DNA modification and functional delivery into human cells using *Escherichia coli* DH10B

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ABSTRACT

The availability of almost the complete human genome as cloned BAC libraries represents a valuable resource for functional genomic analysis, which, however, has been somewhat limited by the ability to modify and transfer this DNA into mammalian cells intact. Here we report a novel comprehensive *Escherichia coli*-based vector system for the modification, propagation and delivery of large human genomic BAC clones into mammalian cells. The GET recombination inducible homologous recombination system was used in the BAC host strain *E.coli* DH10B to precisely insert an EGFPneo cassette into the vector portion of a ~200 kb human BAC clone, providing a relatively simple method to directly convert available BAC clones into suitable vectors for mammalian cells. GET recombination was also used for the targeted deletion of the *asd* gene from the *E.coli* chromosome, resulting in defective cell wall synthesis and diaminopimelic acid auxotrophy. Transfer of the *Yersinia pseudotuberculosis* invasin gene into *E.coli* DH10B *asd⁻* rendered it competent to invade HeLa cells and deliver DNA, as judged by transient expression of green fluorescent protein and stable neomycin-resistant colonies. The efficiency of DNA transfer and survival of HeLa cells has been optimized for incubation time and multiplicity of infection of invasive *E.coli* with HeLa cells. This combination of *E.coli*-based homologous recombination and invasion technologies using BAC host strain *E.coli* DH10B will greatly improve the utility of the available BAC libraries from the human and other genomes for gene expression and functional genomic studies.

INTRODUCTION

The availability of a complete array of fully sequenced cloned BACs that span most of the human genome is a remarkable development in biology, and represents a valuable resource for studying gene expression and other chromosomal functions (1–3). However, the utility of these BACs is limited by the difficulty in manipulating and transferring them into mammalian cells. BACs can contain as much as 350 kb of genomic DNA, and will therefore carry the complete coding sequence of most human genes (average size 30–60 kb) as well as the surrounding regulatory DNA for correct spatio-temporal gene expression. Furthermore, functional chromosome structures such as centromeres are formed on DNA that is several hundred kilobases in size, which has proven difficult to transfer intact without significant rearrangements.

The propagation of BAC libraries in recombination-deficient *Escherichia coli* DH10B imparts greater stability to the large BAC clones but has made their further manipulation using direct homologous recombination very difficult. A relatively simple and straightforward method has recently been demonstrated that provides transiently inducible recombination proficiency to *E.coli* DH10B by expression of the bacteriophage lambda *gam* gene and *E.coli recE* and *recT* from an arabinose promoter on plasmid pGETrec (4). The GET recombination system has been used to insert reporter constructs and disease causing mutations into BAC inserts containing the beta-globin locus (5,6). Additional strategies for the homologous recombination of BAC clones have also been reported (7,8).

The delivery of large DNA into mammalian cells also presents technical difficulties, particularly the 'naked DNA' stage where purified large DNA may undergo mechanical degradation, and considerable efforts have been directed to develop efficient delivery methods. *Escherichia coli* BM2710 has been demonstrated to invade and deliver plasmid DNA into HeLa, COS-1 and CHO cells when they are expressing the *Yersinia pseudotuberculosis* invasin gene, which binds to mammalian integrin receptors and results in internalization in primary vesicles, and are auxotrophic for diaminopimelic acid (DAP), resulting in a cell wall deficiency (9,10). Other attenuated bacterial pathogens have also been used as gene delivery vectors (11–15). Recently, the transfer of plasmids up to 15 kb into CHO-K1 cells has been achieved by donor controlled conjugation from *E.coli* DH5α containing the broad host range RK2 plasmid (16). Additionally, the HSV family of viruses have been used for the infectious delivery of

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genomic BACs up to the ~150 kb viral packaging limit with efficiencies ranging from 25 to 100% into the human MRC-5V2 and mouse primary hepatocytes (17–19).

Here we present a novel combination of GET recombination and bacterial invasion of mammalian cells, providing a comprehensive *E.coli*-based vector system designed for the modification, propagation and delivery of genomic BAC clones using their host *E.coli* DH10B. GET recombination has been used here to delete the *asd* gene in *E.coli* DH10B, resulting in DAP auxotrophy, and to precisely insert the EGFPneo cassette from plasmid pEGFP-N2 into the vector portion of a 200 kb BAC clone. After transfer of a plasmid coding for the *Y.pseudotuberculosis* invasin gene into this *E.coli* DH10B *asd⁻* host, invasion and transfer of both plasmid and BAC DNA into HeLa cells was demonstrated by green fluorescent protein (GFP) and neomycin expression.

MATERIALS AND METHODS

Plasmids and bacterial strains

Escherichia coli DH10B is F⁻ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80dlacZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G (20). Plasmid pEGFP-N2 (4.7 kb) (BD Biosciences Clontech, Palo Alto, CA) carries the EGFP reporter gene driven by the CMV promoter and a kanamycin/neomycin (kan/neo) gene that confers resistance to kanamycin (Km) and neomycin analogs in prokaryotes and eukaryotes, respectively. Plasmid pGB2Ωinv (7.6 kb) contains the *Y.pseudotuberculosis* invasin (*inv*) gene and carries resistance to both spectinomycin and streptomycin on the non-autotransferable low-copy plasmid pGB2 (9). Plasmid pGETrec contains the arabinose inducible recombination genes, *recE*, *recT* and *gam*, as described previously (4). The 3 kb pZeoSV2(+) plasmid carrying the prokaryotic EM7 promoter-zeo^r cassette was obtained from Invitrogen Corporation, Carlsbad, CA.

Preparation and purification of PCR products

The EM7 promoter-zeo^r cassette from pZeoSV2(+) was amplified using primers asdZF (5′-GAG ACC GGC ACA TTT ATA CAG CAC ACA TCT TTG CAG GAA AAA AAC GCT TAc ggt gca ttg gaa cgg acc g-3′) and asdZR (5′-CTC CTG TAT TAC GCA CTA ACA GGG GCG GCA TCG CGC CCC AGA TTT AAT GAt gca ata aac aag ttt cga g-3′). The resultant 590 bp PCR product was designated zeo590. The zeo590 fragment was designed to delete the *E.coli* DH10B *asd* gene after the A from the ATG start codon to seven bases downstream after the TAA stop codon.

The EGFP-kanamycin/neomycin (EGFP-km^r-neo^r) cassette from pEGFP-N2 was amplified using primers GK-F (5′-TTC CGG TCA CAC CAC ATA CGT TCC GCC ATT CCT ATG CGA TGC ACA TGC TGT ATG CCG GTA taa ccg tat tac cgc cat gc-3′) and GK-R (5′-AGA CTT CCG TTG AAC TGA TGG ACT TAT GTC CCA TCA GGC TTT GCA GAA CTT TCA GCG GTA acg acc caa cac cgt gcg tt-3′). The resultant 3.8 kb PCR product was designated EGFPneo. Upper-case characters indicate homologous nucleotide sequences to either the 5′ or 3′ end of the targeted locus. Lower-case characters indicate nucleotides complementary to

the template that is used for the amplification of the PCR product. The PCR products were gel purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA) and resuspended in 10 mM Tris–HCl, pH 8.5 at a concentration of ~300 ng/μl.

Construction of the *E.coli* DH10B *asd⁻*

GET recombination in *E.coli* DH10B was performed as described (4). Approximately 600 ng of purified PCR product zeo590 was electroporated into L-arabinose-induced electro-competent DH10B (pGETrec) cells using a Gene Pulser II unit set at 2.5 kV, 200 Ω and 25 μF (Bio-Rad Laboratories, Hercules, CA). After recovery for 90 min at 37°C in 1 ml of LB medium containing 0.5 mM DAP, cells were plated on LB agar containing 0.5 mM DAP and 25 μg/ml Zeo. Zeo^r colonies were genotyped by plating onto LB, LB containing DAP, and LB containing DAP and 25 μg/ml Zeo. Zeo^r DAP auxotrophs (*asd⁻*) were verified by PCR across the *asd* locus using primers asd1 (5′-TGGTGAAAGATGTGCCAAGA-3′) and asd2 (5′-TCCCGGTAAATCATGAAACA-3′). Plasmid pGETrec was eliminated by streaking DH10B *asd⁻* colonies onto LB plates containing DAP, Zeo and the inducer L-arabinose, and omitting ampicillin (Ac) selection. Single colonies that require DAP, were resistant to Zeo and sensitive to Ac were identified as DH10B *asd⁻* with plasmid pGETrec eliminated.

Retrofitting of the EGFPneo cassette on the 200 kb hgUroS-1 BAC

The 200 kb hgUroS-1 BAC clone containing the complete human Uroporphyrinogen-III synthase gene was kindly provided by Drs Gerardo Aizencang and Robert Desnick (21). Purified PCR product EGFPneo was electroporated into L-arabinose-induced electrocompetent DH10B (pGETrec, hgUroS-1). The subsequent electroporation and selection steps were carried out with the following brief modifications. After recovery in 1 ml of LB medium at 37°C for 90 min, cells were plated on LB agar containing 12.5 μg/ml chloramphenicol (Cm) and 30 μg/ml Km, and resistant colonies verified by PCR amplification across the recombination junction using primers KF (5′-ATCTGGGAAGTGACGGA-CAG-3′) and KR (5′-CAGCATCGCAACCGCATCAG-3′) (4). The modified hgUroS-1-EGFPneo was subsequently re-electroporated into DH10B cells that did not contain pGETrec. BAC DNA from this *E.coli* was digested with *Sal*I and *Not*I following manufacturers protocols and subjected to pulsed field gel electrophoresis (PFGE) [Bio-Rad CHEF-DRII, 1% (w/v) agarose gel, 150 V, switch time of 0.1–30 s, 12°C, 0.5× TBE, for 21 h].

Invasion of HeLa cells with *E.coli* DH10B

Saturated overnight or late-log cultures of *E.coli* DH10B *asd⁻* (pGB2Ωinv) (invasive) or DH10B *asd⁻* (non-invasive) containing the pEGFP-N2 or BAC hgUroS-1-EGFPneo were grown at 37°C in brain heart infusion (BHI) broth with 0.5 mM DAP and appropriate antibiotics. Overnight, bacteria (in 10 ml) at a concentration of ~1 × 10⁹ c.f.u./ml (OD₆₀₀ ~1.2) were pelleted and resuspended in 1 ml of RPMI 1640 medium (Cellgro by Mediatech, Inc., Herndon, VA). Aliquots of bacteria at the desired multiplicity of infection (MOI) were then diluted in RPMI 1640 to give a final volume of 2 ml, vortexed and overlaid onto monolayer cultures of 1 × 10⁵

HeLa cells per well (in a 6-well plate) that had been washed twice with PBS. The plates were centrifuged at 1000 r.p.m. for 10 min before being incubated at 37°C in 5% CO_2 for the desired incubation time. Subsequently, the cells were washed twice with PBS to remove non-adherent bacteria, fresh medium containing 20 µg/ml gentamicin (Gm) added, and further incubated for 48 h.

GFP-expressing cells were viewed directly in 6-well plates using an Axiovert 135 fluorescent microscope (Carl Zeiss, Inc., Germany) equipped with a Sony DKC-5000 CCD camera. Subsequently, cells were trypsinized, resuspended in PBS, and 4×10^4 cells were quantified for GFP expression cells using a FACScan flow cytometer with CellQuest software (Beckton-Dickinson, Mountain View, CA). Stable G418-resistant colonies were obtained by replating HeLa cells 48 h after invasion and addition of G418 (1 mg/ml) 24 h later. Entry of bacteria into HeLa cells was determined using the Gm protection assay (22). After each incubation time point (10, 30, 60 or 120 min), the cells were washed twice with PBS to remove non-adherent bacteria before adding fresh RPMI 1640 medium containing 80 µg/ml Gm and incubated for a further 30 min at 37°C in a 5% CO_2 atmosphere incubator. Cells were washed twice with PBS and internalized bacteria released from the monolayer by a 5 min incubation with 1% (w/v) Triton X-100 and titered on BHI plates containing 0.5 mM DAP and the appropriate antibiotics.

RESULTS

Modification of *E.coli* DH10B using GET recombination

In order to increase the utility of the available genomic BAC resources, the GET recombination system was developed to permit the targeted modification of BAC inserts and vectors in host strain *E.coli* DH10B (4). We have used a novel application of the GET recombination system for the targeted deletion of the aspartate β-semialdehyde (*asd*) gene from the *E.coli* chromosome, converting *E.coli* DH10B into a DAP auxotroph that lyses upon cell division in the absence of DAP supplementation (12,23). A 590 bp PCR fragment (zeo590) containing the complete zeocin-resistance (zeoʳ) gene was amplified from pZeoSV2(+) using PCR primers asdZF and asdZR, which each contain 50 bp of DNA that is homologous to the *E.coli asd* locus. Electroporation of zeo590 into arabinose-induced *E.coli* DH10B (pGETrec) resulted in homologous recombination of the 50 bp flanking DNA with the *asd* locus, and replacement of the *asd* coding sequences with the zeoʳ fragment (Fig. 1A). The correct targeting of this homologous recombination was verified by PCR amplification across the *asd* locus using primers asd1 and asd2 which flank the chromosomal region replaced by zeo590, which amplify a 691 bp PCR fragment from correctly targeted cells (Fig. 1C, lanes 2 and 3, two independent isolates) and a 1.3 kb PCR fragment (Fig. 1C, lane 3) from untargeted cells. The pGETrec plasmid continued to persist in the *asd⁻* DH10B cells in the absence of Ac selection, in contrast to that reported by Muyrers *et al.* (24). Therefore, L-arabinose induction of the strong P_{BAD} promoter on the pGETrec plasmid in the absence of selection was used to eliminate the plasmid.
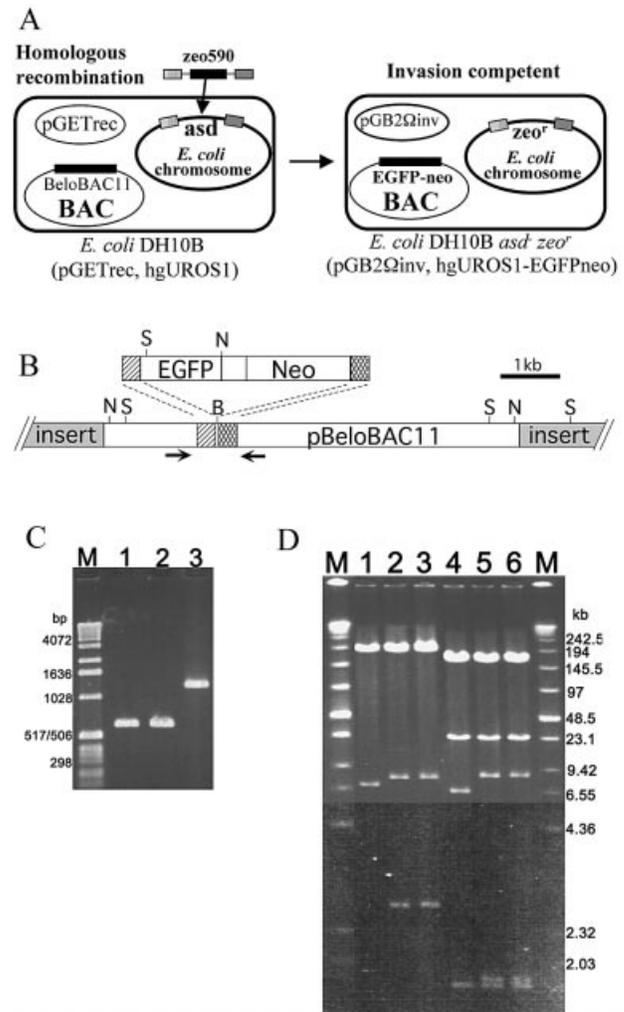

Figure 1. GET recombination in *E.coli* DH10B. (**A**) Modification of the *E.coli* chromosome. The zeo590 PCR product contains the zeo-resistance gene flanked at both ends with 50 bp sequences (stippled boxes) that are homologous to regions of the *asd* gene. Introduction of zeo590 into arabinose-induced *E.coli* (pGETrec) results in precise replacement of the *asd* gene with the zeo-resistance gene. After elimination of pGETrec, introduction of pGB2Ωinv encoding the *Y.pseudotuberculosis inv* gene confers the ability to invade mammalian cells and deliver either pEGFP-N2 or a modified BAC clone. (**B**) Modification of a 200 kb BAC clone. PCR product, EGFPneo, which contains the EGFP-kmʳ-neoʳ cassette flanked on either side by 60 bp of DNA homologous to pBeloBAC11 vector centered on the *Bst*11701 site. Homologous recombination results in insertion of DNA encoding for EGFP and neomycin resistance. Arrows represent primers KF and KR which flank the recombination junction. B, *Bst*11071; N, *Not*I; S, *Sal*I. (**C**) PCR analysis of *E.coli* DH10B *asd⁻*. Primers asd1 and asd2 amplify across the *asd* locus in *E.coli* DH10B. Lanes 1 and 2, the predicted 691 bp PCR product confirming correct targeted replacement of the *asd* locus in two independent zeoʳ colonies. Lane 3, the 1.3 kb fragment predicted for an intact *asd* locus. Lane M, marker X (Roche Diagnostics Corporation, IN). (**D**) Restriction analysis of the modified BAC clone. Lane 1, unmodified BAC digested with *Not*I, showing 6.9 kb *Not*I fragment. Lanes 2 and 3, independent isolates of modified BAC digested with *Not*I showing 7.8 and 2.7 kb *Not*I fragments, which confirm the correct targeting of EGFPneo into the vector portion of the BAC clone. Lane 4, unmodified BAC digested with *Sal*I showing a 6.7 kb *Sal*I fragment. Lanes 5 and 6, independent isolates of modified BAC digested with *Sal*I, showing 8.2 and <2 kb *Sal*I fragments, which confirm the correct targeting of EGFPneo into the vector portion of the BAC clone. The 200 kb *Not*I and 175 and 24 kb *Sal*I fragments representing the BAC insert have not changed during the homologous recombination. Higher contrast was used on the gel photograph below the 6.55 kb marker for clarity of the lower molecular weight ethidium bromide stained bands.

Modification of a 200 kb genomic BAC clone

In order to assay the functional delivery of BAC DNA into mammalian cells, we have used the GET recombination system to insert DNA encoding the GFP and G418 resistance into the pBeloBAC11 vector of a 200 kb human BAC that contains the human UroS-1 gene (hgUROS-1) (21). A 3.8 kb PCR fragment (EGFPneo) containing the genes for GFP and neomycin was amplified from pEGFP-N2 using PCR primers GK-F and GK-R, which each contain 60 bp of flanking DNA that is homologous to the 120 bp of DNA centered on the unique *Bst*1107I site on pBeloBAC11 (Fig. 1B). Insertion of DNA into this *Bst*1107I site has been shown to not disrupt BAC replication and maintenance (4). After electroporation of EGFPneo DNA into arabinose-induced DH10B (pGETrec, hgUroS-1), DNA from Km^r colonies were screened by PCR amplification across the recombination junction using primers KF and KR (Fig. 1B). Amplification of the predicted 4.0 kb fragment confirmed the correct targeting of EGFPneo within the vector portion of the hgUroS-1 BAC (data not shown). BAC DNA isolated from two Km^r colonies were analyzed by *Not*I and *Sal*I restriction digestions using PFGE, which further confirmed the correct targeting of the BAC vector (Fig. 1D). Furthermore, the stability of the BAC insert during this recombination procedure is demonstrated by the consistent 200 kb *Not*I and 175 and 24 kb *Sal*I insert bands in both independent recombinant BAC isolates (Fig. 1D). This modified BAC clone was designated hgUroS-1-EGFPneo.

Plasmid delivery and expression in human cells

The *Y.pseudotuberculosis* invasin gene on pGB2Ωinv (9) was introduced into *E.coli* DH10B *asd*⁻ carrying pEGFP-N2 (Fig. 1A). F-derived BACs and pUC-based high copy plasmids are compatible with pGB2Ωinv and can therefore co-exist in the DH10B host. Although the optimal growth temperature for invasion of mammalian cells by *Y.pseudotuberculosis* is at 28°C, invasin expression in *E.coli* is regulated in a temperature-independent manner (25). Therefore, the invading *E.coli* were grown overnight at 37°C, which simplifies the invasion procedure, and were observed to improve the efficiency in delivering pEGFP-N2 into HeLa cells (data not shown).

The *E.coli* DH10B *asd*⁻ (pEGFP-N2, pGB2Ωinv) grown at 37°C was overlaid onto target HeLa and HT1080 cells, and the transfection efficiency monitored by the expression of GFP using both fluorescence microscopy and fluorescence-activated cell sorting (FACS). Figure 2 shows an increase in GFP expression in response to increasing MOI of bacteria incubated with HeLa cells for 2 h. MOIs of 2000 (Fig. 2B) and 4000 (Fig. 2C) produced GFP expression in ~12% of the HeLa cells. The DH10B *asd*⁻ (pEGFP-N2) that did not contain the *Y.pseudotuberculosis* invasin (*inv*) gene resulted in minimal GFP expression even at an MOI of 4000 (Fig. 2D). Invasion of HT1080 cells with DH10B *asd*⁻ (pGB2Ωinv, pEGFP-N2) with MOIs ranging from 500 to 4000 using identical conditions to HeLa transfection resulted in 2.66% of the cells expressing GFP at an MOI of 1500 (data not shown).

In order to assess the ability of this invading *E.coli* to stably transfect HeLa cells, HeLa colonies resistant to 1 mg/ml G418 were selected from cells invaded with DH10B *asd*⁻ (pGB2Ωinv, pEGFP-N2). A representative stable transfected colony is shown in Figure 2E, with 62.5% of the cells positive for GFP expression, as visualized by fluorescence microscopy after 32 days of growth and quantified by FACS. Thus, this *E.coli* DH10B-based delivery system can both transiently and stably transfect HeLa cells.

Rapid internalization of invasive *E.coli* DH10B into HeLa cells

The co-incubation of *E.coli* with HeLa cells for 2 h, as shown above (Fig. 2A–D), resulted in a significant loss of viability of the HeLa cells, especially at the higher MOIs. Therefore, in order to maximize HeLa cell viability, the minimal co-incubation time required for efficient invasion and plasmid delivery into HeLa cells was determined using a Gm plating assay (22). HeLa cells were incubated with *E.coli* at an MOI of 2000 for varying time points and cells washed to remove uninvaded *E.coli*. The bacteriocidal antibiotic Gm, which does not penetrate HeLa cell membranes, was added to kill any remaining adherent *E.coli* (22). HeLa cells were harvested and lysed, and viable *E.coli* in the cells measured by plating on selective media. Surprisingly, invasion of *E.coli* reached a maximum at the shortest time point tested (10 min), and remained approximately constant for up to 120 min (Fig. 3A). Uninvasive bacteria [DH10B *asd*⁻ (pEGFP-N2)] showed only minimal background levels (please note the log scale used on the histogram in Fig. 3A). This result shows that *E.coli* invasion is efficient at short exposure times, which greatly increases the overall viability of the HeLa cells. Based on these results, an incubation time of 30 min with late-log invasive *E.coli* was chosen to optimize invasion efficiency (Fig. 3A) yet maintain good HeLa cell viability (>75%).

Invasive *E.coli* delivers plasmid and BAC DNA into HeLa cells

The transfection efficiency of HeLa cells using invasive *E.coli* DH10B *asd*⁻ (pGB2Ωinv, pEGFP-N2) was performed for a co-incubation time of 30 min, which greatly increases HeLa cell viability. Less than 0.4% of HeLa cells expressed GFP when incubated with non-invasive *E.coli* DH10B *asd*⁻ (pEGFP-N2) at an MOI of 4000. At increasing MOIs of invasive *E.coli* and HeLa cells, the percentage of HeLa cells expressing GFP increases, from ~2% at an m.o.i of 500 to 8.9% at an MOI of 4000, 23-fold over background (Fig. 3B). Using similar conditions, the transfection efficiency of the invasive *E.coli* was investigated for the genomic BAC hgUroS-1-EGFPneo, which we modified using GET recombination (Fig. 1B and D). This BAC has a relatively large 200 kb insert containing the entire human Uroporphyrinogen-III synthase gene and surrounding regulatory sequences (21). This BAC was transferred into the invasive *E.coli* to create DH10B *asd*⁻ (pGB2Ωinv, hgUroS-1-GFPneo), which was co-incubated with HeLa cells at a range of MOIs for 30 min (Fig. 3B). The background level of GFP expression using non-invasive *E.coli* *asd*⁻ DH10B (hgUroS-1-GFPneo) was lower (<0.2%) than that observed for *E.coli* DH10B *asd*⁻ (pGB2Ωinv, pEGFP-N2), likely due to the much lower copy number per *E.coli* of the BAC relative to pEGFP-N2. FACS analysis 48 h after transfection showed that the percentage of HeLa cells expressing GFP from BAC hgUroS-1-GFPneo increased with increasing MOI, reaching a maximum of 2.8% at an MOI of 4000, 15-fold over background (Fig. 3B).
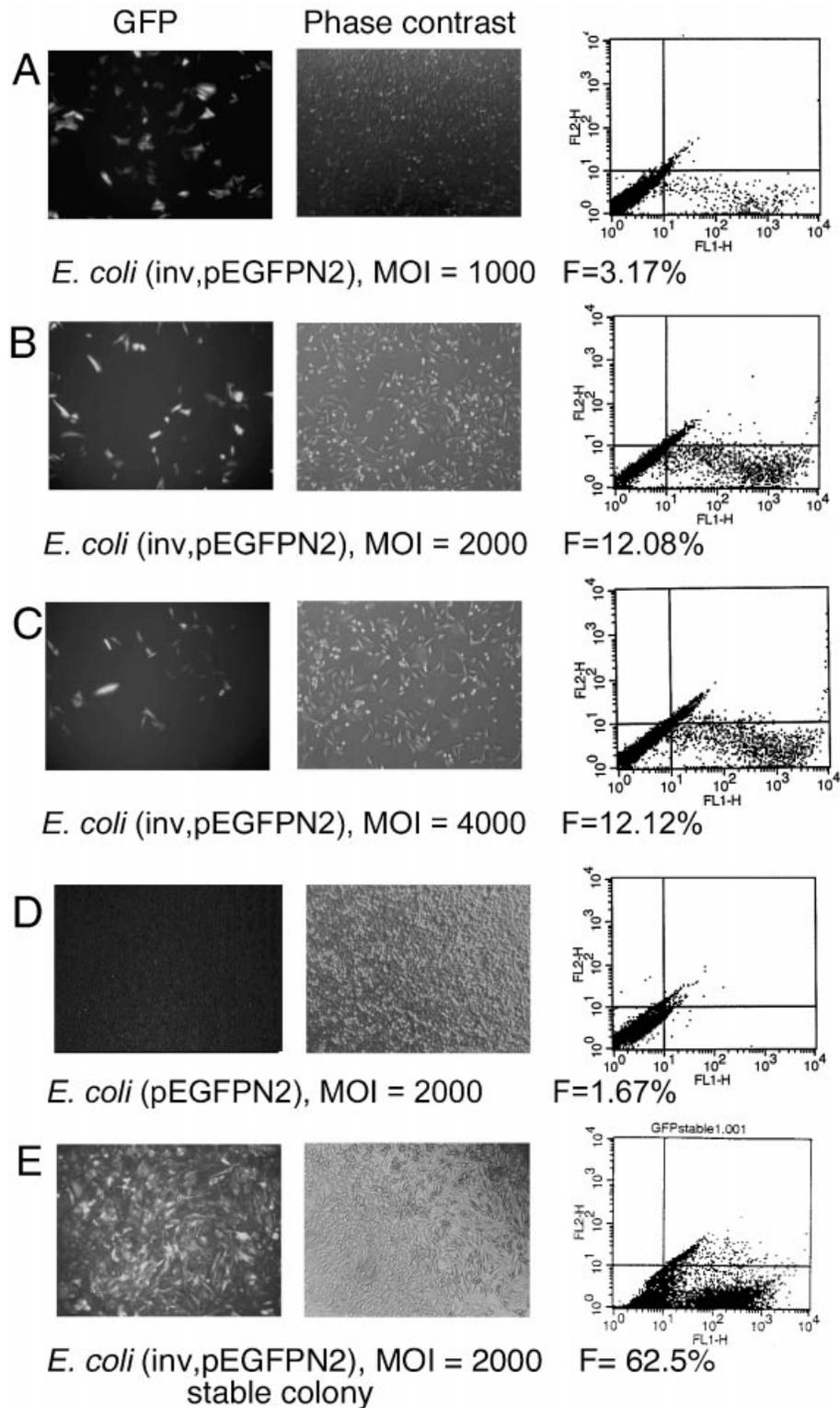
Figure 2. Invasion of HeLa cells by *E.coli* DH10B *asd⁻* (pGB2Ωinv, pEGFPN2). Overnight culture of invasive DH10B *asd⁻* bacteria or non-invasive DH10B *asd⁻* control were overlaid onto monolayer cultures of HeLa cells at the desired MOI for 120 min. HeLa cells were washed to remove extracellular bacteria before being incubated at 37°C for 48 h prior to detection of GFP expression. (**A–E**) Left, photomicrograph showing GFP fluorescence; center, phase contrast microscopy (red filter) showing same field of cells; right, FACS analysis of cells sorted for GFP (*x*-axis) and orange auto-fluorescence (*y*-axis); cells in lower right quadrant represent GFP-positive cells (*F*, % GFP-expressing cells). (A) MOI = 1000, *F* = 3.17. (B) MOI = 2000, *F* = 12.08. (C) MOI = 4000, *F* = 12.12. (D) Non-invasive *E.coli*, MOI = 2000, *F* = 1.67. (E) HeLa colony stably expressing GFP. *F* = 62.5% after G418 selection for 32 days.
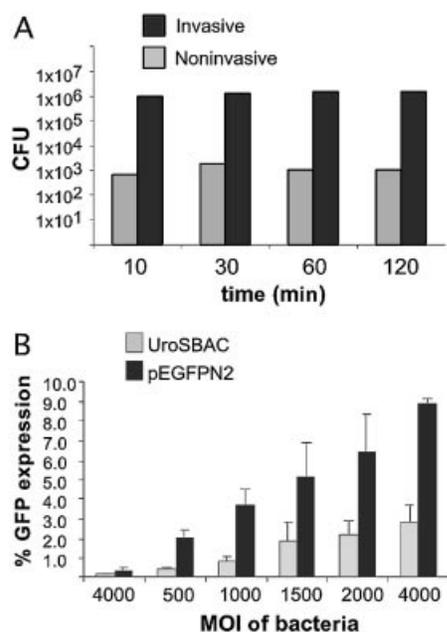
Figure 3. Invasive *E.coli* is internalized and delivers plasmid and a 200 kb BAC clone into HeLa cells. (**A**) Internalization of the *E.coli* DH10B *asd*⁻ upon exposure to HeLa cells for various time points. Mean values are calculated from two independent experiments. (**B**) Percentage of GFP expression in HeLa cells by invading *E.coli* harboring either pEGFP-N2 or hgUroS-1 BAC. Mean values are calculated from three independent experiments. An MOI of 4000 using uninvasive bacteria is shown in leftmost histograms.

DISCUSSION

We have shown that *E.coli* DH10B can be engineered to (i) permit targeted modification of plasmid and BAC DNA and (ii) deliver this modified DNA to human cells. *Escherichia coli* presents several advantages for use as a gene manipulation and delivery vector. *Escherichia coli* is widely used for the cloning and propagation of DNA, with protocols for growth, transformation, selection and DNA purification standard in most molecular biology laboratories. Genomic BAC libraries, which represent the building blocks of current genome assemblies, are propagated in *E.coli* DH10B, which has a natural potential to carry extrachromosomal DNA of over 1 Mb in size (26). However, the recombination deficiency of *E.coli* DH10B has limited the manipulation of these large BAC clones (>100 kb) using conventional recombinant DNA techniques. Here we have demonstrated the use of the GET recombination inducible system (4) to insert selectable markers and reporter genes (from pEGFP-N2) directly into the vector portion of an ~200 kb BAC clone, providing a relatively simple and widely applicable method for the conversion of available genomic BAC clones directly into vectors suitable for transfection into human cells.

The utility of these modified BACs has also been limited by lack of an efficient delivery system into mammalian cells. Here we have developed a strategy using the *E.coli* DH10B BAC host strain directly as a delivery vector. Using a novel application of the GET recombination system, we have created a targeted deletion of the *asd* gene from the *E.coli* DH10B chromosome, resulting in DAP auxotrophy and defective cell wall synthesis. Transformation of plasmid

pGB2Ωinv expressing the *Y.pseudotuberculosis* invasin gene renders these *E.coli* competent to invade mammalian cells and deliver plasmid and BAC DNA. pGETrec can be used to directly replace the *asd* gene with the invasin gene in the *E.coli* chromosome in one step, making this second transformation with pGB2Ωinv unnecessary and eliminating any biohazard risk from inadvertent plasmid transfer to non-auxotrophic bacteria.

Incubation of the *E.coli* DH10B *asd*⁻ (pGB2Ωinv) carrying pEGFP-N2 with both HeLa and HT1080 cells resulted in the delivery and transient expression of GFP in as high as 12% of cells, and isolation of stable G418-resistant colonies. Using the Gm protection assay (22), we found that incubation for as little as 10 min allowed for optimal internalization of bacteria while greatly increasing the viability of recipient cells. Incubation of *E.coli* DH10B *asd*⁻ (pGB2Ωinv) carrying the ~200 kb modified BAC clone resulted in ~2.8% of HeLa cells expressing GFP. This *E.coli*-based DNA vector represents the first comprehensive system capable of modification, propagation and delivery of full-sized BACs on the order of several hundred kilobases in size, and will greatly increase the utility of available BAC libraries for functional genomics and gene expression studies. These studies will provide important information regarding the regulation of temporal and tissue-specific gene expression for the development of improved gene therapy vectors (27).

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