut-F and alkSmut-R (Table 1). Three 50 μl reactions with 500–1000 ng template were subjected to 15 rounds of error-prone amplification to form a library with a low mutation rate. PCR amplicons were ligated into pACYC-$P_L\lambda$ and then transformed into DH10β cells already containing pZE-P_{alkB}-GFP. Colonies were scraped directly from transformation plates and frozen as a glycerol stock. In the second round of directed evolution, epPCR was performed on *alkSm4*. Libraries were on the order of 30,000 mutants each.

2.5.2. Fluorescence activated cell sorting

A 500 μL aliquot of each mutant library (containing mutated *alkS* on plasmid pACYC-$P_L\lambda$-alkS) was induced as described using hexane as the alkane inducer. Cells were resuspended at a density of 10^6 cells/ml and sorted using a BD FACS Aria to collect cells that generated fluorescence levels one standard deviation above the wildtype *alkS*-based biosensor mean that also used hexane as the inducer (Bonner et al., 1972).

2.5.3. Individual biosensor isolation and screening

Sorted libraries were plated on LB-kan-cam plates supplemented with 5% v/v hexane. Cells were selected based on hexane-induced green fluorescence under blue light, picked, grown overnight, and saved as glycerol stocks. Induction proceeded as described above using a BD Fortessa flow cytometer for fluorescence response analysis. Mutants displaying desirable characteristics were re-transformed into *E. coli*, and the alkane response spectrum of biological triplicates was tested using water, pentane, hexane, heptane, octane, nonane, decane, undecane, and dodecane (C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, and C_{12}) as inducers. Positive and negative controls contained either plasmid pZE-$P_L\lambda$-GFP or plasmid pACYC-$P_L\lambda$-alkS and were induced with water.

3. Results and discussion

Two iterative rounds of directed evolution on the AlkSp protein were used to alter the alkane-response profile of the AlkS-P_{AlkB} system. Mutants from each library were fully characterized through sequence analysis and an induction test. Since short-chain hydrocarbons are volatile, 8.33% v/v inducer and constant agitation in sealed tubes were utilized to ensure consistent inducer saturation of the aqueous culture media. Both absolute induction level (as measured by GFP fluorescence) and alkane preference were altered through two progressive rounds of mutation and selection (Fig. 2a). AlkSp mutants 3 and 4 from the first generation imparted a roughly two fold higher output on hexane compared to WT AlkSp. Second generation mutants (based on mutant 4 from round 1) had an even further increased response to alkane induction, as seen in particular by AlkSp mutants 4-4 and 4-5. For the case of AlkSp mutant 4-5, there is a 3 to 5 fold increase in fluorescence over wild-type AlkSp for a wide range of short-chain alkanes from C_5 to C_9. It is of note that every isolated mutant displaying improved selectivity towards short-chain alkanes was created using EpPCR with a low mutation rate, implying that too many mutations can destabilize the protein or cause loss of function. If mutation rate is high, structurally or catalytically important regions of the protein are not conserved, leading to reduced fluorescence response.

Our analysis shows that AlkSp mutant 4 was the best performing first generation mutant. First generation mutants tended to have increased alkane responsiveness across-the-board relative to WT. Sequence analysis shows that all first generation mutants possess the same Q410K (C1228A) and silent (C1392G) mutations, and AlkSp mutants 3 and 4, the two highest performing first generation mutants, share a S470T (T1408A) mutation (Tables 2 and 3). Thus, the S470T amino acid substitution is likely responsible for the increased output observed from these mutants. Conserved domain analysis demonstrates that the AlkSp N-terminal region is similar to the MalT transcription factor, an *E. coli* transcription factor activated in the presence of maltose (Marchler-Bauer et al., 2011). As the S470T mutation lies within the MalT-like region, we hypothesize this mutation occurs in the alkane-binding pocket of the AlkSp, rendering this protein more responsive. The substitution of serine by threonine provides the residue with a bulkier, branched side chain, possibly enticing smaller alkane molecules enter the binding domain.

Second generation mutants exhibited altered specificity towards alkanes compared to wildtype (Fig. 2a). AlkSp mutants 4-2,

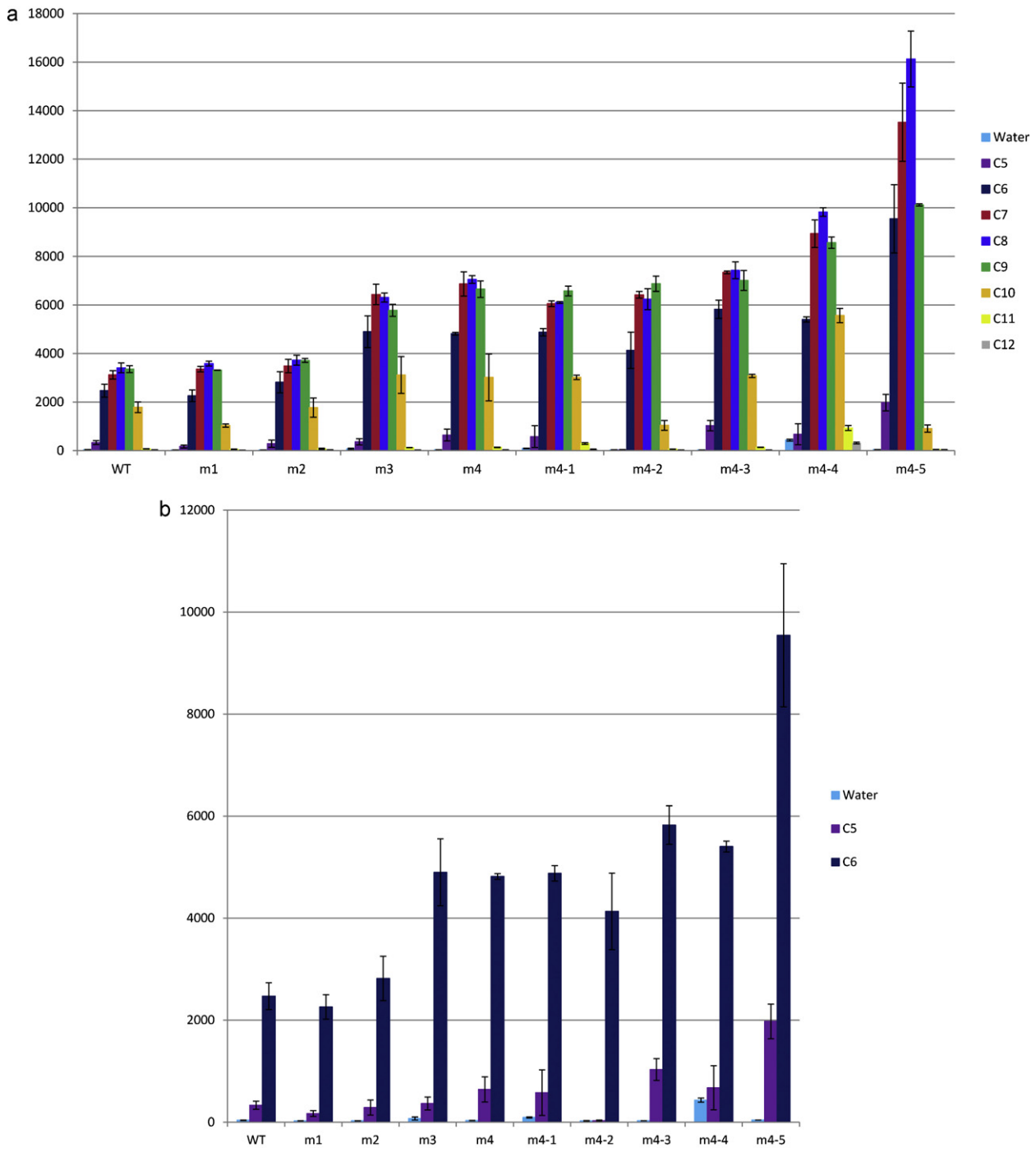

Fig. 2. Fluorescence response for AlkSp mutants. (a) Biosensor systems were characterized based on their fluorescence response upon induction by a range of alkanes: pentane (C_5) through dodecane (C_{12}). Experimental conditions are outlined in the Section 2. WT refers to the wild-type AlkSp, m1 through m4 are first generation mutants and m4-1 though m4-5 are second generation mutants. First generation mutants, m3 and m4, exhibited up to a twofold improvement in response to short-chain alkanes. Second generation mutants continue this trend with mutant m4-5 exhibiting up to a five-fold increase in induced response. Error bars represent the standard deviation of measurements between biological triplicates. (b) Subset of data from (a) illustrating improvement of biosensor systems towards hexane and pentane.

4-3, and 4-5 showed increased preference towards shorter alkane inducers (Fig. 2b), while AlkSp mutant 4-4 has a novel response towards longer-chain alkane inducers, specifically dodecane. As expected given the diverse specificity profiles, there is less repetition among the mutations obtained in this second round. However, at least one mutation occurs near the AlkSp C-terminus for each mutant. We hypothesize that these mutations are not directly associated with the alkane-binding pocket but rather affect the overall structure of the protein. Mechanism aside, these results clearly demonstrate the capacity to alter the substrate response range and magnitude of this synthetic regulator through directed evolution.

In conclusion, this work presents the first synthetic construction and evolution of an alkane-inducible biosensor for short-chain alkanes. Up to a 5-fold improvement in reporter output was achieved using this directed evolution approach. Alkane preference profiles were additionally altered illustrating the flexibility of this system for future, specific alkane sensing applications. This work has implications for synthetic biology, biosensor technology, and

Table 2
DNA mutations for isolated alkS mutants. The DNA mutations of the nine fully characterized *alkS* mutants are listed. Underlines indicate silent mutations.

m1	m2	m3	m4	m4-1	m4-2	m4-3	m4-4	m4-5
T51A	C1228A	C1228A	C1228A	C1228A	A162T	C1228A	C1228A	C273T
C1228A	C1392G	C1392G	C1392G	C1392G	G553A	A1229T	C1392G	T545A
C1392G	T2481C	T1408A	T1408A	T1408A	A654C	C1392G	T1408A	G966T
			A1636G	A1636G	C1228A	T1408A	A1636G	C1228A
			G1929A	G1929A	C1392G	A1636G	G1929A	C1392G
			T2388C	A2387G	T1408A	G1929A	T2091C	T1408A
				T2388C	A1636G	A2325T	T2388C	A1636G
					G1803A	A2349T	C2531G	G1929A
					G1929A	T2388C		T2356A
					T2046A			T2388C
					A2364T			C2494T
					T2388C			

Table 3
Protein mutations for isolated alkS mutants. The protein mutations of the nine fully characterized AlkSp mutants are listed. The S470T mutation (deduced to be critical in establishing preference to short-chain alkanes) is highlighted in bold.

m1	m2	m3	m4	m4-1	m4-2	m4-3	m4-4	m4-5
D17E	Q410K	Q410K	Q410K	Q410K	A185T	Q410K	Q410K	L182H
Q410K		**S470T**	**S470T**	**S470T**	E218D	**S470T**	**S470T**	Q410K
			K546E	K546E	Q410K	K546E	K546E	**S470T**
				D796G	**S470T**	E775D	T844R	K546E
					K546E			S786T
					E788D			L832F

for use as an inducible expression system for recombinant protein production in bacteria.

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