Engineered of *Escherichia coli* for targeted delivery of transgenes to HER2/neu-positive tumor cells

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**Abstract**
Targeting of non-phagocytic tumor cells and prompt release of gene cargos upon entry into tumors are two limiting steps in the bacterial gene delivery path. To tackle these problems, the non-pathogenic *Escherichia coli* strain BL21(DE3) was engineered to display the anti-HER2/neu affibody on the surface. After co-incubation with tumor cells for 3 h, the anti-HER2/neu affibody-presenting *E. coli* strain was selectively internalized into HER2/neu-positive SKBR-3 cells. The invasion efficiency reached as high as
30%. Furthermore, the bacteria were equipped with the phage ϕX174 lysin gene E-mediated autolysis system. Carrying the transgene (e.g., eukaryotic green fluorescent protein, GFP), the tumor-targeting bacteria were subjected to the thermal shock to trigger the autolysis system upon entry into HER2/neu-positive cells. Flow cytometric analysis revealed that 3% of infected cells expressed GFP 24 h post thermal induction. Overall, the results show a promise of the proposed approach for developing bacteria as a delivery carrier.

Introduction
Gene therapy appears to be a promising method for therapeutic treatment of diseases. For most applications, it lies in the use of gene transfer vectors to deliver therapeutic cargos to the desired location. In general, these delivery vectors are classified into viral and non-viral vehicles (Vassaux et al., 2006). As a non-viral vector, bacteria have been employed to transfer plasmids that carry genetic materials into mammalian cells via a process called bactofection (Palffy et al., 2006). Many bacterial strains implemented for gene therapy include *Listeria monocytogenes* (Pilgrim et al., 2003), *Salmonella typhimurium* (Zhao et al., 2005), *Shigella flexneri* (Sizemore et al., 1995), *Vibrio cholerae* (Yu et al., 2004), and *Yersinia enterocolitica* (Al-Mariri et al., 2002). These pathogenic bacteria are capable of penetrating non-phagocytic mammalian cells. Some of them can elicit an inhibitory effect on tumor growth when multiplying inside the malignant cells (Vassaux et al., 2006). This unique trait, along with low cost and easy operation, makes these bacteria an ideal non-viral gene delivery vehicle.

Despite the above-mentioned merits, these bacteria generally lack the selectivity for targeting tumors. Moreover, their innate virulence factors might cause serious infection and induce autoimmune reaction in the host (Vassaux et al., 2006). Therefore, genetic attenuation of the bacteria is usually required (Vassaux et al., 2006). As the best-described laboratory tool in molecular biology, non-pathogenic *Escherichia coli* strains have been applied for gene therapy (Grillot-Courvalin et al., 1998; Fajac et al., 2004). One known example is the expression of invasin (encoded by *inv*) from *Yersinia pseudotuberculosis* and listeriolysin O (encoded by *hlyA*) from *L. monocytogenes* in *E. coli*. Production of invasin allows *E. coli* strain to invade cells expressing β1-integrins (Critchley et al., 2004). Meanwhile, HlyA functions to disrupt lysosomal membranes, leading to release of the internalized strain into cell cytoplasm (Cossart et al., 1989).

Targeted gene therapy is a subject receiving many intensive studies (Palffy et al., 2006). The prerequisite for bactofection is that bacteria vectors have the ability to selectively recognize tumors. After triggering the entry pathway, the bacteria are internalized and subsequently lysed. As a consequence, harbored plasmids are released and transported to the nucleus where the transgene is expressed. However, many challenges still remain to overcome to improve the efficiency of bactofection.

It is our ultimate goal to develop a bacterium-based gene delivery system for targeted gene therapy. As a first attempt, tumor cells which overexpress human epidermal growth factor receptor-2 (HER2/neu) were chosen for targeting. HER2/neu is the second member of the human epidermal growth factor receptor family (Hung and Lau, 1999). Enormous amplification of
HER2/neu could result in the loss of the normal control mechanism of cells, consequently leading to the progression of aggressive tumors (Citri and Yarden, 2006). To selectively target HER2/neu-positive cells, *E. coli* strain was engineered to display a recognition motif on its surface. Two types of recognition motifs were employed, including anti-HER2/neu ML39 single-chain antibody fragment (ScFv) and a bivalent anti-HER2/neu affibody (denoted ZH2). The former was created by chain shuffling (Schier et al., 1996), while the latter was derived from IgG-binding domain of *staphylococcal* protein A and was extensively screened from a mutant library (Orlova et al., 2006). Moreover, a programmed lysis system was constructed to liberate the plasmid cargo in *E. coli* by controlled production of phage φX174 gene E (Paukner et al., 2006). By these approaches, the engineered bacterial strain was shown to mediate the expression of green fluorescent protein (GFP) in HER2/neu-positive tumor cells.

### Materials and Methods

#### Bacterial Strains and Cell Lines

The bacterial strains, plasmids, and primers used in this study are listed in Table I. *E. coli* BL21(DE3) strain and its derivative, BL21-GFP, were used for the experiments. BL21-GFP strain carries a genomic copy of GFP under control of the T7 promoter. This strain was generated by integrating plasmid pϕT7-GFPuv (see below) into the genome of BL21(DE3) strain as previously described (Chiang et al., 2008a). Bacteria bearing plasmids were grown in Luria–Bertani (LB) medium (Miller, 1972) supplemented with 50 µg/mL ampicillin, 20 µg/mL chloramphenicol, or 10 µg/mL streptomycin at 30°C. The growth of bacteria was measured turbidimetrically at 550 nm (OD550). Upon reaching 0.3 at OD550, 50 µM L-arabinose, or IPTG, or both were added to induce protein production. After induction for 4 h, bacteria were harvested, washed, and resuspended in phosphate buffer (pH 7.4) until use.

**Table I. Bacterial strains, plasmids, and primers applied in this study.**

<table>
<thead>
<tr>
<th>Relevant characteristic (sequence)</th>
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<tr>
<td>1. P&lt;sub&gt;T7&lt;/sub&gt;, T7 promoter; LoA, lpp-ompA; P&lt;sub&gt;BAD&lt;/sub&gt;, L-arabinose BAD promoter; φXE, φ174 gene E; P&lt;sub&gt;CMV&lt;/sub&gt;, human cytomegalovirus promoter.</td>
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<td><strong>Strain</strong></td>
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<td>BL21(DE3)</td>
<td>dcm gal ompT hsdS&lt;sup&gt;(i&lt;sub&gt;6&lt;/sub&gt; m&lt;sub&gt;16&lt;/sub&gt;)&lt;/sup&gt;</td>
</tr>
<tr>
<td>BL21-GFP</td>
<td>as BL21(DE3) but φ80::P&lt;sub&gt;T7&lt;/sub&gt;-GFP</td>
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<td><strong>Plasmid</strong></td>
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<td>pET28-GFPuv</td>
<td>carry P&lt;sub&gt;T7&lt;/sub&gt;-GFP</td>
</tr>
<tr>
<td>pPhi80-Tc</td>
<td>carry φ80 attachment (att) site</td>
</tr>
<tr>
<td>pϕT7-GFPuv</td>
<td>carry P&lt;sub&gt;T7&lt;/sub&gt;-GFP and φ80 att site</td>
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<td>pACgp67B-H</td>
<td>carry ML39 ScFv</td>
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<tr>
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<td>pET-Her2M carry (P_{T7}-ML39) ScFv</td>
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<td>pLOA carry (P_{T7}-LoA)</td>
<td>Wang and Chao (2006)</td>
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<td>pLOA-ZH carry (P_{T7}-LoA-ZH2)</td>
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<td>pBAD33 carry (P_{BAD})</td>
<td>Guzman et al. (1995)</td>
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<td>pBAD-LoAZH2 carry (P_{BAD}-LoA-ZH2)</td>
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<td>pPL452 carry (\lambda P_RP_L)</td>
<td>Christopher et al. (1996)</td>
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<td>pPL452M carry mutant (\lambda P_RP_L) (m(\lambda P_RP_L))</td>
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<td>pPL-(\varphi XE) carry (m\lambda P_RP_L-\varphi XE)</td>
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<td>pGB2(\Omega)-inv-hly</td>
<td>Grillot-Courvalin et al. (1998)</td>
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<td>pCL1920 carry pSC101 origin</td>
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<td>pCL-(\varphi XE)-hlyA pCL1920 with (m\lambda P_RP_L-\varphi XE) and (hlyA)</td>
<td>This study</td>
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<tr>
<td>pBac-EGFP carry (P_{CMV_GFP})</td>
<td>Lab collection</td>
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Primer YC1101 CGGCCATGGCCAGGTGCAG
YC1102 GAAGAATTCCGCACCTAGGACGGTG
YC1103 CCATTGAATTCAGCCATGAAGATGGC GAAC
YC1104 GTTACCAAGCTTAGAAACTGTATTTCA TCCCAG
YC1105 GTAAAATAGCCAACACGCACGGTGTT AGATATTATAC
YC1106 GATAAATATCTAACCACCCGCGTGGTGCTATTAC
YC1107 GAAAATAGCCAACACGCACGGGTT TAGATATTATAC
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<tr>
<td>YC1109 TTGCGCATATGGTACGCTGGACTTTGT</td>
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<tr>
<td>YC1110 TTTGAATTCAGACATTTTATCACTT</td>
<td></td>
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<tr>
<td>YC1111 ATGTTGAGCTCCGGGAGGCATTAAC</td>
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<tr>
<td>YC1112 TACAACTCGAGTTATTCGATTGGATTA</td>
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Human breast cancer cell lines, MDA-MB-231 and SKBR-3, were kindly provided by Dr. M.C. Kao (China Medical University, Taiwan). These cells were maintained in DEME/F12 medium (HyClone Lab, Inc., Logan, UT) supplemented with 10% fetal bovine serum (FBS) and cultured at 37°C in a humidified atmosphere of 5% CO₂. Cell concentration was counted by a hemocytometer. Cells were resuspended and seeded into a 24-well plate (1 × 10⁵ cells per well), 12-well plate (2 × 10⁵ cells per well), or culture dishes (1 × 10⁶ cells per dish). The culture medium was changed every 2 days until cell confluence reached 80%.

**Plasmid Construction**

Plasmid pϕT7-GFPuv was created by ligation of a DNA fragment, removed from plasmid pET28-GFPuv by SacI–SphI digestion, into plasmid pPhi80-Tc (Chiang et al., 2008a). This plasmid carries a DNA cassette consisting of the T7 promoter-driven GFP. The anti-HER2/neu ML39 ScFv was amplified from plasmid pACgp67B-Her2mP (Addgene, Inc., Cambridge, MA) using polymerase chain reaction (PCR) with primer YC1101 and YC1102. The ML39 ScFv-containing DNA was then digested with NcoI–EcoRI and ligated into plasmid pET-20b (Novagene Co., Darmstadt, Germany) to obtain plasmid pET-Her2. Meanwhile, the misL gene of S. typhimurium LT2 was obtained by PCR with primer YC1103 and YC1104. After EcoRI–HindIII cleavage, the resulting DNA was incorporated into plasmid pET-Her2 to produce plasmid pET-Her2M (Supplementary Fig. 1A).

The ZH2 DNA motif was synthesized and confirmed with DNA sequencing by Mission Biotech Co. (Nangang, Taiwan). It contains two identical domains of ZHER2:342 (Orlova et al., 2006) separated by a linker. Incorporation of this synthesized DNA into plasmid pLOA (Wang and Chao, 2006) resulted in plasmid pLOA-ZH. Subsequently, the DNA was recovered from plasmid pLOA-ZH by HindIII–XbaI cleavage and spliced into plasmid pBAD33 (Guzman et al., 1995) to give plasmid pBAD-LoAZH2 (Supplementary Fig. 1B).

In addition, plasmid pCL-ϕXE2-hlyA was constructed in several steps as follows. To reduce the basal expression level at 37°C, the λPRM and PR promoter of plasmid pPL452 (Christopher et al., 1996) was mutated according to the previous report (Jechlinger et al., 2005). This was carried out by
site-directed mutagenesis (Chiang et al., 2008b) to give plasmid pPL452M with two pairs of primers, YC1105/YC1106 and YC1107/YC1108. From the phage ϕX174 DNA, the gene E was first amplified by PCR with primer YC1109 and YC1110. The PCR DNA was pretreated with EcoRI–NdeI and ligated into plasmid pPL452M to produce pPL-ϕXE. Moreover, the hlyA gene of L. monocytogenes was synthesized by PCR from plasmid pGB2Ω-inv-hly (Grillot-Courvalin et al., 1998) using primer YC1111 and YC1112. After digestion with XhoI–SmaI, the PCR DNA was incorporated into plasmid pPL-ϕXE to give plasmid pPL-ϕXE-hlyA. The DNA containing ϕX174 gene E and hlyA was recovered from plasmid pPL-ϕXE-hlyA by PstI–NruI cleavage. Subsequent splice of the recovered DNA into plasmid pCL1920 gave plasmid pCL-ϕXE2-hlyA (Supplementary Fig. 1C).

**Analysis of Bacterial Surface by Immunostaining and Flow Cytometry**

Bacterial strain was grown and induced by adding L-arabinose. After induction for 4 h, bacteria were harvested, washed, and resuspended in phosphate buffer (pH 7.4). Bacteria with the number of $1 \times 10^9$ were withdrawn and treated with 500 μL 0.25% trypsin–EDTA (HyClone Lab, Inc.) at 37°C for 2 min. The digestion was terminated with 500 μL 10% FBS/DMEM-F12 medium on ice. The uninduced, induced, and trypsinized bacteria were fixed in 3.7% paraformaldehyde for 30 min. The BSA-blocked bacterial cells were then washed three times with PBS and incubated with 1:200 diluted mouse anti-6xHis monoclonal antibody (Genemark Technology Co. Ltd, Tainan, Taiwan) at room temperature for 1 h. The bacteria were rinsed and exposed to a 200-fold dilution of the FITC-conjugated anti-mouse IgG (Jackson Immuno Research, West Grove, PA) for 1 h. Washed with PBS, bacterial cells were mounted on glass slides for analysis by fluorescence microscopy (IX71 Olympus, Tokyo, Japan). Meanwhile, 10,000 bacteria were employed for measurement of green fluorescence by BD FACSCanto (BD Bioscience, Mountain View, CA). Data were further analyzed by Diva software (De Novo Software, Los Angeles, CA) and FlowJo (TreeStar, Ashland, OR).

**Infection of Tumor Cells With Bacteria**

Human tumor cells were seeded into 12- and 24-well plates with cover slips for flow cytometry assay and microscopy observation, respectively. The harvested bacteria were washed twice with serum-free medium, diluted in the serum-free medium, and then added to each well to achieve the required multiplicity of infection (MOI). The incubation was carried out in a humidified atmosphere of 5% CO₂ at 37°C for 3 h. The culture plates were washed three times with PBS to remove unbound bacteria and then incubated at 42°C for 60 min to apply the thermal induction. Moreover, 50 μg/mL gentamicin was applied for 1 h at 37°C to eliminate the unassociated bacteria. The treated cells were incubated in a humidified CO₂ incubator at 37°C until observation.

**Assessment of Bacterial Invasion Efficiency**

After infection with bacteria for 3 h, tumor cells were washed with PBS twice, collected, and then treated with 0.2% EDTA/PBS. The harvested cells were washed twice with PBS and then fixed in 1% paraformaldehyde. The percentage of 10,000 cells that emitted bacteria-producing GFP was
determined by BD FACSCanto. Data were further analyzed by Diva software and FlowJo.

**Analysis of Bacterial Internalization by Fluorescence and Confocal Microscopy**

After bacterial infection, cells were washed with PBS and fixed in 3.7% paraformaldehyde for 30 min at room temperature. Cells were washed three times with PBS and subsequently blocked with 3% BSA in PBS. For clear observation, cells were stained with 5 µg/mL phalloidin–TRITC (Sigma–Aldrich, St. Louis, MO) and 1 µg/mL DAPI (Sigma–Aldrich). Washed with PBS, cells were mounted on glass slides for observation by fluorescence microscopy.

Alternatively, the BSA-blocked cells were stained with 9G6 anti-HER2/neu primary antibody (Santa Cruz Biotechnol, Santa Cruz, CA) for 1 h at room temperature. After washing three times with PBS, FITC-conjugated anti-mouse IgG was added to cells for 1 h. Stained cells were subsequently washed with PBS and mounted on glass slides for analysis by confocal microscopy (LCS SP2 Leica, Wetzlar, Germany). For each sample, a composite image of 20 sections with a 0.8 µm shift in the z-axis was taken and combined by LCS AF (Leica).

**Assessment of Internalized Bacteria in Tumor Cells**

The average number of bacteria that invade one cell was assayed at a MOI of 200:1. After infection, tumor cells were washed with PBS and lysed in 0.2% Triton X-100 and. Serial dilutions of the cell lysate were plated on LB agars. Incubated at 37°C for overnight, the bacterial forming units were counted. The assay was performed in triplicate.

**Analysis of GFP Expression in Tumor Cells by Flow Cytometry**

Tumor cells were incubated with bacteria for 3 h as described. The cells were collected and fixed in 1% paraformaldehyde at 0, 24, 48, and 72 h. The number of cells emitting fluorescence was then quantified by BD FACSCanto. Data were further analyzed by Diva software and FlowJo. The experiment was performed in triplicate and 20,000 cells were assayed each time.

**Results**

**Targeting of HER2/neu-Positive Cells by E. coli**

In this study, two E. coli strains were mainly used (Table I). A green fluorescence-emitting BL21-GFP strain, derived from BL21(DE3) strain, was first transformed with plasmid pET-Her2M to obtain BL21-GFP/pET-Her2M strain. Plasmid pET-Her2M carries the C-terminal fusion of S. typhimurium misL with anti-HER2/neu ML39 ScFv (Schier et al., 1996). S. typhimurium misL is similar to E. coli autotransporter (Maurer et al., 1997), known as AIDA (adhesin involved in diffuse adherence), and enables to expose the fusion passenger on the bacterial surface (Ruiz-Perez et al., 2002). Subsequently, tumor cells were co-incubated with either BL21-GFP or BL21-GFP/pET-Her2M strain to examine their interaction. As shown in Fig. 1, the fluorescence signal was detected in HER2/neu-positive SKBR-3 cells that were infected by BL21-GFP/pET-Her2M strain but undetectable in the cell infected with BL21-GFP strain. However, these ML39 ScFv-presenting bacteria (e.g., BL21-GFP/pET-Her2M strain) were not invasive after administration of bactofection for 12 h. In addition, no signals could be observed in
HER2/neu-negative MDA-MB-231 cells exposed to both bacterial strains (data not shown).

Figure 1. Targeting of tumor cells by ML39 ScFv-presenting bacteria with fluorescence microscopy. BL21-GFP/pET-Her2M strain was cultivated and induced by IPTG to express MisL-ML39 ScFv and GFP. As a control, BL21-GFP strain was grown and induced by IPTG to produce GFP. Infection of tumor cells with either BL21-GFP/pET-Her2M or BL21-GFP strain was carried out at a MOI of 200:1. After infection for 3 h, the gentamicin protection assay was carried out to eliminate free bacteria. For clear visualization by fluorescence microscopy, cell nucleus (blue) and cytoskeleton (red) were stained by DAPI and phalloidin, respectively. After infection with BL21-GFP/pET-Her2M strain, the fluorescence images of MDA-MB-231 and SKBR-3 cells were acquired in the green, red, and blue channels. The panels shown on the right represent the overlay of three images.

Selective Internalization of ZH2-Presenting Bacteria by HER2/neu-Positive Cells

As illustrated above, ML39 ScFv-displayed bacteria are able to target but incapable of invading HER2/neu-positive cells. To seek an alternative, plasmid pBAD-LoAZH2 was constructed to fuse the anti-HER2/neu motif ZH2 with the surface-anchoring domain consisting of lpp and ompA. This construction resulted in the fusion gene with a C-terminal 6xHis tag. The usefulness of lpp and ompA has been well illustrated for directing the fused passenger motif onto bacterial surface (Wang and Chao, 2006). Plasmid pBAD-LoAZH2 was then transformed into BL21(DE3) strain to obtain BL21(DE3)/pBAD-LoAZH2 strain. As shown in Fig. 2A, the fusion protein comprising Lpp-OmpA-ZH2 was found in the induced BL21(DE3)/pBAD-LoAZH2 strain with the expected molecular weight (33 kDa). The fusion protein became undetectable when the induced bacteria were pretreated with 0.25% trypsin–EDTA for 2 min at 37°C. Meanwhile, immunostaining of bacterial surface display of the 6xHis tag was carried out using anti-6xHis antibody. Fig. 2B shows that the induced BL21(DE3)/pBAD-LoAZH2 strain was surrounded by green fluorescence whereas the signal became eliminated when the bacterial strain was pretreated by trypsin. Similarly, the fluorescence was absent in the uninduced BL21(DE3)/pBAD-LoAZH2 strain. By flow cytometry, more than 20% of the induced bacteria population was found to exhibit green fluorescence over the background level (Fig. 2C). Overall, it suggests the presence of ZH2 on bacterial surface.

Figure 2. Analysis of Lpp-OmpA-ZH2 with the 6xHis tag produced by bacteria.

A: As described, BL21(DE3)/pBAD-LoAZH2 and BL21(DE3) strain were grown and induced by L-arabinose for protein production. At the end of cultivation, the bacteria were harvested and disrupted by sonication. After centrifugation, the supernatant was removed and the precipitate (insoluble part) was collected and analyzed by performing 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as reported previously (Chiang et al., 2008b).

Keys: M, protein marker; lane 1, insoluble fraction of BL21(DE3) strain; lane 2, insoluble fraction of uninduced BL21(DE3)/pBAD-LoAZH2 strain; lane 3,
The functionality of ZH2 displayed on bacterial surface was further investigated. First, BL21-GFP strain was transformed with plasmid pBAD-LoAZH2 to give BL21-GFP/pBAD-LoAZH2 strain. Subsequently, bactofection of tumor cells with either BL21-GFP or BL21-GFP/pBAD-LoAZH2 strain was conducted in a similar manner. After administration of the gentamicin treatment, green fluorescence was detected in SKBR-3 cells that were infected by BL21-GFP/pBAD-LoAZH2 strain (Fig. 3A). This signal became absent when the cells were infected with trypsin-treated BL21-GFP/pBAD-LoAZH2 strain (data not shown). In addition, no green fluorescence could be observed in MDA-MB-231 cells infected with both strains. Overall, the result suggests that the bacteria are selectively internalized into HER2/neu-positive cells in a ZH2-dependent way. This argument was further confirmed with the Z-section analysis of confocal microscopy. The merged image clearly indicates the localization of the green fluorescence-emitting bacteria in the cytoplasmic space of SKBR-3 cells (Fig. 3B).
are shown below (X–Z section) and to the right (Y–Z section) of the merge panel. It reveals the appearance of the internalized bacteria (green) in the cytoplasm surrounded by HER2/neu receptors (red).

The efficiency of bacterial invasion was determined by counting the percentage of cells emitting green fluorescence. Therefore, co-incubation of tumor cells with BL21-GFP or BL21-GFP/pBAD-LoAZH2 strain was conducted for 3 h. After treatment with gentamicin, cells displaying green fluorescence were then detected by flow cytometry. As shown in Fig. 4, 30% of SKBR-3 cells that were infected by BL21-GFP/pBAD-LoAZH2 strain displayed fluorescence whereas the signal was absent in the BL21-GFP strain-infected cells. Moreover, no signals were detected in MDA-MB-231 cells that were infected with both strains. The average number of invaded bacteria in each SKBR-3 cell was also determined by counting the viable intracellular bacteria. As analyzed, it was estimated to have 13 intracellular bacteria per cell.

**Figure 4.** The invasion efficiency of bacteria in HER2/neu-positive cells.

Bacterial strains were grown and induced in a similar way. Tumor cells were co-incubated with either BL21-GFP/pBAD-LoAZH2 or BL21-GFP strain at a MOI of 200:1 for 3 h. After treatment with gentamicin, cells displaying green fluorescence were detected by flow cytometry as described.

**Programmed Autolysis of Bacteria**

Next, plasmid pCL-ϕXE2-hlyA was constructed to carry hlyA gene and the phage ϕX174 gene E under the control of heat-inducible λP₅P₅ promoter (Christopher et al., 1996). The latter gene was employed to lyse bacteria for release of plasmid cargos, while the hlyA gene was implemented to disrupt endosomal membranes. Accordingly, plasmid pCL-ϕXE2-hlyA was transformed into BL21(DE3)/pBAD-LoAZH2 strain to obtain BL21(DE3)/pBAD-LoAZH2/pCL-ϕXE2-hlyA strain. The resulting strain was then grown in shake flasks and subjected to the thermal shock. As shown in Fig. 5, the growth of the ϕX174 gene E-expressing strain (BL21(DE3)/pBAD-LoAZH2/pCL-ϕXE2-hlyA) dropped abruptly 1 h after the thermal induction. In sharp contrast, the control strain (BL21(DE3)/pBAD-LoAZH2) grew unaffectedly. Moreover, 2 h after receiving the thermal induction, a part of both bacteria was withdrawn and re-cultured in shake flasks containing the fresh growth medium at 37°C. As a result, BL21(DE3)/pBAD-LoAZH2 stain continued to grow at the preshift rate whereas
BL21(DE3)/pBAD-LoAZH2/pCL-ϕXE2-hlyA strain completely stopped growing. The result suggests the active function of ϕX174 gene E.

Figure 5. Growth curve of engineered *E. coli*. BL21(DE3)/pBAD-LoAZH2 and BL21(DE3)/pBAD-LoAZH2/pCL-ϕXE2-hlyA strain were grown in LB at 37°C and their growth was followed by the turbidity measurement. In parallel, the culture temperature was shifted from 37 to 42°C as indicated by the arrow. After 1 h, the culture temperature was then returned to 37°C throughout the experiment. Additionally, a part of the heat-shocked strains was withdrawn and re-cultivated at 37°C in fresh medium. Symbols: ●, BL21(DE3)/pBAD-LoAZH2 strain at 37°C; ●, BL21(DE3)/pBAD-LoAZH2 strain receiving thermal shock; ○, BL21(DE3)/pBAD-LoAZH2/pCL-ϕXE2-hlyA strain at 37°C; □, BL21(DE3)/pBAD-LoAZH2/pCL-ϕXE2-hlyA strain receiving thermal shock; ▲, re-culturing of the heat-shocked BL21(DE3)/pBAD-LoAZH2 strain; △, re-culturing of the heat-shocked BL21(DE3)/pBAD-LoAZH2/pCL-ϕXE2-hlyA strain.

The developed bacterial vector was further examined for its feasibility in control-and-released of produced GFP. Therefore, BL21-GFP/pBAD-LoAZH2 strain was transformed with plasmid pCL-ϕXE2-hlyA to give BL21-GFP/pBAD-LoAZH2/pCL-ϕXE2-hlyA strain. The resulting strain was utilized to infect SKBR-3 cells. After the administration of gentamicin, cells were subjected to the thermal shock. As a result, most of internalized bacteria no longer elicited the green fluorescence (Fig. 6). It indicates that bacteria within the cells are lysed by the programmed induction of ϕX174 gene E, thereby releasing the green fluorescence that fades away in the cell cytoplasm.

Figure 6. Analysis of GFP release by programmed autolysis of *E. coli* using confocal microscopy. Likewise, infection of SKBR-3 cell with BL21-GFP/pBAD-LoAZH2/pCL-ϕXE2-hlyA strain at a MOI of 200:1 was carried out. The infected cells were then challenged by heat, subjected to DAPI- and phalloidin-staining, and analyzed by confocal microscopy. The upper row shows the fluorescence images obtained in green, red, and blue channels. The three images were merged to give the panel shown on the right. Magnification of the inset with individual and merged images is given in the lower row. Furthermore, two three-dimensional reconstruction sections are shown below (X–Z section) and to the right (Y–Z section) of the merge panel. It revealed the appearance of the internalized bacteria in an intact (green) or broken form (pale blue) as indicated in the cytoplasm.

Delivery of a Eukaryotic Expression Plasmid by ZH2-Presenting Bacteria
Finally, the usefulness of bacterial carriers for targeted delivery of transgens was investigated. Plasmid pBac-EGFP was then transformed into BL21(DE3)/pBAD-LoAZH2/pCL-ϕXE2-hlyA strain to obtain BL21(DE3)/pBAD-LoAZH2/pCL-ϕXE2-hlyA/pBac-EGFP strain. Note that plasmid pBac-EGFP contains the enhanced GFP gene under the control of the human CMV promoter. Bactofection of tumor cells was conducted in a similar manner. After co-incubation for 3 h, gentamicin was administrated and followed by the thermal shock. The percentage of tumor cells expressing eukaryotic GFP was monitored by flow cytometry along the time course. After 72 h, almost no GFP expression could be detected in MDA-MB-231 cells (Fig. 7). In contrast, the percentage of SKBR-3 cells expressing GFP increased over time. With the thermal induction, the expression level of GFP in infected SKBR-3 cells reached the maximum 1 day after infection. Without the thermal induction, the maximal expression of GFP was obtained 2 days after bactofection.

Figure 7. In vitro gene transfer assessment by flow cytometry. Co-incubation of tumor cells with BL21(DE3)/pBAD-LoAZH2/pCL-ϕXE2-hlyA/pBac-EGFP strain at a MOI of 200:1 was carried out as described. The expression of the human CMV promoter-driven GFP in cells was determined by flow cytometry at 0, 24, 48, and 72 h after bacterial infection. The data were representative of three independent experiments and were shown in graphs for better comparison. The statistical analysis by Students' t-test was performed. Symbols: *, Students' t-test ($P < 0.1$); **, Students' t-test ($P < 0.05$); MDA-MB-231 cells without (open hatched bar) or with thermal induction (gray hatched bar); SKBR-3 cells without (open bar) or with thermal induction (gray bar).

**Discussion**

The concept of applying live bacteria to treat cancer is not new (Pawelek et al., 2003). However, recent advances in molecular genetics have promoted an emerging and intensive study on bacterial gene therapy. For direct gene transfer by bacteria, the first limiting step lies in the selective entry of genetic cargos into tumor cells (Palffy et al., 2006). As an initial attempt, non-invasive *E. coli* strain was engineered for surface display of the anti-HER2/neu ML39 ScFv (Schier et al., 1996) via *S. typhimurium* misL (Ruiz-Perez et al., 2002). After infection, the ML39 ScFv-presenting *E. coli* strain was found to selectively adhere to SKBR-3 cells and no invaded bacteria could be observed (Fig. 1). In contrast, the previous study reported the selective delivery of ML39 ScFv complexed with DNAs into HER2/neu-positive cells (Li et al., 2001). It seems likely that the association of cells with ML39 ScFv-presenting bacteria is not sufficient to trigger the receptor-mediated entry mechanism. It has been well illustrated that anti-HER2/neu affibody, either conjugated with nanoparticles and liposomes or condensed with DNAs, enables to mediate drug and gene delivery to HER2/neu-positive cells (Alexis et al., 2008; Canine
et al., 2009; Puri et al., 2008). Therefore, we attempted to display the anti-HER2/neu affibody on the surface of E. coli strain. This was achieved by C-terminal fusion of a bivalent affibody to the anchoring domain Lpp-OmpA. The Lpp region directs the transfer of the passenger motif to bacterial outer membrane in which the passenger is anchored with the aid of the OmpA region (Lee et al., 2003). Upon induction, the bacteria were able to express the hybrid protein (Fig. 2) and exhibited selective invasion of HER2/neu-positive cells (Fig. 3). The invasion of cells by bacteria was then abolished when the induced bacteria were pretreated with trypsin. Apparently, anti-HER2/neu affibody appears to be more efficient to activate the cell entry mechanism than ML39 ScFv. The reason for this variation remains unclear. Moreover, the anti-HER2/neu affibody-mediated internalization of bacteria was found to be time-dependent and to reach the maximum within 3 h (data not shown). This is consistent with the invasion kinetics of the anti-HER2/neu affibody-conjugated materials reported previously (Alexis et al., 2008). As recognized, two pathways are implemented by pathogenic bacteria to enter non-phagocytic cells (Vassaux et al., 2006). One referred to as the zipper mechanism relies on the direct contact between bacterial ligands and cellular receptors. The attachment then results in the local cytoskeletal rearrangement and uptake of bacteria in an enclosed vacuole. In addition, the translocation mechanism of HER2/neu receptor to cell nucleus has been recently proposed (Giri et al., 2005). The transport path starts with endocytic internalization of the receptor. Interacting with the transport receptor importin, the receptor binds to nuclear pore protein and then travels to the nucleus. Taken together, it suggests that endosomal entry of bacteria into cells proceeds in an anti-HER2/neu affibody-dependent manner. This is indeed reflected by the observation of heterogeneous distribution of internalized bacteria in HER2/neu-overexpressing cells (Fig. 3).

The second limiting step for targeted bactofection resides in the free release of delivery cargos carried by bacterial vectors (Patlffy et al., 2006). To this end, the thermo-inducible phage ϕX174 gene E was used for autolysis of bacterial vector. This lysin gene functions to cause bacterial inner and outer membrane to fuse, leading to the formation of a transmembrane tunnel through which the cytoplasmic content is expelled (Paukner et al., 2006). Consequently, bacteria stopped growing upon the induced production of ϕX174 gene E (Fig. 6). Re-culturing of these lysin gene-afflicted bacteria gave no clear increase in turbidity, indicating the formation of bacterial ghosts as suggested (Paukner et al., 2006). In a similar fashion, this approach was applied in vitro using the tumor-targeting E. coli strain with the programmed autolysis system. As depicted in Fig. 7, early detection of GFP expression in infected cells was obtained when the thermal induction was conducted. Approximately 3% of HER2/neu-positive cells expressed the CMV promoter-driven GFP 24 h after bactofection and the expression persisted for 2 days. It should be noted that the efficiency in inducing bacterial lysis by ϕX174 gene E can vary with the cell physiological state. As suggested, the diaminopimelate auxotrophic E. coli strain undergoes lysis upon entry into cells due to impaired cell wall synthesis (Grillot-Courvalin et al., 1998). Combining these two strategies might improve bacterial autolysis for efficient release of plasmid cargos.
The invasion efficiency of ZH2-presenting bacteria into HER2/neu-positive cells was as high as 30% (Fig. 4). However, 3% of invaded cells were able to express the transgene (e.g., CMV promoter-regulated GFP). It was reported that the invasiveness of Mycobacterium tuberculosis InvX-presenting E. coli into HeLa cells reached only 0.8% (Casali et al., 2002). By expression of either Staphylococcus aureus fibronectin-binding protein A or L. monocytogenes internalin A, Lactococcus lactis strain was capable of delivering GFP into human epithelial cells. Approximately 1% of infected cells expressing GFP were reported (Innocentin et al., 2009). In addition, the invasion efficiency of E. coli that expressed Y. pseudotuberculosis inv and L. monocytogenes hylA varied with cell lines. The percentage of GFP-expressing cells after infection ranged from 2% to 8% (Grillot-Courvalin et al., 1998). Overall, it clearly indicates the existence of unseen limitations in bactofection. To address this problem, one important issue is how to overcome the inefficient translocation of plasmid entities to cell nucleus. It is possible to attach the plasmid cargos with a nuclear localization signal peptide to conquer the barrier (Vaysse et al., 2006). Finally, the presence of bacterial DNAs naturally rich in CpG dinucleotides on plasmids is known to cause transcriptional silencing of the transgene in cells. The exploitation of minicircle plasmid DNA deprived of bacterial DNA or CpG-depleted plasmid provides two useful solutions to this problem (Chen et al., 2003; Yew et al., 2002).

The biological effect of the anti-HER2/neu affibody on tumor cells was recently investigated. Continuous treatment of SKBR-3 cells with the affibody for 8 weeks shows an inhibitory effect on the cell proliferation (Ekerljung et al., 2008). However, such an inhibitory effect was not observed for SKBR-3 cells infected with ZH2-displayed bacteria. This is probably because the bactofection treatment was conducted in a short-term and non-continuous way. Nevertheless, in this study the proposed approach by engineering bacteria for targeted delivery of transgenes shows a great promise. The usefulness of this bacterial vector for targeted cancer therapy is currently under investigation in our lab.

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