Gram negative shuttle BAC vector for heterologous expression of metagenomic libraries

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ABSTRACT

Bacterial artificial chromosome (BAC) vectors enable stable cloning of large DNA fragments from single genomes or microbial assemblages. A novel shuttle BAC vector was constructed that permits replication of BAC clones in diverse Gram-negative species. The "Gram-negative shuttle BAC" vector (pGNS-BAC) uses the F replicon for stable single-copy replication in *E. coli* and the broad-host-range RK2 mini-replicon for high-copy replication in diverse Gram-negative bacteria. As with other BAC vectors containing the *oriV* origin, this vector is capable of an arabinose-inducible increase in plasmid copy number. Resistance to both gentamicin and chloramphenicol is encoded on pGNS-BAC, permitting selection for the plasmid in diverse bacterial species. The *oriT* from an IncP plasmid was cloned into pGNS-BAC to enable conjugal transfer, thereby allowing both electroporation and conjugation of pGNS-BAC DNA into bacterial hosts. A soil metagenomic library was constructed in pGNS-BAC-1 (the first version of the vector, lacking gentamicin resistance and *oriT*), and recombinant clones were demonstrated to replicate in diverse Gram-negative hosts, including *Escherichia coli*, *Pseudomonas* spp., *Salmonella enterica*, *Serratia marcescens*, *Vibrio vulnificus* and *Enterobacter nimipressuralis*. This shuttle BAC vector can be utilized to clone genomic DNA from diverse sources, and then transfer it into diverse Gram-negative bacterial species to facilitate heterologous expression of recombinant pathways.

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1. Introduction

BAC vectors using the modified F plasmid are commonly used for construction and analysis of genomic libraries; greatly facilitating research that relies upon the stable maintenance of very large DNA inserts (Shizuya et al., 1992). Early versions of BAC vectors (e.g., pBELOBAC11) provided excellent stability of recombinant clones, but due to the single copy number of the BAC replicon the concentration of plasmid DNA necessary for library construction or clone analysis

required large culture volumes to achieve sufficient vector DNA (Kim et al., 1996). The introduction of an additional origin of replication (*oriV*) from a broad host-range RK2 plasmid permitted a stable but inducible copy system, wherein the copy number was controlled by an arabinose-inducible replicator protein (TrfA) inserted into the *E. coli* chromosome (Wild et al., 2002; Wild and Szybalski, 2004a). With an inducible copy number, BAC clones can be maintained at a single copy under control of the F replicon and then induced to multiple copies (50- to 150-fold induction) by the addition of 0.01% arabinose to the culture medium. These vectors were further developed by Szybalski's lab to a new class of pBAC/oriV "copy-control tightly regulated expression vectors" (Wild and Szybalski, 2004b).

While providing significant advantages, these commercially available inducible BAC vectors (e.g., CopyRight v2.0 BAC, Lucigen Corp., Middleton, WI) are limited to replication within an *E. coli* host. For sequence-based mapping and molecular analysis, maintenance in *E. coli* is sufficient. However, for construction and functional screening of metagenomic libraries it is advantageous to transfer recombinant clones into multiple bacterial expression hosts to improve heterologous expression of cloned metagenomic DNA (Craig et al., 2010; Handelsman et al., 1998; Rondon et al., 2000). Various shuttle vectors have been used to transfer recombinant clones into alternative

heterologous hosts, such as *Streptomyces* and *Pseudomonas* spp. (Martinez et al., 2004; Wang et al., 2000). By incorporating into a BAC vector both *oriV* and *trfA*, which is the mini-replicon necessary for RK2 plasmid replication (Thomas et al., 1981), a much greater host range can be achieved, including most Gram-negative bacterial species.

A previous phylogenetic analysis of a soil metagenomic library indicated the very low prevalence of 16S rRNA genes from Gram-positive phyla (Liles et al., 2003), reflecting the poor lysis of Gram-positive cells when attempting to clone large DNA fragments. Therefore, a Gram-negative shuttle BAC vector would be particularly advantageous in cloning DNA derived from the diverse Gram-negative bacteria preferentially represented within large-insert metagenomic libraries. With any environmental sample, cloning into a shuttle vector would allow for conjugal transfer and heterologous expression of metagenomic cloned DNA in multiple bacterial hosts. One such example is pRS44, a RK2-based broad-host-range cloning vector (Aakvik et al., 2009). Unlike the pGNS-BAC vector, which has the complete RK2 mini-replicon contained within the vector (i.e., *oriV* and *trfA*), the pRS44 vector system requires transposon-mediated insertion of the *trfA* gene within the desired host species. Each heterologous bacterial host may have unique transcriptional regulatory factors that can affect expression of cloned genes; thus, the pGNS-BAC vector increases the probability of identifying clones with specific functions by expanding the range of genomic library hosts for recombinant gene expression.

2. Materials and methods

2.1. Bacterial strains and media

E. coli strain DH10B was used as the primary host for transformations. Cultures were grown at 37 °C in Luria–Bertani broth or agar plates supplemented with the appropriate antibiotics. Concentrations of antibiotics were 12.5 μg/ml chloramphenicol (Cm) and 30 μg/ml gentamicin sulfate (Gm). *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Pseudomonas fluorescens*, *Salmonella enterica*, *Serratia marcescens*, *Vibrio vulnificus*, and *Enterobacter nimipressuralis* were used as recipients to test the host range of pGNS-BAC-1 (Table 1).

2.2. Construction of pGNS-BAC-1

The P$_{araBAD}$ promoter of plasmid pJW544 drives expression of the *trfA* gene, and the TrfA replication initiation protein then binds to *oriV* iterons (Perri et al., 1991). A BamHI restriction site within the promoter was destroyed by restriction digestion and subsequent fill-in with Klenow DNA polymerase and dNTPs. Plasmid DNAs were extracted from *E. coli* cultures using a Promega Wizard Plus SV Minipreps kit (Madison, WI). Restriction and DNA sequence analysis was conducted to confirm loss of the BamHI site, and induction of plasmid copy number with 0.01% arabinose was performed to confirm

that the copy-inducible phenotype was still functional. The resulting plasmid was named pGNS-BAC-1 (Fig. 2A).

2.3. Soil metagenomic library construction

To determine if recombinant BAC clones in the vector pGNS-BAC-1 were capable of replication within Gram-negative bacterial hosts, a small-insert BAC library was constructed from bacterial cells that were first extracted from the soil prior to DNA isolation (Liles et al., 2008). Briefly, the extracted and washed bacterial cells were incorporated into agarose plugs, lysed, and then high molecular weight (HMW) metagenomic DNA was electrophoresed from the plug. Purification by a formamide denaturation step (70% final concentration) resulted in removal of associated nuclease activity from the HMW DNA and improved cloning efficiency (Liles et al., 2008). The formamide-treated metagenomic DNA was partially restriction digested with HindIII, electroeluted from an agarose gel, and ligated into a HindIII digested and dephosphorylated pGNS-BAC-1 vector. The ligated vector and insert DNA was transformed into *E. coli* strain DH10B, and transformants were selected on LB containing 12.5 μg/ml Cm. Transformants were robotically picked into a 96-well format and stored in 10% glycerol at −80 °C.

2.4. Electroporation of BAC DNA into bacterial strains

Random clones were selected from the soil metagenomic library in pGNS-BAC-1. DNA was extracted using a manual alkaline lysis protocol and characterized by restriction fragment length polymorphism (RFLP) analysis using HindIII (Promega). Clones with insert DNA were transformed into electrocompetent *Serratia marcescens*, *V. vulnificus*, and *Pseudomonas putida* (1 mm gap cuvette, 1.8 kV, 600 Ω, 10 μF). Cells were grown in SOC recovery medium for 1 h at 37 °C and plated on LB agar supplemented with Cm. id DNA was extracted and subjected to RFLP analysis as above to test for the presence of the recombinant BAC DNA in each bacterial host.

2.5. Construction of pGNS-BAC

A Gm resistance cassette was obtained from plasmid pBSL141 (Alexeyev et al., 1995) as a NheI restriction fragment and ligated into an Eco47III site of the vector pGNS-BAC-1. Transformants were selected on LB containing both Cm and Gm. Restriction digests with EcoRV established the presence of the Gm-resistance cassette, resulting in the plasmid pGNS-BAC-2.

A cloning region from the vector pSMART BAC v2.0 (Lucigen Corporation, Middleton, WI) containing the counter-selectable *sacB* gene and pUC19 origin of replication was cloned into pGNS-BAC-2 to reduce the background of transformants without inserts (i.e., by *sacB*-mediated counter-selection) and to provide a very high copy number for preparation of empty vector DNA (pUC19 origin). The cloning region was PCR amplified using flanking primers, purified, blunt-ended, and ligated to a filled-in HindIII restriction site of the pGNS-

Table 1
Bacterial strains and plasmids.

Bacteria	Plasmids of interest	Plasmid-encoded antibiotic resistance or other characteristic	Source
E. coli strain DH10B	pJW544	BAC vector, Cm^R *oriV*	Wild and Szybalski (2004a,b)
E. coli strain DH10B	pGNS-BAC-1	BAC vector, Cm^R, *oriV*, BamHI site-minus	This study
E. coli strain SM10	pGNS-BAC	BAC vector, Cm^R, Gm^R, *oriV*, *oriT*, *sacB*	This study
Pseudomonas putida	pGNS-BAC-1 or pGNS-BAC	Cm^R, or Cm^R and Gm^R	This study
Pseudomonas aeruginosa	pGNS-BAC-1 or pGNS-BAC	Cm^R, or Cm^R and Gm^R	This study
Pseudomonas stutzeri	pGNS-BAC-1 or pGNS-BAC	Cm^R, or Cm^R and Gm^R	This study
Pseudomonas fluorescens	pGNS-BAC-1 or pGNS-BAC	Cm^R, or Cm^R and Gm^R	This study
Salmonella enterica	pGNS-BAC-1 or pGNS-BAC	Cm^R, or Cm^R and Gm^R	This study
Vibrio vulnificus	pGNS-BAC-1 or pGNS-BAC	Cm^R, or Cm^R and Gm^R	This study
Enterobacter nimipressuralis	pGNS-BAC-1 or pGNS-BAC	Cm^R, or Cm^R and Gm^R	This study

BAC-2 vector. The ligation was transformed into electrocompetent *E. coli* strain DH10B and plated onto LB containing Cm and Gm. Transformants were screened for sucrose sensitivity. Plasmid DNA was extracted from sucrose-sensitive clones and restriction digested with HindIII to confirm the addition of the cloning region to pGNS-BAC-2. The resulting BAC vector was designated as pGNS-BAC-3.

To introduce the ability to conjugally transfer the BAC vector, the *oriT* (*mob*) gene from pLOF-Km was PCR amplified using the primers *mobF* (5'-GATC**CTCGAG**GGATCCTTTTTGTCCG) and mobR (5'-GATC**CTCGAG**CAGCCGACCAGGCT) (Herrero et al., 1990). The PCR primers include 5'-XhoI restriction sites (bold). After amplification and XhoI digestion, the amplicon was ligated into the XhoI site of pGNS-BAC-3, transformed into *E. coli* strain DH10B, and selected on LB containing Cm and Gm. Clones containing the *oriT* were identified via PCR using the mobF and mobR primers, and the resultant plasmid was verified by restriction digestion with Sau3AI. The final vector construct, pGNS-BAC-4, also referred to as the pGNS-BAC vector (Fig. 2B), was stored as a glycerol stock at −80 °C. The pGNS-BAC vector was sequenced completely, and the sequence was deposited within the GenBank database (accession number HQ245711).

2.6. Conjugal transfer of BAC vector DNA into bacterial strains

The pGNS-BAC vector was electroporated (1 mm gap cuvette, 1.8 kV, 600 Ω, 10 μF) into *E. coli* strain SM10, which permits conjugal transfer of *oriT*-containing plasmids (Simon et al., 1983). Cells were grown in SOC recovery medium for 1 h at 37 °C and plated on LB agar supplemented with Cm (12.5 μg/ml) and Gm (30 μg/ml). *E. coli* strain SM10 having the pGNS-BAC vector was used as the donor for conjugation experiments. Confirmation of the ability of *oriT* to mediate conjugal transfer was performed using *S. marcescens* as the recipient. LB broth supplemented with Cm and Gm was used to grow the donor, and LB broth without antibiotics was used to grow the recipient. Cultures were grown overnight at 37 °C with aeration. Donor and recipient were mixed in a ratio of 1:4 (50 μl and 200 μl respectively) and treated with 1 ml of 10 mM MgSO$_4$. After mixing thoroughly, centrifugation was carried out at 15,000×*g* for 10 min. One milliliter of supernatant was discarded, and the cells were resuspended in the remaining liquid and spread on a nitrocellulose membrane placed on the surface of an LB agar plate. Following incubation at 37 °C for 4 h the membrane was transferred to an LB agar plate containing 1 mM IPTG and incubated at 37 °C for another 12 h. Cells were then washed off the membrane with 3 ml of 10 mM MgSO$_4$ and collected in a tube. Different dilutions of this cell mixture were then spread on LB agar containing Cm and Gm (to select against the recipient) and colistin (10 μg/ml, to select against the donor). This procedure allows exclusive selection of the *S. marcescens* (or other recipient host) transconjugants.

Transconjugants were selected and screened for the presence of pGNS-BAC vector DNA by isolating plasmid DNA from the recipient hosts after varying times of cultivation, in the presence and absence of Cm and/or Gm and/or 0.01% arabinose. The presence of plasmid DNAs was confirmed by restriction analysis. The cell counts of donor, recipient, and transconjugants were estimated by plating a range of serial dilutions on suitable media.

2.7. Amplification of the pGNS-BAC vector in E. coli strain DH10B and Serratia marcescens

Two sets of *E. coli* and *S. marcescens* cultures containing the pGNS-BAC vector were grown overnight in 10 ml of LB containing 12.5 μg/ml Cm. Cultures were grown with shaking for 30 min and then one set of cultures was induced with 50 μl of 2% ʟ-Arabinose (Sigma) [0.01% final concentration]. After 4 h of induction the DNA was extracted using Wizard plus SV Minipreps DNA Purification System (Promega). DNA was prepared from 4.5 ml of induced and not induced cultures and

eluted from the column with 50 μl of nuclease free water. To evaluate the integrity and relative amount of DNA purified from each culture, a restriction digestion with NcoI (cuts twice within pGNS-BAC) was performed at 37 °C. After 1 h of incubation the restriction digest was electrophoresed through a 0.6% agarose gel and restriction fragments were visualized by staining with EtBr.

2.8. Increase in MIC of Cm and Gm conferred by pGNS-BAC

Minimum inhibitory concentration (MIC) testing using the macrodilution method was carried out to test the degree of resistance to gentamicin or chloramphenicol conferred by pGNS-BAC on *E. coli* or *S. marcescens*. Both bacterial species were tested in the presence and absence of the BAC vector and with or without addition of 0.01% arabinose to the cation-adjusted Mueller–Hinton broth (CAMHB) (Table 2). Antibiotic stock solutions of Gm (960 μg/ml), Cm (400 μg/ml), and arabinose (0.01% and 0.02%) were made using CAMHB. The final concentration range tested for Gm was from 7.5 μg/ml with a twofold consecutive increase up to 960 μg/ml and likewise for Cm from 3.125 μg/ml up to 400 μg/ml. The experiment was conducted in triplicate, with inclusion of the controls: 1) bacterial strains without vector DNA, 2) bacterial growth without any antibiotics added, and 3) media only. Tubes were incubated overnight at 37 °C and turbidity was measured the next day to determine the MIC of the antibiotic.

A gradient agar plate method was used to confirm the increase in antibiotic resistance as a result of plasmid amplification (Bryson and Szybalski, 1952). A Cm gradient agar plate with antibiotic concentration ranging from no added Cm to 500 μg/ml Cm in the presence and absence of 0.01% arabinose was used to streak overnight cultures of *E. coli* and *S. marcescens*. Plates were incubated at 37 °C and observed and recorded after 24 h.

3. Results

3.1. pGNS-BAC-1 construction and analysis

The pGNS-BAC-1 plasmid was tested as a shuttle vector under control of either of its two origins of replication (i.e., F and RK2) (Table 1). The pGNS-BAC-1 vector is maintained in *E. coli* as a single-copy plasmid by repressing the RK2 origin of replication with the addition of 0.1% glucose to the growth medium. Induction of plasmid copy number in *E. coli* was achieved by supplementation with 0.01% arabinose. The pGNS-BAC-1 vector was electroporated into *P. putida*, *P. aeruginosa*, *P. stutzeri*, *P. fluorescens*, *S. enterica*, *S. marcescens*, *V. vulnificus*, and *E. nimipressuralis* (Table 1). Isolated colonies from each transformation were used to inoculate LB broth cultures containing Cm, and the plasmid DNAs extracted from each host revealed a banding pattern identical to the pGNS-BAC-1 plasmid (data not shown). In some cases, the DNA isolated from non-*E. coli* hosts (e.g., *S. marcescens*) was retransformed into *E. coli*, yielding Cm-resistant clones with a pGNS-BAC-1 restriction profile (data not shown).

Table 2
MIC for Cm and Gm conferred by pGNS-BAC.

Bacterial strain	pGNS-BAC	Arabinose	MIC (μg/ml)	
			Cm	Gm
E. coli	+	−	25	60
E. coli	+	+	200	480
E. coli	−	−	6.25	15
E. coli	−	+	6.25	15
S. marcescens	+	−	12.5	30
S. marcescens	+	+	200	240
S. marcescens	−	−	6.25	15
S. marcescens	−	+	6.25	15
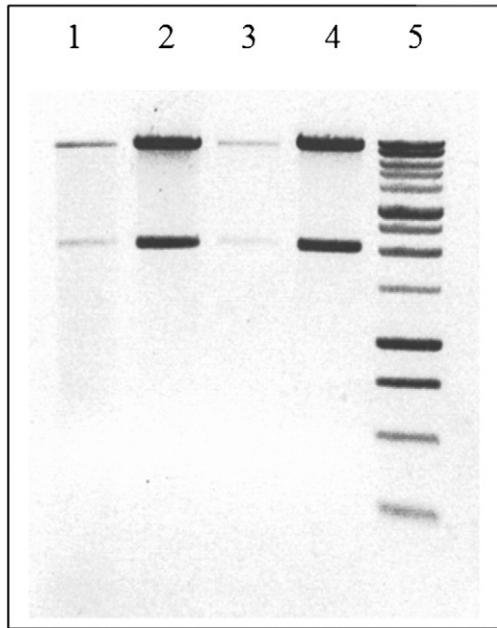
Fig. 1. Isolation of BAC vector DNA from *E. coli* and *S. marcescens*. Lanes 1–2, *S. marcescens* containing pGNS-BAC without (lane 1) or with arabinose induction (lane 2); lanes 3–4, *E. coli* strain DH10B containing pGNS-BAC without (lane 3) or with arabinose induction (lane 4); lane 5, molecular weight marker (Bench Top 1 kb DNA ladder, Promega). By comparing the DNA band intensity in lane 1 relative to lane 2 and lane 3 relative to lane 4 the degree of arabinose-mediated plasmid copy-induction can be estimated.

3.2. Construction of a soil metagenomic library and lateral transfer of recombinant clones

To determine if recombinant pGNS-BAC-1 clones can also stably replicate in different bacterial hosts, a metagenomic library was constructed within pGNS-BAC-1. Metagenomic DNA was extracted from soil at the Bonanza Creek Experimental Forest near Fairbanks, AK, and was partially restriction digested and ligated into pGNS-BAC-1. *E. coli* transformants were picked into 96-well plates, and random clones were analyzed by RFLP to identify large-insert containing clones. Random clones containing DNA inserts of approximately 75.0 kb, 79.8 kb, 83.9 kb, and 86.0 kb were electroporated into *S. marcescens*, *V. cholerae*, and *E. nimipressuralis*. Cm-resistant

transformants were successfully isolated for each of the clones in each of the bacterial hosts. The range of transformation efficiencies for the clones containing inserts relative to the empty pGNS-BAC-1 vector was 94.9% to 259% for *S. marcescens*, 45.7% to 76.2% for *V. cholerae*, and 55.9% to 104.9% for *E. nimipressuralis*. Recovery of recombinant clones with intact inserts from the transformants in *S. marcescens* was confirmed by pulsed field gel electrophoresis.

3.3. pGNS-BAC construction and analysis

Although pGNS-BAC-1 was maintained in multiple Gram-negative bacteria, its utility as a shuttle vector was limited due to the presence of only a single antibiotic resistance gene and an inability to be conjugally transferred to recipient hosts. Therefore, a Gm resistance cassette and an *oriT* were added to pGNS-BAC-1.

An improved multiple cloning region with a removable counter-selectable marker was also added to pGNS-BAC-1 to provide much lower background during transformations, resulting in the final pGNS-BAC vector construct. Cells containing intact pGNS-BAC vector are sucrose-sensitive due to the presence of the *sacB* gene within the cloning region. This region is removed as a restriction fragment during preparation of the vector for cloning. The final vector size is 11.9 Kb, and recombinant clones are sucrose-resistant (data not shown). The complete sequence of pGNS-BAC was determined and annotated and submitted to GenBank (Accession number HQ245711).

E. coli strain SM10 containing pGNS-BAC was mixed with *S. marcescens* to test its ability to be conjugally transferred and to replicate within a bacterial host other than *E. coli*. Transconjugants that were Cm^R and Gm^R were readily obtained ($>1 \times 10^5$ transconjugants µg^{-1} DNA). Representative transconjugants were inoculated into broth cultures with and without antibiotic selection, and after 12 to 16 h of growth, plasmid DNAs were isolated and restriction digested to determine plasmid yield and stability. Plasmid DNAs corresponding to the pGNS-BAC restriction profile were observed by RFLP. Fig. 1 shows the comparison of pGNS-BAC DNA isolated from *E. coli* strain DH10B and *Serratia marcescens* with and without arabinose induction.

In the absence of arabinose copy-induction, *E. coli* (pGNS-BAC) had an MIC for Cm of 25 µg/ml and an MIC for Gm of 60 µg/ml, whereas *S. marcescens* (pGNS-BAC) had an MIC for Cm of 12.5 µg/ml and an MIC for Gm of 30 µg/ml (Table 2). In the presence of arabinose, *E. coli* (pGNS-BAC) had an MIC for Cm of 200 µg/ml and 480 µg/ml for Gm, and *S. marcescens* (pGNS-BAC) had an MIC for Cm of 200 µg/ml and an MIC for Gm of 240 µg/ml (Table 2). Thus, both *E. coli* and *S. marcescens*
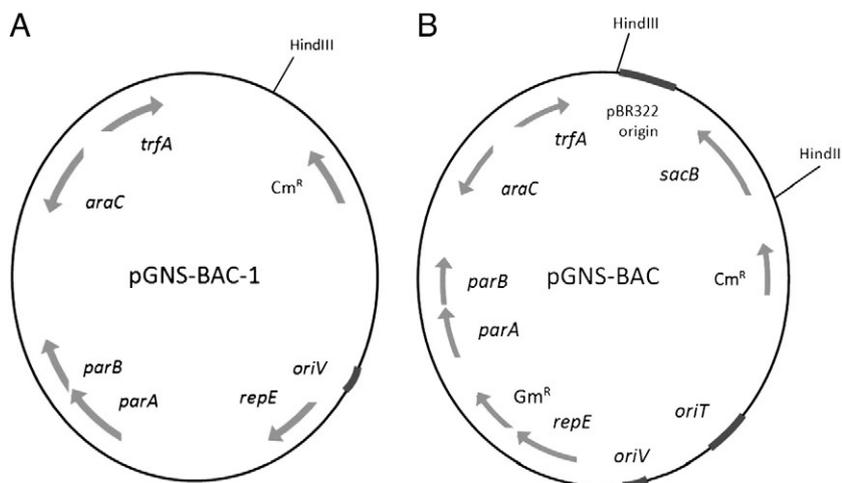
Fig. 2. Annotated plasmid map for pGNS-BAC-1 (panel A) and pGNS-BAC (panel B).
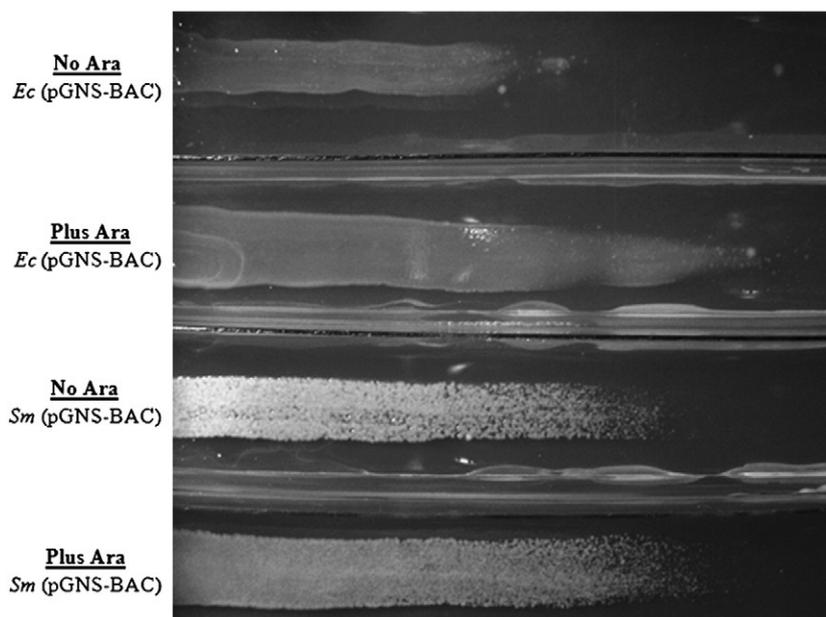
Fig. 3. Growth of *E. coli* with the pGNS-BAC vector (*Ec* (pGNS-BAC)) and *S. marcescens* with the pGNS-BAC vector (*Sm* (pGNS-BAC)) in the presence (Plus Ara) and absence (No Ara) of arabinose, and on a Cm gradient agar plate ranging from no added Cm (left) to 500 μg/ml Cm (right).

harboring pGNS-BAC had a ca. 32-fold increase in resistance to Cm as a result of arabinose-mediated copy-induction, and a similar increase in resistance to Gm in the presence of arabinose (32-fold for *E. coli*, and 16-fold for *S. marcescens*). However, in the absence of the pGNS-BAC vector no arabinose-induced changes in MIC levels were observed (Table 2).

On a Cm gradient agar plate ranging from no added Cm to 500 μg/ml Cm, both *E. coli* and *S. marcescens* with the pGNS-BAC vector exhibited higher resistance to Cm in the presence of arabinose (Fig. 3). Comparatively less growth was observed in the absence of arabinose. This confirms that pGNS-BAC plasmid amplification leads to an increase in the level of antibiotic resistance in multiple bacterial hosts.

4. Discussion

The pGNS-BAC vector provides the ability to clone DNA inserts and maintain recombinant clones at single copy in *E. coli*, utilizing the well-described stability of the F plasmid. The addition of arabinose results in induction of pGNS-BAC copy number mediated by *trfA* located on the plasmid. Copy-induction greatly increases plasmid DNA yield and could improve heterologous expression of cloned DNA via a gene-dosage mechanism (Rine et al., 1983). The RK2 mini-replicon that affords the copy-inducible phenotype in *E. coli* also permits replication in a phylogenetically diverse range of Gram-negative bacteria. Large-insert clones within the first version of the shuttle vector pGNS-BAC-1 were demonstrated to be capable of transfer and replication within diverse *Proteobacteria* species. The final pGNS-BAC vector construct has a significantly expanded host range compared to pGNS-BAC-1 due to the addition of genes for Gm resistance and its ability to be conjugally transferred.

This inducible-copy and Gram-negative shuttle vector can be employed for metagenomic analysis of diverse environments, most of which contain abundant Gram-negative species, as well as to heterologously express specific genetic pathways. Construction of a soil metagenomic library in the pGNS-BAC vector provides the ability to transfer entire libraries, or specific recombinant clones, into bacterial hosts that may be more closely related to the bacterial taxa from which the cloned DNA was derived. Ideally, metagenomic libraries from a given source DNA could be constructed in both pGNS-

BAC and a Gram-positive shuttle vector, potentially providing the widest possible range of heterologous expression hosts.

The rapidly advancing science of metagenomics requires molecular tools to enhance the heterologous expression of cloned DNAs. The metagenomic libraries constructed in pGNS-BAC will have all of the properties valued in previous libraries, such as stable maintenance of large inserts, with added features that have great potential to facilitate manipulation and expression of recombinant clones in a variety of different Gram negative hosts.

Acknowledgements

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