doi:10.1016/j.jmb.2005.10.076 J. Mol. Biol. (2006) 355, 619–627

JMB

Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

Environmentally Controlled Invasion of Cancer Cells by Engineered Bacteria

J. Christopher Anderson[1,3], Elizabeth J. Clarke[3], Adam P. Arkin[1,2*] and Christopher A. Voigt[2,3]

[1]*Howard Hughes Medical Institute, California Institute of Quantitative Biology Department of Bioengineering University of California, 717 Potter Street, Room 257 Berkeley, CA 94720, USA*

[2]*Physical Biosciences Division E.O. Lawrence Berkeley National Laboratory, 1 Cyclotron Road, MS 977-257 Berkeley, CA 94710, USA*

[3]*Biophysics Program Department of Pharmaceutical Chemistry, California Institute of Quantitative Biology The University of California San Francisco, 600 16th St. San Francisco, CA 94107 USA*

**Corresponding author*

Bacteria can sense their environment, distinguish between cell types, and deliver proteins to eukaryotic cells. Here, we engineer the interaction between bacteria and cancer cells to depend on heterologous environmental signals. We have characterized invasin from *Yersinia pseudotuburculosis* as an output module that enables *Escherichia coli* to invade cancer-derived cells, including HeLa, HepG2, and U2OS lines. To environmentally restrict invasion, we placed this module under the control of heterologous sensors. With the *Vibrio fischeri lux* quorum sensing circuit, the hypoxia-responsive *fdhF* promoter, or the arabinose-inducible *araBAD* promoter, the bacteria invade cells at densities greater than 10^8 bacteria/ml, after growth in an anaerobic growth chamber or in the presence of 0.02% arabinose, respectively. In the process, we developed a technique to tune the linkage between a sensor and output gene using ribosome binding site libraries and genetic selection. This approach could be used to engineer bacteria to sense the microenvironment of a tumor and respond by invading cancerous cells and releasing a cytotoxic agent.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: invasin; synthetic biology; cancer; therapeutic bacteria; *lux*

Introduction

Recent efforts to design and construct organisms for biotechnological applications, such as metabolic engineering and bioremediation,[1] have led to developing a toolbox of modular and robust parts including biosensors and genetic circuits. The output of these systems is interfaced to control cellular behaviours such as biofilm formation,[2] chemotaxis,[3] and differentiation.[4] Here, we link heterologous environmental sensors to the expression of a protein that enables *Escherichia coli* to invade mammalian cells. Reprogramming the interaction between a bacterium and mammalian cell will enable the forward engineering of bacteria for therapeutic purposes including live vaccines,[5] probiotics,[6] and anti-tumor agents.[7]

The construction of a number of synthetic biosensors and genetic circuits has been reported. Recently, Hellinga and co-workers computationally designed maltose binding protein variants that bind various unnatural ligands, including TNT.[8] In addition, Buskirk *et al*. linked small molecules to translation by designing RNA aptamers that bind tetramethylrosamine and activate gene expression.[9] To process environmental inputs, synthetic genetic circuits have been constructed that function as logic blocks,[10] an oscillator,[11] a bistable "toggle" switch,[12] and a pulse generator.[13] Synthetic eukaryotic protein circuits have also been constructed that enable the integration of multiple inputs.[14]

There has been an effort to link the sensors and circuits to control cellular behaviour through the activation of an "output interface".[2] For example, biofilm formation was induced by activating a toggle switch genetic circuit controlling *traA*. By linking this switch to quorum sensing or the activation of RecA, biofilm formation was induced at high cell densities or when exposed to UV light, respectively.[2] In addition, Arnold and co-workers

Abbreviations used: UTR, untranslated region; cfu, colony-forming units; MOI, multiplicity of infection.

E-mail address of the corresponding author: aparkin@lbl.gov

0022-2836/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.

developed a synthetic population control circuit by linking quorum sensing to cell death by inducing the synthesis of a toxic protein.[15]

Emerging applications of synthetic biology are the design of bacteria to produce therapeutic agents and the use of live bacteria as targeted delivery systems.[5,6] Towards this latter goal, it will be important to control the interaction of a bacterium with a mammalian cell and to regulate this interaction in response to environmental stimuli.[5,7,16–28] Bacteria have numerous systems to interact with and manipulate eukaryotic cells. Redundancies of these systems and their complex regulatory control complicate the engineering of natural bacteria. In contrast, the *inv* gene encoding invasin from *Yersinia pseudotuberculosis* represents a single-gene output interface for initiating adhesion and invasion of mammalian cells when expressed in *E. coli*. Invasin binds tightly to β1-integrins present on the surface of many cell lines and induces bacterial uptake by stimulating Rac-1.[29] In the gut, *Yersinia* uses invasin to identify and invade M cells, which uniquely express β1-integrins on their apical surface.[30] Transfer of *inv* to *E. coli* is sufficient to induce the invasion of mammalian cell lines that express β1-integrins.[31] Moreover, the therapeutic potential of inv^+ *E. coli* has been explored by constructing strains that can deliver proteins[17,19] and plasmids[18,16] into mammalian cells.

Here, we demonstrate that invasin-mediated internalization does not require additional known adhesion molecules. In addition, inv^+ *E. coli* can invade a broad range of tumor cells including epithelial, hepatocarcinoma, and osteosarcoma lines. Towards the goal of engineering therapeutic bacteria, we show that bacterial internalization can be synthetically linked to cell density, hypoxia, and inducible inputs (Figure 1). This is achieved by placing *inv* under the control of the quorum sensing *lux* operon, an anaerobically induced *fdhF* promoter, or an arabinose-inducible *araBAD* promoter.

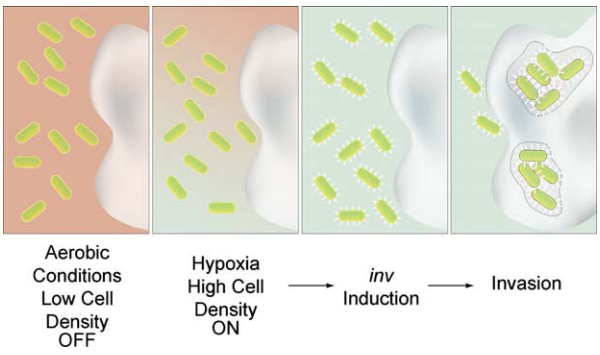

Figure 1. Design for induction-dependent invasion of a cancer cell. Under conditions of low cell-density or normal aerobic growth, engineered bacteria are non-invasive. Above a critical cell density or in a hypoxic environment, sensors are activated resulting in the synthesis of invasin from *Y. pseudotuberculosis* and the invasion of HeLa cells.

Connecting the invasin output to environmental inputs required engineering beyond gene fusion. Initial construction of *fdhF* and *araBAD*-controlled invasin resulted in constitutive phenotypes. To overcome this problem, we constructed ribosome binding site libraries and developed a genetic selection to identify clones with inducible phenotypes. This combinatorial strategy readily afforded the desired phenotypes and should be broadly applicable for any output module amenable to positive selection.

Results

Modularity of invasin

Invasin is a long rigid protein that is anchored in the outer membrane and extends 18 nm from the bacterial cell surface.[32] Binding of β1-integrins does not require additional bacterial proteins to confer invasion since latex beads coated with invasin are taken up by mammalian cells.[33] Nevertheless, bacteria use multiple strategies to interact with mammalian cells.[34] *E. coli* MC1061 synthesize type I pili encoded by the *fim* operon, which bind to mammalian surface carbohydrates. This strain does not express any other known adhesion modules, such as curli[35] or P pili. To determine whether type I pili play a significant role in invasin-mediated internalization, a *fim* deletion strain (CAMC600) was constructed.

To constitutively express invasin, the *inv* gene was inserted into a medium-copy plasmid under the control of a *tet* promoter (pAC-TetInv) (Figure 2(a)). Strain MC1061 lacks Tet repressor, so bacteria harbouring pAC-TetInv constitutively produce invasin. Invasiveness towards HeLa cells was assayed by gentamicin protection (Methods) and was reported as the fraction of added bacteria recovered from lysis. In this assay, 8(±5)% of inv^+ *E. coli* (pAC-TetInv) were recovered (Figure 2(b)). In contrast, invasion by *E. coli* MC1061 without invasin was below the detection limit of the assay ($\sim 10^{-5}$). To determine the role of type I pili on invasion, we transformed CAMC600 with pAC-TetInv and examined its ability to invade HeLa cells. This strain retained the ability to invade (1.5(±0.5)%) (Figure 2(b)). Therefore, the invasive phenotype of *inv* is modular and does not require other *E. coli* adhesion systems.

We next examined the range of host cells for inv^+ *E. coli*. In addition to HeLa cells, we examined the human cancer cell lines U2OS (osteosarcoma) and HepG2 (hepatocarcinoma). After incubation with U2OS cells, 2.9(±0.6)% of inv^+ *E. coli* were recovered (Figure 2(b)). Similarly, 0.2(±0.1)% of MC1061 harbouring pAC-TetInv were recovered from HepG2 cells. When invasin-deficient *E. coli* were incubated with either cell line, <0.001% of the bacteria were recovered. Small differences in the relative efficiency of invasion of the three cell lines may reflect different levels of β1 integrin expression

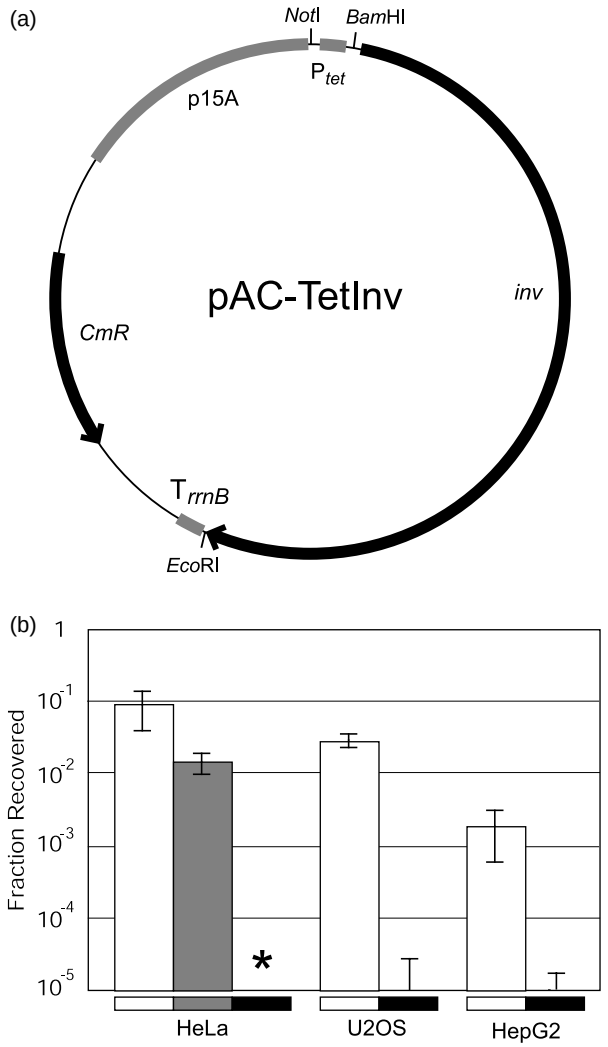

Figure 2. Invasin as an output module. (a) The *inv* gene was expressed under the control of a constitutive *tet* promoter. (b) Fraction of bacteria recovered from gentamicin protection assay invading HeLa, U2OS, or HepG2 cells. Constitutively expressed invasin is shown in white (pAC-TetInv). Cells with no invasin are shown in black, and cells expressing invasin but lacking type I pili are shown in grey (CAMC600/pAC-TetInv). Assays in which no bacteria were recovered are indicated with an asterisk.

or the accessibility of β1 integrin on the cell surface. Although the efficiency varies, these experiments demonstrate that inv^+ *E. coli* are capable of invading cancer cell lines of diverse origin. Nevertheless, not all mammalian cells are susceptible to invasion. *Y. pseudotuburculosis* specifically invades M cells over enterocytes during infection of rat epithelium.[30] Also, inv^+ *E. coli* will only invade cells actively expressing β1-integrins, such as those at the leading edge of an epithelial sheet.[16]

Inducible control of invasion

To demonstrate the inducible control of bacterial invasion, we placed the invasin gene under the control of the *araBAD* operon (Figure 3(a)). The arabinose operon of *E. coli* encodes three genes involved in arabinose catabolism and the transcription factor AraC. Regulation of the *araBAD* promoter involves both arabinose-dependent activation and DNA looping-mediated repression by *AraC*.[36] This operon is absent in MC1061, so arabinose is not metabolized by this strain. Moreover, this promoter is used extensively for arabinose-inducible overexpression of foreign proteins.

The *araBAD* promoter and AraC gene were inserted into: (1) pAC-TetInv replacing the *tet* promoter, and (2) into a single-copy BAC plasmid (bacterial artificial chromosome) pBAC874t. These constructs resulted in invasive phenotypes independent of arabinose concentration (not shown). Thus, background transcription from the *araBAD* promoter is sufficient for invasin synthesis, even at single-copy. To reduce the basal expression of invasin, we constructed ribosome binding site variants and used genetic selection to identify library members that responded to promoter activation. We replaced the 5′ untranslated region (UTR) of invasin with random sequence flanking a partial ribosome binding site and randomized the first position of the first and second codons (Figure 3(b)). These variants were fused to the *araBAD* promoter in pBAC874t affording 10^6 library members. To identify induction-dependent members of this library, we first applied a positive selection to identify members of the library that could invade HeLa cells after growth under inducing conditions. A negative screen was then used to identify the subset of these clones that did not invade in the absence of induction. We added 10^8 bacteria grown in the presence of arabinose to 10^5 HeLa cells and enriched for clones with active ribosome binding sites, as indicated by a gentamicin protection assay measuring invasiveness. Approximately 1000 colonies were recovered after lysis of the HeLa cells.

From the pool of *araBAD* clones recovered from positive selection, we screened 24 *araBAD* colonies for loss of invasiveness after growth in media lacking arabinose. Of these, three clones did not invade HeLa cells, and one clone, pBACr-AraInv, was characterized. In the absence of arabinose, invasion by MC1061 cells harbouring pBACr-AraInv was undetectable, but 2.3(±0.7)% were recovered after growth in the presence of 0.02% arabinose. Growth in the presence of arabinose did not affect the invasiveness of MC1061 cells with no plasmid or pAC-TetInv (Figure 3(c)). pBACr-AraInv encodes a non-canonical member of the library, GTCGGAGTCCCTCGTGTTGTTTTCCA GCCAATCAGTGGAGTTGGATCCCCCGGAGCC CCCCatgctg with an apparent duplication of the 5′ UTR (putative first two codons in lowercase, conserved ribosome binding site underlined; Figure 3(b)). Deletion of the sequence 5′ of the BamHI site did not affect invasiveness in the presence or absence of arabinose.

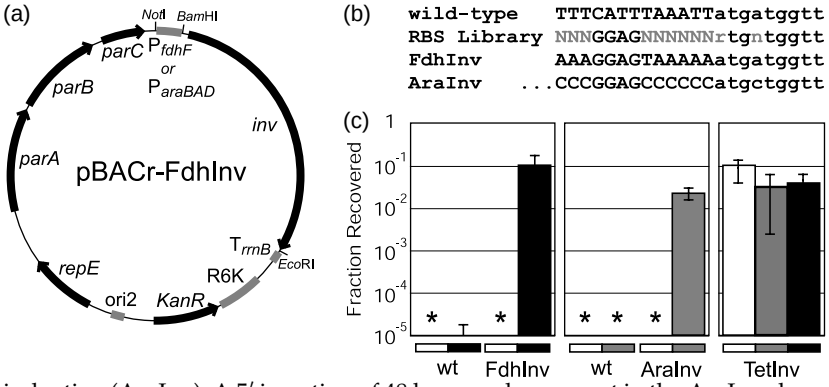

Figure 3. Inducible invasion. (a) *fdhF* and *araBAD* gene fusions with invasin were constructed in a single-copy plasmid. (b) A library of *inv* ribosome binding variants were screened for selective induction under anaerobic growth or arabinose induction. Sequences are shown for the wild-type ribosome binding site, a library of variants (N=A, T, C, or G, R=A or G), and two recovered library members selective for anaerobic induction (FdhInv) and arabinose induction (AraInv). A 5′ insertion of 48 bp was also present in the AraInv clone. (c) Fraction of bacteria recovered for cells with no plasmid (wt), with the AraInv or FdhInv isolates, or pAC-TetInv (TetInv) grown with no induction in white, anaerobic induction in black, and arabinose induction in grey. Assays in which no bacteria were recovered are indicated with an asterisk.

Linkage of bacterial invasion with hypoxia

One potential application of inv^+ *E. coli* is therapeutic bacteria for the treatment of cancer. By restricting the expression of *inv* to tumor sites, invasion could be confined to malignant cells. The hypoxic environment could provide a cue for detection of tumors and the induction of cancer cell invasion.[37] Previous microarray analysis identified several genes in *E. coli* whose expression is strongly induced after the transition from aerobic to anaerobic growth.[38] Of these, formate dehydrogenase (*fdhF*) is one of the most strongly induced genes.[39] Therefore, this promoter was chosen to link invasion with the transition to an anaerobic environment.

When invasin was placed under the control of the *fdhF* promoter, a constitutive invasive phenotype was observed for both medium and single-copy plasmids, as was observed for the *araBAD* constructs. To create a hypoxia-inducible system, a 10^6-member ribosome binding site library was constructed and screened as before. After induction in an anaerobic chamber, positive selection of inv^+ bacteria was performed by gentamicin protection. For the negative screen, 64 *fdhF* colonies recovered from positive selection were grown under aerobic conditions and then assayed by gentamicin protection for loss of invasiveness. From this screen, we identified two *fdhF* variants that did not invade HeLa cells. One of these clones, pBACr-FdhInv, was investigated further. During aerobic growth, invasion of HeLa cells by MC1061 cells harbouring pBACr-FdhInv was undetectable, but 10(±5)% of added bacteria were recovered after growth for 2 h in the anaerobic chamber (Figure 3(c)). Growth in the anaerobic chamber did not affect the invasiveness of MC1061 cells harbouring no plasmid or pAC-TetInv indicating that the different phenotypes observed with pBACr-FdhInv were the result of promoter induction and not a physiological effect. Sequencing of pBACr-FdhInv revealed the sequence AAAGGAGTAAAAAatgatg (putative first two codons in lowercase, conserved ribosome binding site underlined; Figure 3(b)).

Cell-density-dependent invasion

A remarkable range of bacterial species, including non-pathogens, localize to tumors after intravenous (IV) injection, such as *E. coli*, *Vibrio cholerae*, *Clostridium*, *Bifidobacterium*, *Salmonella*, and *Listeria monocytogenes*.[20,40] This occurs for a wide variety of solid tumors, including bladder, brain, and breast cancers.[40] These microbes impart their selectivity for tumors by exploiting the hypoxic microenvironment, poor immune surveillance, and the increased availability of nutrients.[41,21,22] After a *Salmonella* IV injection in mice, the concentration of bacteria in tumors is approximately 10^9 colony forming units (cfu)/g tissue, whereas in liver it is 10^6 cfu/g and in muscle it is 10^3 cfu/g.[23] This provides an additional cue that could be exploited to restrict infection to the tumor microenvironment. Here, we link the capacity to invade mammalian cells with the density of bacteria by placing invasin under the control of a quorum sensing genetic circuit.

The bacterium *Vibrio fischeri* contains a genetic circuit (*lux*) that enables the bacteria to distinguish between the low cell densities found in the ocean (10^3 cfu/ml) and the high cell density found in the squid light organ (10^{10} cfu/ml).[42] The *lux* quorum system was used previously to synthetically link a biological response to cell density.[2,15] The circuit encodes the transcriptional activator LuxR and an enzyme LuxI that catalyzes the synthesis of *N*-3-oxohexanoyl-L-homoserine lactone (AI-1). AI-1 freely diffuses through the cell membrane and accumulates in the media as the cell density increases. At high density, AI-1 activates LuxR, which in turn upregulates gene expression of both *luxI* and *luxR*.[42] Positive feedback results in an ultrasensitive switch that rapidly transitions

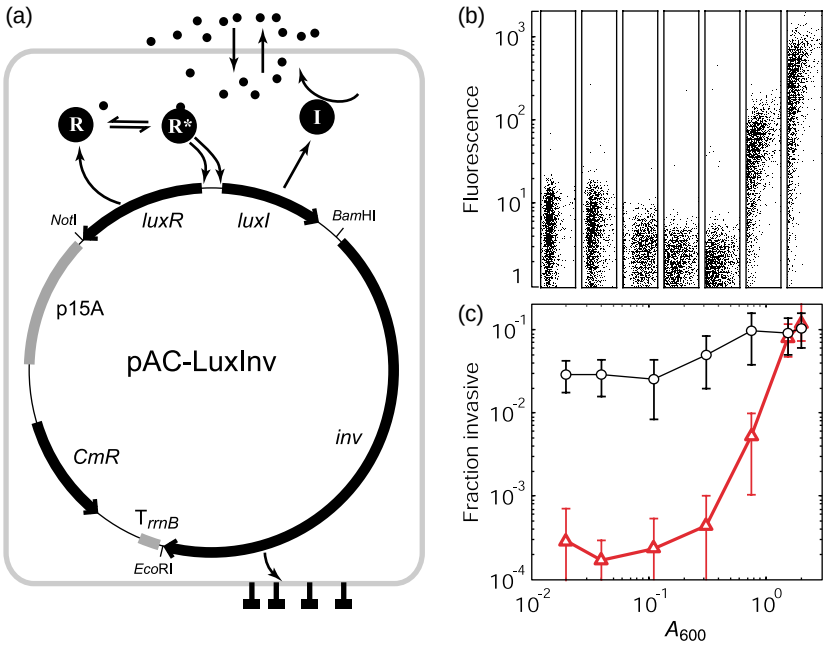

Figure 4. Cell-density-dependent activation of *inv* and *gfp*. (a) LuxI synthesis results in the production of *N*-3-oxohexanoyl-L-homoserine lactone (AI-1). The transcription factor LuxR is activated by high concentrations of AI-1 resulting in the activation of *luxI* and *inv*. (b) Flow cytometric analysis of MC1061 cells harboring *lux* GFP reporter plasmid pAC-LuxGfp. (c) Fraction of recovered invading bacteria is shown as a function of cell density for invasin expressed constitutively (pAC-TetInv, black circles) and under quorum control (pAC-LuxInv, red triangles). Error bars are given as one standard deviation around the mean for five independent experiments. The detection limit for this assay is $\sim 10^{-5}$.

from the off to on state at a critical cell density. The *lux* quorum circuit was shown previously to be active in *E. coli* and does not cross-react with homologous enterobacterial systems.[43]

To place invasin under quorum control, a plasmid was created where *inv* is fused to the *lux* P_R promoter downstream of *luxI* (pAC-LuxInv) (Figure 4). Bacteria harbouring invasin were grown to various cell densities and their abilities to invade HeLa cells were quantified by gentamicin protection. When *inv* was expressed from a constitutive promoter, invasion efficiency was independent of cell density (Figure 4(c)). In contrast, when *inv* is under quorum control (pAC-LuxInv) it is detectable only at high densities of bacteria. The half-maximum efficiency was observed at 3×10^8 cfu/ml ($A_{600} = 0.7$). This transition occurs between the densities observed for *Salmonella* within tumors (10^9) and healthy tissues (10^3–10^6) in mouse experiments.[23] After the quorum system is fully activated, the observed multiplicity of infection (MOI) is comparable to that of the constitutive construct. To monitor the activation of the quorum circuit directly, we replaced the *inv* gene with green fluorescent protein (GFP) (pAC-LuxGfp) and measured fluorescence as a function of cell density using flow cytometry. The transition for GFP fluorescence was identical to that observed for invasion ($A_{600} > 0.7$).

Discussion

We have described the design of bacteria able to invade mammalian cells selectively at high cell density, after anaerobic growth, or after chemical induction. This was achieved by combining an output interface module encoded by the *inv* gene from *Y. pseudotuberculosis* with the *lux* operon of *V. fisheri*, the *fdhF* promoter, or the arabinose operon. As an output module, *inv* afforded invasion of multiple cancer cell types including cervical carcinoma, hapatocarcinoma, and osteosarcoma.

Invasin presents a scaffold upon which protein engineering could be used to alter its function or enable it to bind new targets. For example, Isberg and co-workers identified invasin mutants that confer binding to host cells but not invasion.[33] It may also be possible to mutate the invasin binding domain to adhere to surface proteins and carbohydrates indicative of other cell types. This binding domain is structural plastic with respect to peptide.[44]

In this work individual promoters communicate an input signal to an output response. Genetic logic circuits or response regulatory networks could integrate multiple inputs to achieve more accurate environmental sensing. However, engineering the necessary artificial fusions between sensory promoters and output genes is complicated by mismatches in rates of transcription and translation, especially as the complexity of the system increases. Induced and non-induced states have promoter-specific rates of transcription, and in some instances, the induced transcription rate is insufficient for output gene expression. In other instances, high non-induced transcription rates result in a constitutive phenotype.[2,45] One of two strategies could reduce basal expression: the transcription rate could be lowered through promoter mutation, or else the promoter could be left unaltered, and the translation rate of the output gene reduced.[46] In the latter case, the mRNA synthesized no longer affords sufficient protein to observe a phenotype.

We adopted the strategy of mutating the ribosome binding in order to reduce basal expression of invasin. Previously, Lee and co-workers randomized positions −3 to −13 of an *lpp-lac* promoter driven *lacZ* gene and identified variants with greater than tenfold range in activity. The sequence identity of the ribosome binding site and flanking bases, the position of the ribosome binding site, and the first base of the start codon all influenced expression levels.[47] We hypothesized that saturation mutagenesis of the *inv* 5′UTR would similarly produce a wide range of expression levels with a subset displaying the correct level of activity for induction-dependent responses. To minimize nonfunctional sequences, we restricted mutagenesis to the flanking bases of a consensus ribosome binding site. Nevertheless, we recovered only 1×10^{-5} of library members from positive selection. This necessitated genetic selection to identify the rare functional clones. For other phenotypes not amenable to positive selection, it will be necessary to use smaller libraries. Future analysis of other 5′ UTR libraries may reveal a more efficient strategy for creating diversity.

It is surprising that the ribosome binding site variants identified by selection show less background activity than the wild-type sequence. Lacking a canonical Shine-Delgarno sequence, the sequence present in wild-type invasin should afford a lower rate of protein synthesis. This result reiterates the difficulty in predicting the activity of a ribosome binding site *de novo*.

Specific invasion of tumor cells is only one component of an anti-cancer bacterium. Once inside target cells, a cytotoxic or immunostimulatory response must instigate destruction of the tumor. Various bacteria have been engineered for this effect including *Salmonella* that metabolize a chemotherapeutic prodrug at tumor sites,[24] strains of *Clostridium* that secrete TNFα,[25,26] and strains of BCG and *Salmonella* that secrete a repertoire of mammalian cytokines.[27] In addition, the use of *E. coli* as a non-pathogenic chassis will require modifications otherwise unnecessary with evolved pathogens. The short half-life of *E. coli* in the bloodstream and strong anti-LPS response will require modifications to the lipopolysaccharide structure,[48] O-antigen, or capsule.[49]

It has been proposed to use bacteria for a number of therapeutic applications; for example, as live vaccines[5] and gene delivery vectors.[28] These goals share a common need to be able to genetically program gene expression to occur in a particular environmental niche in the body. Here, we demonstrate that environmental stimuli can be synthetically linked to the adhesion and invasion of cancer cells. This strategy could be applied to processes other than invasion, such as the synthesis of displayed antigens or the release of a therapeutic protein. The combination of genetic modules encoding sensing, circuitry, and actuator functions presents a general platform by which therapeutic functions can be programmed into bacteria.

Methods

Molecular biology

All manipulations were performed in derivatives of *E. coli* strains MC1061 or EC100D™ pir-116 (Epicentre, Madison, Wis.) in 2YT liquid media or LB agar plates supplemented with antibiotics at 25 μg/ml at 37 °C. DNA-modifying enzymes were purchased from New England Biolabs. Oligonucleotides were synthesized by Sigma-Genosys (The Woodlands, TX) and used unpurified. PCR was performed with the Roche High Fidelity PCR kit. The *lux* operon was derived from plasmids pKE705 and pKE555.[50] *Y. pseudotuberculosis* genomic DNA was prepared using the Sigma Genomic DNA purification kit from a culture of ATCC 79833 (Mannassis, VA). HeLa and HepG2 cells were obtained from the UCSF Cell Culture Facility (San Francisco, CA) and U2OS cells were a gift from Keith Yamamoto. Cells were grown in DMEM media supplemented with 10% (v/v) FCS and 1% (v/v) streptomycin/penicillin solution.

Construction of CAMC600

A gene knockout cassette was constructed by PCR amplification of a 5′ flanking sequence of the *fim* operon with oligonucleotides ca598F (5′-GAGTTGGTCTCAGATCAAAAATCACGCAATC-3′) and ca598R (5′-CGAAAGGTCTCAGACGCTTCTGAGTGAACTTAAATG-3′) and a 3′ flanking sequence with ca600F (5′-GAGTTGGTCTCAGGTCGAAATCACAGGACATTGC-3′) and ca600R (5′-CGAAAGGTCTCAAATTCGTCTGGCGACGGGTCAGGGTG-3′). A FRT-KanR-FRT cassette from plasmid pKD13[51] was PCR amplified with oligonucleotides ca589F (5′-GAGTAGGTCTCTCGTCGTGTAGGCTGGAGCTGCTTC-3′) and ca599R (5′-CGAAAGGTCTCAGACCATTCCGGGGATCCGTCGACC-3′) and inserted between the two flanking sequences by ligation after BsaI digestion of the three products. The cassette was amplified with oligonucleotides ca598F and ca600R and recombined into the genome of MC1061 by the methods described by Datsenko and Wanner.[51] The kanamycin resistance cassette was eliminated by Flp-mediated recombination[51] to generate an antibiotic-sensitive *fim* deletion strain.

Invasion assays

Invasion/protection assays were performed as described.[52] For time-course assays, saturated aliquots of bacteria were diluted 500-fold in 2YT media and grown for 2 h with shaking at 30 °C. Each hour, aliquots were removed and stopped by cooling on ice. After 7 h, each aliquot was diluted to an MOI of 5, and 50 μl were added to 1 ml of DMEM media in 24 well plates containing a confluent culture of mammalian cells. This low MOI ensures that the number of invasion events observed averaged less than one per HeLa cell. After 1 h incubation at 37 °C, the wells were washed once in DMEM media and then incubated for 1 h with 1 ml of DMEM supplemented with 100 μg/ml gentamicin. Subsequently the wells were washed three times with DMEM and lysed with 1 ml of 1% Triton X-100. Dilutions of lysate were spread on LB agar plates and grown 24 h. Percent invasion was determined as the ratio of recovered bacteria divided by the number of CFU present in the original dilute culture as determined by titer. Values were averaged over five repetitions of the experiment. Data

was averaged by linear interpolation based on absorbance using MATLAB (Mathworks, Inc.).

Plasmid construction

Medium-copy plasmids

To construct plasmid pAC-TetInv, the *inv* gene (GenBank accession no. M17448) was PCR amplified from *Y. pseudotuberculosis* genomic DNA with oligonucleotides ca783F (5′-CTGAAGGATCCGTTTGACGTAT GACAGGTATGC-3′) and Rinv (5′-AGGGTCGAATTC TTATATTGACAGCGCACAGA-3′), digested with BamHI and EcoRI, and inserted into similar sites of pAC581 (Figure 2). This plasmid contains a p15A origin of replication, a chloramphenicol resistance gene, a *tet* promoter and a T_{rrnB} terminator derived from pBAD/*Myc*-HisA (Invitrogen). The *lux* operon was assembled from plasmids pKE705 and pKE555. pKE705 was digested with XhoI and XbaI and the 580 bp fragment was ligated to a 2980 bp XbaI/BglII fragment of pKE555. The ligation product was amplified by PCR with oligonucleotides ca742F (5′-CAGTCGGATCCTTAA TTTTTAAAGTATGGGCAATC-3′) and ca721R (5′-CAC TGGAATTCGTAATGACAGATAATTTTACTC-3′). The full-length *lux* operon gene was digested with BamHI and EcoRI and inserted into similar sites of pPROBE-*gfp*[LAA][53] resulting in plasmid pPROBE-luxIR. Plasmid pAC-LuxInv was constructed by PCR amplification of the *lux* operon in pPROBE-luxIR with oligonucleotides ca837F (5′-GGTATGCGGCCGCTTAATTTTTAAAGT ATGGGCAATC-3′) and ca837R (5′-CACTTGGATCCG TAATGACAGATAATTTTACTC-3′) and inserted into the NotI and BamHI sites of pAC-TetInv. In this manner, the *tet* promoter was replaced with the *lux* operon. Plasmid pAC-LuxGfp was constructed by PCR amplification of the *lux* and *gfp* genes of plasmid pPROBE-luxIR with oligonucleotides ca837F and ca803R (5′-GCAACG GTCTCGAATTCCCTTAGCTCCTGAAAATCTCGC-3′), digestion with NotI and BsaI, and insertion into the NotI and EcoRI sites of plasmid pAC-TetInv.

Single-copy plasmids

Plasmid pBAC874t encodes the origin and partition genes from pBeloBAC11, a kanamycin resistance gene, an R6K origin of replication, a *tet* promoter and a T_{rrnB} terminator (Figure 3(a)). Two components of this plasmid, β-lactamase and pUC origin, are deleted after insertion of foreign sequences, thus changing the copy number. In MC1061, derivatives of this plasmid replicate at single-copy, but the R6K origin of replication confers high-copy replication in *pir-116* strains. Promoter cassettes were inserted into the NotI and BamHI sites of this plasmid and *inv* variants were inserted into the BamHI and EcoRI sites.

Selection of ribosome variants of invasin

An *fdhF* promoter variant of pBAC874t was constructed from a PCR product of MG1655 genomic DNA with oligonucleotides ca853F (5′-GGTATGCGGCCGCGGG TATCAGGCAGGTCC-3′) and ca853R (5′-CACTTG GATCCAATAGGGGCAAACCGTGACGAC-3′). The *araC* and *araBAD* cassette was amplified with oligonucleotides ca872F (5′-GGTATGCGGCCGCCATAATGT-GCCTGTCAAATG-3′) and ca872R (5′-CGTCAGGAT CCGGGTATGGAGAAACAGTAG-3′) from plasmid pBAD860. The BamHI site present in the wild-type *araBAD* promoter has been mutated to GGATCT in this plasmid. Both PCR cassettes were digested with NotI and BamHI and inserted into similar sites of pBAC874t to afford plasmids pBACr-Fdh and pBACr-Ara. Invasin was PCR-amplified from pAC-TetInv with oligonucleotides ca877F (5′-GAGTTGGATCCNNNGGAGNNNNNN RTGNTGGTTTTCCAGCCAATCAGTG-3′) and ca606R (5′-GTCGACGGCGCTATTCAGATCCTC-3′), digested with BamHI and EcoRI, and inserted in similar sites of pBACr-Fdh and pBACr-Ara affording ribosome binding site libraries.

The *fdhF* promoter was induced by growth for 2 h to an $A_{600}=1$ in a controlled environment chamber 855AC equipped with a catalyst heater (Plas Labs, Lansing, MI) in 2YT supplemented with kanamycin under an atmosphere of 85% N_2, 10% H_2, and 5% CO_2. The *araBAD* promoter was induced by growth for 2 h to $A_{600}=1$ in 2YT media supplemented with kanamycin and 0.02% (w/v) arabinose. HeLa cells grown in 24 well plates were infected with 10 μl of induced library members, and selection was performed as described for gentamicin protection assays. Cell lysates were concentrated by centrifugation at 16,000 *g* and plated on LB agar plates. For negative screens, colonies were grown in 2YT media supplemented with kanamycin. Aliquots (1.5 μl) of each culture were added to 96 well plates containing HeLa cells. Gentamicin protection assays were performed as described above. Each well was lysed with 50 μl of water for 5 min, and then 150 μl of DMEM supplemented with 10% 2YT and kanamycin was added. Cultures were grown for 6 h and monitored visually for color change and turbidity. Secondary screens were performed by growing the bacteria under the appropriate conditions to $A_{600}=1$, diluting 1000-fold, and assaying 50 μl by gentamicin protection.

Cytometry

Serial dilutions of bacteria harbouring pAC-LuxGfp were grown in parallel with invasion-expressing cells. Analysis was performed on a Becton Dickinson FACSCalibur™.

Acknowledgements

We thank Professor E. P. Greenberg for *lux* plasmids, J. S. Weissman for use of the anaerobic chamber, and K. R. Yamamoto for U2OS cells. J.C.A. is a Damon Runyon Cancer Research Foundation fellow. C.A.V. acknowledges support from the Sloan Foundation and a Sandler Family Award. A.P.A. acknowledges support from the Howard Hughes Medical Institute and from the National Institutes of Health (5R01GM63525-3).

References

1. Ferber, D. (2004). Microbes made to order. *Science*, **303**, 158–161.
2. Kobayashi, H., Kaern, M., Araki, M., Chung, K., Gardner, T. S., Cantor, C. R. & Collins, J. J. (2004).

Programmable cells: interfacing natural and engineered gene networks. *Proc. Natl Acad. Sci. USA*, **101**, 8414–8419.
3. Jung, K. H., Spudich, E. N., Trivedi, V. D. & Spudich, J. L. (2001). An archaeal photosignal-transducing module mediates phototaxis in *Escherichia coli*. *J. Bacteriol.* **183**, 6365–6371.
4. Basu, S., Gerchman, Y., Collins, C. H., Arnold, F. H. & Weiss, R. (2005). A synthetic multicellular system for programmed pattern formation. *Nature*, **434**, 1130–1134.
5. Garmory, H. S., Leary, S. E., Griffin, K. F., Williamson, E. D., Brown, K. A. & Titball, R. W. (2003). The use of live attenuated bacteria as a delivery system for heterologous antigens. *J. Drug Target.* **11**, 471–479.
6. Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D. & Keasling, J. D. (2003). Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nature Biotechnol.* **21**, 796–802.
7. Pawelek, J. M., Low, K. B. & Bermudes, D. (2003). Bacteria as tumour-targeting vectors. *Lancet Oncol.* **4**, 548–556.
8. Looger, L. L., Dwyer, M. A., Smith, J. J. & Hellinga, H. W. (2003). Computational design of receptor and sensor proteins with novel functions. *Nature*, **423**, 185–190.
9. Buskirk, A. R., Landrigan, A. & Liu, D. R. (2004). Engineering a ligand-dependent RNA transcriptional activator. *Chem. Biol.* **11**, 1157–1163.
10. Guet, C. C., Elowitz, M. B., Hsing, W. & Leibler, S. (2002). Combinatorial synthesis of genetic networks. *Science*, **296**, 1466–1470.
11. Elowitz, M. B. & Leibler, S. (2000). A synthetic oscillatory network of transcriptional regulators. *Nature*, **403**, 335–338.
12. Gardner, T. S., Cantor, C. R. & Collins, J. J. (2000). Construction of a genetic toggle switch in *Escherichia coli*. *Nature*, **403**, 339–342.
13. Basu, S., Mehreja, R., Thiberge, S., Chen, M. T. & Weiss, R. (2004). Spatiotemporal control of gene expression with pulse-generating networks. *Proc. Natl Acad. Sci. USA*, **101**, 6355–6360.
14. Dueber, J. E., Yeh, B. J., Chak, K. & Lim, W. A. (2003). Reprogramming control of an allosteric signaling switch through modular recombination. *Science*, **301**, 1904–1908.
15. You, L., Cox, R. S., III, Weiss, R. & Arnold, F. H. (2004). Programmed population control by cell-cell communication and regulated killing. *Nature*, **428**, 868–871.
16. Fajac, I., Grosse, S., Collombet, J. M., Thevenot, G., Goussard, S., Danel, C. & Grillot-Courvalin, C. (2004). Recombinant *Escherichia coli* as a gene delivery vector into airway epithelial cells. *J. Control Release*, **97**, 371–381.
17. Grillot-Courvalin, C., Goussard, S., Huetz, F., Ojcius, D. M. & Courvalin, P. (1998). Functional gene transfer from intracellular bacteria to mammalian cells. *Nature Biotechnol.* **16**, 862–866.
18. Narayanan, K. & Warburton, P. E. (2003). DNA modification and functional delivery into human cells using *Escherichia coli* DH10B. *Nucl. Acids Res.* **31**, e61.
19. Critchley, R. J., Jezzard, S., Radford, K. J., Goussard, S., Lemoine, N. R., Grillot-Courvalin, C. & Vassaux, G. (2004). Potential therapeutic applications of recombinant, invasive *E. coli*. *Gene Ther.* **11**, 1224–1233.
20. Yazawa, K., Fujimori, M., Amano, J., Kano, Y. & Taniguchi, S. (2000). *Bifidobacterium longum* as a delivery system for cancer gene therapy: selective localization and growth in hypoxic tumors. *Cancer Gene Ther.* **7**, 269–274.
21. Bettegowda, C., Dang, L. H., Abrams, R., Huso, D. L., Dillehay, L., Cheong, I. *et al*. (2003). Overcoming the hypoxic barrier to radiation therapy with anaerobic bacteria. *Proc. Natl Acad. Sci. USA*, **100**, 15083–15088.
22. Zhao, M., Yang, M., Li, X. M., Jiang, P., Baranov, E., Li, S. *et al*. (2005). Tumor-targeting bacterial therapy with amino acid auxotrophs of GFP-expressing *Salmonella typhimurium*. *Proc. Natl Acad. Sci. USA*, **102**, 755–760.
23. King, I., Bermudes, D., Lin, S., Belcourt, M., Pike, J., Troy, K. *et al*. (2002). Tumor-targeted Salmonella expressing cytosine deaminase as an anticancer agent. *Hum. Gene Ther.* **13**, 1225–1233.
24. Nemunaitis, J., Cunningham, C., Senzer, N., Kuhn, J., Cramm, J., Litz, C. *et al*. (2003). Pilot trial of genetically modified, attenuated Salmonella expressing the *E. coli* cytosine deaminase gene in refractory cancer patients. *Cancer Gene Ther.* **10**, 737–744.
25. Nuyts, S., Van Mellaert, L., Theys, J., Landuyt, W., Bosmans, E., Anne, J. & Lambin, P. (2001). Radio-responsive recA promoter significantly increases TNFalpha production in recombinant clostridia after 2 Gy irradiation. *Gene Ther.* **8**, 1197–1201.
26. Dang, L. H., Bettegowda, C., Huso, D. L., Kinzler, K. W. & Vogelstein, B. (2001). Combination bacteriolytic therapy for the treatment of experimental tumors. *Proc. Natl Acad. Sci. USA*, **98**, 15155–15160.
27. Murray, P. J., Aldovini, A. & Young, R. A. (1996). Manipulation and potentiation of antimycobacterial immunity using recombinant bacille Calmette-Guerin strains that secrete cytokines. *Proc. Natl Acad. Sci. USA*, **93**, 934–939.
28. Drabner, B. & Guzman, C. A. (2001). Elicitation of predictable immune responses by using live bacterial vectors. *Biomol. Eng.* **17**, 75–82.
29. Wong, K. W. & Isberg, R. R. (2005). Emerging views on integrin signaling via Rac1 during invasin-promoted bacterial uptake. *Curr. Opin. Microbiol.* **8**, 4–9.
30. Clark, M. A., Hirst, B. H. & Jepson, M. A. (1998). M-cell surface beta1 integrin expression and invasin-mediated targeting of *Yersinia pseudotuberculosis* to mouse Peyer's patch M cells. *Infect. Immun.* **66**, 1237–1243.
31. Isberg, R. R., Voorhis, D. L. & Falkow, S. (1987). Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. *Cell*, **50**, 769–778.
32. Hamburger, Z. A., Brown, M. S., Isberg, R. R. & Bjorkman, P. J. (1999). Crystal structure of invasin: a bacterial integrin-binding protein. *Science*, **286**, 291–295.
33. Dersch, P. & Isberg, R. R. (1999). A region of the *Yersinia pseudotuberculosis* invasin protein enhances integrin-mediated uptake into mammalian cells and promotes self-association. *EMBO J.* **18**, 1199–1213.
34. Niemann, H. H., Schubert, W. D. & Heinz, D. W. (2004). Adhesins and invasins of pathogenic bacteria: a structural view. *Microbes Infect.* **6**, 101–112.
35. Hammar, M., Arnqvist, A., Bian, Z., Olsen, A. & Normark, S. (1995). Expression of two csg operons is required for production of fibronectin- and congo red-binding curli polymers in *Escherichia coli* K-12. *Mol. Microbiol.* **18**, 661–670.
36. Schleif, R. (2003). AraC protein: a love-hate relationship. *Bioessays*, **25**, 274–282.
37. Henning, T., Kraus, M., Brischwein, M., Otto, A. M. & Wolf, B. (2004). Relevance of tumor microenvironment for progression, therapy and drug development. *Anticancer Drugs*, **15**, 7–14.

38. Salmon, K., Hung, S. P., Mekjian, K., Baldi, P., Hatfield, G. W. & Gunsalus, R. P. (2003). Global gene expression profiling in *Escherichia coli* K12. The effects of oxygen availability and FNR. *J. Biol. Chem.* **278**, 29837–29855.
39. Wang, H. & Gunsalus, R. P. (2003). Coordinate regulation of the *Escherichia coli* formate dehydrogenase fdnGHI and fdhF genes in response to nitrate, nitrite, and formate: roles for NarL and NarP. *J. Bacteriol.* **185**, 5076–5085.
40. Yu, Y. A., Shabahang, S., Timiryasova, T. M., Zhang, Q., Beltz, R., Gentschev, I. *et al*. (2004). Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins. *Nature Biotechnol.* **22**, 313–320.
41. Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R. & Ferguson, T. A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science*, **270**, 1189–1192.
42. Sitnikov, D. M., Schineller, J. B. & Baldwin, T. O. (1995). Transcriptional regulation of bioluminesence genes from *Vibrio fischeri*. *Mol. Microbiol.* **17**, 801–812.
43. Surette, M. G. & Bassler, B. L. (1998). Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. *Proc. Natl Acad. Sci. USA*, **95**, 7046–7050.
44. Nakajima, H., Shimbara, N., Shimonishi, Y., Mimori, T., Niwa, S. & Saya, H. (2000). Expression of random peptide fused to invasin on bacterial cell surface for selection of cell-targeting peptides. *Gene*, **260**, 121–131.
45. Weiss, R., Homsy, G. E. & Knight, T. F. (1999). Toward in vivo digital circuits. *DIMACS Workshop on Evolution as Computation*, Princeton University.
46. Lee, S. H., Hava, D. L., Waldor, M. K. & Camilli, A. (1999). Regulation and temporal expression patterns of *Vibrio cholerae* virulence genes during infection. *Cell*, **99**, 625–634.
47. Min, K. T., Kim, M. H. & Lee, D. S. (1998). Search for the optimal sequence of the ribosome binding site by random oligonucleotide-directed mutagenesis. *Nucl. Acids Res.* **16**, 5075–5088.
48. Low, K. B., Ittensohn, M., Le, T., Platt, J., Sodi, S., Amoss, M. *et al*. (1999). Lipid A mutant Salmonella with suppressed virulence and TNFalpha induction retain tumor-targeting *in vivo*. *Nature Biotechnol.* **17**, 37–41.
49. Alexander, C. & Rietschel, E. T. (2001). Bacterial lipopolysaccharides and innate immunity. *J. Endotoxin Res.* **7**, 167–202.
50. Egland, K. A. & Greenberg, E. P. (2001). Quorum sensing in *Vibrio fischeri*: analysis of the LuxR DNA binding region by alanine-scanning mutagenesis. *J. Bacteriol.* **183**, 382–386.
51. Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl Acad. Sci. USA*, **97**, 6640–6645.
52. Leong, J. M., Fournier R., S. & Isberg, R. R (1990). Identification of the integrin binding domain of the *Yersinia pseudotuberculosis* invasin protein. *EMBO J.* **9**, 1979–1989.
53. Miller, W. G., Leveau, J. H. & Lindow, S. E. (2000). Improved gfp and inaZ broad-host-range promoter-probe vectors. *Mol. Plant Microbe Interact.* **13**, 1243–1250.

Edited by J. Karn

(Received 31 August 2005; received in revised form 18 October 2005; accepted 28 October 2005)
Available online 14 November 2005