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Identification of type II toxin–antitoxin modules in *Burkholderia pseudomallei*

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Abstract

Type II toxin–antitoxin (TA) systems are believed to be widely distributed amongst bacteria although their biological functions are not clear. We have identified eight candidate TA systems in the genome of the human pathogen *Burkholderia pseudomallei*. Five of these were located in genome islands. Of the candidate toxins, BPSL0175 (RelE1) or BPSS1060 (RelE2) caused growth to cease when expressed in *Escherichia coli*, whereas expression of BPSS0390 (HicA) or BPSS1584 (HipA) (in an *E. coli* Δ*hipBA* background) caused a reduction in the number of culturable bacteria. The cognate antitoxins could restore growth and culturability of cells.

Introduction

Toxin–antitoxin (TA) modules are found throughout the prokaryotic world and typically consist of a gene pair, in an operon, which code for a toxin and an antitoxin product. Different systems are classed into type 1, 2 or 3, depending on the gene products (Yamaguchi *et al.*, 2011). In the case of type 2 systems, the genes encode protein toxins and antitoxins. The free toxins bind to cellular targets. Depending on the toxin family, these targets include DNA gyrase, ribosomes, elongation factor thermal unstable or uridine diphosphate-*N*-acetylglucosamine (Daines *et al.*, 2007; Schumacher *et al.*, 2009; Mutschler *et al.*, 2011; Yamaguchi & Inouye, 2011; Yamaguchi *et al.*, 2011). Degradation, inhibition or alteration of these targets by the toxin induces either a bacteriostatic or bactericidal response. The activity of the toxin can be blocked by the cognate antitoxin, and the relative levels of toxin and antitoxin determine the metabolic status of the bacterial cell (Rotem *et al.*, 2010).

Although the biological functions of TA modules are still being elucidated, in *Escherichia coli* they have been shown to influence persister cell formation (Korch *et al.*, 2003; Korch & Hill, 2006; Dörr *et al.*, 2010). More recently, Maisonneuve *et al.* (2011) demonstrated that persister frequencies were markedly reduced in an *E. coli* strain lacking 10 TA systems known to encode mRNases. Persisters are phenotypic variants within a population that are believed to be in a state of dormancy with the ability to re-initiate growth (Lewis, 2010). This allows tolerance towards antibiotics and other stresses, which target actively growing cells (Keren *et al.*, 2004).

It has previously been hypothesised that persister cells may have an involvement in chronic recurring bacterial diseases. High-persister mutants have been shown to be selected for in *Candida albicans* infections and in cystic fibrosis patients infected with *Pseudomonas aeruginosa* (Lafleur *et al.*, 2010; Mulcahy *et al.*, 2010). RelBE has been shown to have an involvement in bacterial cell dormancy in a cell density-dependent manner (Tashiro *et al.*, 2012). We considered that this may also be true for *Burkholderia pseudomallei*, the causative agent of the human disease melioidosis. A common feature of melioidosis is the re-emergence of overt disease months or years after apparently successful antibiotic treatment of primary clinical disease (Currie *et al.*, 2000). The purpose of this

study was to identify potential TA systems present in the *B. pseudomallei* K96243 genome.

Materials and methods

Bioinformatic screening

An online software tool called RASTA bacteria http://genoweb1.irisa.fr/duals/RASTA-Bacteria/ was used to screen the *B. pseudomallei* K96243 genome for TA systems. Those genes that scored a value of over 55% were considered as potential candidate TA genes. The NCBI Blast function was used to search for homology between sequences.

Growth conditions and chemicals

Bacteria were normally grown in Luria–Bertani (LB) broth at 37 °C with shaking (200 r.p.m.) or on LB agar plates at 37 °C. Where appropriate, media was supplemented with 100 μg mL^{-1} ampicillin (Sigma-Aldrich) for selection of pBAD/his (Invitrogen) and/or 15 μg mL^{-1} tetracycline for selection of pME6032. Where indicated, glucose or arabinose was also added to a final concentration of 0.2% (w/v) or isopropyl β-D-1 thiogalactopyranoside (IPTG) added to 25 mM final concentration.

Construction of plasmids

pBAD/his (Invitrogen) was used for toxin expression. Genes were PCR amplified from *B. pseudomallei* K96243 DNA using Hot start taq polymerase (Qiagen) and cloned into NcoI- and EcoRI-digested pBAD/his. For antitoxin cloning, genes were PCR amplified and cloned into NcoI- and SacI-digested pME6032. Constructs were checked by sequencing. Oligonucleotides used in PCR amplifications are listed in Table 1.

Construction of an *E. coli hipBA* mutant

The *hipBA* locus in *E. coli* MG1655 was inactivated using the Wanner mutagenesis method (Datsenko & Wanner, 2000). Briefly, a PCR product including the upstream and downstream regions of the *hipBA* locus along with flippase recognition target regions and a chloramphenicol resistance cassette was electroporated into *E. coli* MG1655, which possessed pK46 plasmid expressing the arabinose-inducible λ Red recombinase. Cultures were shifted to 37 °C to eliminate the pK46 plasmid after recombination. Inactivation of *hipBA* was confirmed by PCR with flanking region primers and plating onto LB agar plates supplemented with 50 μg mL^{-1} chloramphenicol.

Table 1. Oligonucleotides used in this study

Primer	Sequence (5′–3′)	Description
BPSL0174 Fw	GAGCTCCGAACTGGAGGATTGAGTAT	Cloning of BPSL0174 into pME6032 via SacI site
BPSL0174 Rv	CCATGGTGTGACGTTCGACGATTA	Cloning of BPSL0174 into pME6032 via NcoI site
BPSS0391 Fw	GAGCTCATGGAATTTCCCATCGCAGTG	Cloning of BPSS0391 into pME6032 via SacI site
BPSS0391 Rv	CCATGGTTATGCGTGCCTAACTTTGCC	Cloning of BPSS0391 into pME6032 via NcoI site
BPSL0175 Fw	GAGCTCGCCATGGCGTTGTCCCTTATGGGTTA CAATTCTCGCAT	Cloning of BPSL0175 into pBAD/his via EcoRI site
BPSL0175 Rv	GCGAATTCTCAATCCTCCAGTTCGCCAGCGATTT	Cloning of BPSL0175 into pBAD/his via SacI site
BPSS1060 Fw	GAGCTCGCCATGGGAATGTCCCTTATGGGTTACAATTCTC	Cloning of BPSS1060 into pBAD/his via EcoRI site
BPSS1060 Rv	GAATTCCTCAATCCTCCAGTTCGCCAGCGATTTGC	Cloning of BPSS1060 into pBAD/his via SacI site
BPSL3261 Fw	GAGCTCGCTAGCATGGCAACCATGCACGATA	Cloning of BPSL3260 into pBAD/his via SacI site
BPSL3261 Rv	CGGAAGCTTCGTCATGACCATGCCTCGTTA	Cloning of BPSL3260 into pBAD/his via NcoI site
BPSS1583 F	CCGAATTCATGGCCATCCTCATCGAGCAC	Cloning of BPSS1583 into pME6032 via SacI site
BPSS1583 Rv	CCATGGTCACCAGTCCTCCCGCTTT	Cloning of BPSL0174 into pME6032 via NcoI site
BPSL0559 Fw	GAGCTCGCCATGGCTATGGCGACGACAAAAAAAGC	Cloning of BPSL0559 into pBAD/his via SacI site
BPSL0559 Rv	CGCGGAATTCTTACATGACGTCGGTCCACTCG	Cloning of BPSL0559 into pBAD/his via EcoRI site
BPSL2333 Fw	GAGCTCGCCATGGCTATGGCCGAGGAAGACCTGTTGTG	Cloning of BPSL2333 into pBAD/his via SacI site
BPSL2333 Rv	GAATTCTCACGGCCATTCTTGGCGCGCATGCTTGA	Cloning of BPSL2333 into pBAD/his via EcoRI site
BPSS0395 Fw	GAGCTCGCCATGGCTATGGAGTGTCGTTGGCGCTTG	Cloning of BPSS0395 into pBAD/his via SacI site
BPSS0395 Rv	GGCGCGGAATTCTTACGCTTGCTCGACATAGC	Cloning of BPSS0395 into pBAD/his via EcoRI site
BPSS0390 Fw	GGAGCTCGCCATGGCTATGAACTCATCGAAGCTGATCC	Cloning of BPSS0390 into pBAD/his via SacI site
BPSS0390 Rv	GAATTCTCACAGGCCGGCGGATTTC	Cloning of BPSS0390 into pBAD/his via EcoRI site
HipBA R	CTTATAATATCCCCTTAAGCGGATAAACTTGCTGTGGAC GTATGACATGGTGTAGGCTGGAGCTGCTT	Inactivation of HipBA
HipBA F	CGGTCATGATTGTCATGCTCATTAACAATGACCAAACCC CATATCTCACATATGAATATCCTCCTTTAGTTCCTA	Inactivation of HipBA

Bacterial growth after toxin and/or antitoxin expression

Overnight cultures were diluted 1 : 100 in fresh LB broth supplemented with ampicillin (toxin), tetracycline (antitoxin) or both ampicillin and tetracycline and grown until reaching an $OD_{590\,nm}$ of approximately 0.1 when cultures were divided and supplemented with glucose to repress toxin or antitoxin expression, with arabinose to induce toxin expression, with IPTG to induce antitoxin expression or with both arabinose and IPTG to induce toxin and antitoxin expression. Cultures were grown at 37 °C, with shaking, and at intervals, the $OD_{590\,nm}$ was measured and viable cells enumerated on LB agar containing ampicillin.

Resuscitation assay

For the resuscitation assay with washing, we followed the protocol above but after growth for 2 h in the presence of arabinose the cultures were centrifuged at 3220 ***g*** for 10 min, and then the cell pellet re-suspended in LB before the addition of IPTG.

Live/dead staining

After growth for 3 h in the presence of glucose or arabinose, cultures were diluted to an $OD_{590\,nm}$ of 0.5 and stained using LIVE/DEAD Baclight (Invitrogen), following the manufacturer's instructions. Isopropanol-killed bacteria were used as negative controls. Fluorescent bacteria were visualised with a CETI inverted fluorescent microscope using green and red filters at a 40× objective. ProgRes CF software and a Jenoptik camera were used for image capture.

Density-dependent assay

Overnight cultures were diluted 1 : 100 in LB broth supplemented with ampicillin and grown until reaching an $OD_{590\,nm}$ of approximately 0.2. Suitable dilutions (1 : 10 and 1 : 100) of this culture were prepared in LB supplemented with antibiotic. Arabinose was added, the cultures incubated, and after 2 h, cells enumerated on agar as described above.

Results

Bioinformatic screening identified candidate TA systems

To identify candidate TA genes in *B. pseudomallei* K96243, the software tool RASTA bacteria was used (Sevin & Barloy-Hubler, 2007). A total of 67 ORFs were identified. We compared this list to the TA predictions reported by Makarova *et al.* (2009) and to the TA database (Shao *et al.*, 2011). Eight loci were picked for further study: BPSL0174-175, BPSL0558-0559, BPSL2333-2334, BPSL3260-3261, BPSS0390-0391, BPSS0394-0395, BPSS1060-1061 and BPSS1583-1584. Five loci (BPSL0558-0559, BPSL0174-0175, BPSL3260-3261, BPSS1060-161 and BPSS0390-0391) are located on genomic islands (GI 3, GI 2, GI 11, GI 15 and GI 13, respectively) in *B. pseudomallei*. The putative TA loci are differentially distributed amongst different *B. pseudomallei* strains. BPSS1583-1584 is present in all sequenced and partially sequenced *B. pseudomallei* strains available on the NCBI database, BPSL2333-3334 and BPSS0394-0395 are in the majority of strains, whereas the other loci are restricted to some strains. BPSS0390-0391 is present in only three genomes. However, homologues of BPSS0390-0391 were present in the related species *Burkholderia thailandensis*, *Burkholderia ambifaria* and *Burkholderia xenovorans.*

BPSS0390, BPSS1060 or BPSL0175 expression has an effect on growth when expressed in *E. coli* MG1655. BPSS1584 expression only effects growth in a Δ*hipBA* background

To assess functionality of these TA systems in *E. coli*, we expressed the toxin from an arabinose-inducible promoter and using a plasmid from a different incompatibility group co-expressed antitoxin from an IPTG-inducible promoter. Candidate toxins were cloned into pBAD and transformed into *E. coli* MG1655. When the culture had reached the early log phase ($OD_{590\,nm}$ approximately 0.1), either 0.2% glucose (to repress expression) or 0.2% arabinose (to induce expression) was added. When glucose was added, all of the cultures grew in a similar manner. The induction of BPSS0390, BPSS1060 or BPSL0175 expression resulted in a marked reduction in the rate of increase in optical density (Fig. 1f–h). BLAST searching the sequences for the three encoded proteins, revealed homology to known TA toxins in other bacterial species and will be referred to as HicA, RelE1 and RelE2, respectively, hence forth. BPSS0390 shared 78% sequence identity with HicA from *Acinetobacter baumannii*, and RelE1 and RelE2 show 54% sequence identity with RelE from *Klebsiella pneumonia.* The induction of the remaining five genes by the addition of arabinose had no effect on bacterial growth (Fig. 1a–e).

We considered that the lack of phenotype after the addition of arabinose could be due to the presence of a homologous TA system in the *E. coli* host, resulting in the inactivation of ectopically expressed toxin by the endogenous antitoxin. We constructed a *ΔhipBA* mutant of *E. coli* MG1655 (due to homology to the BPSS1584-1583 locus) and retested the phenotype following induction of

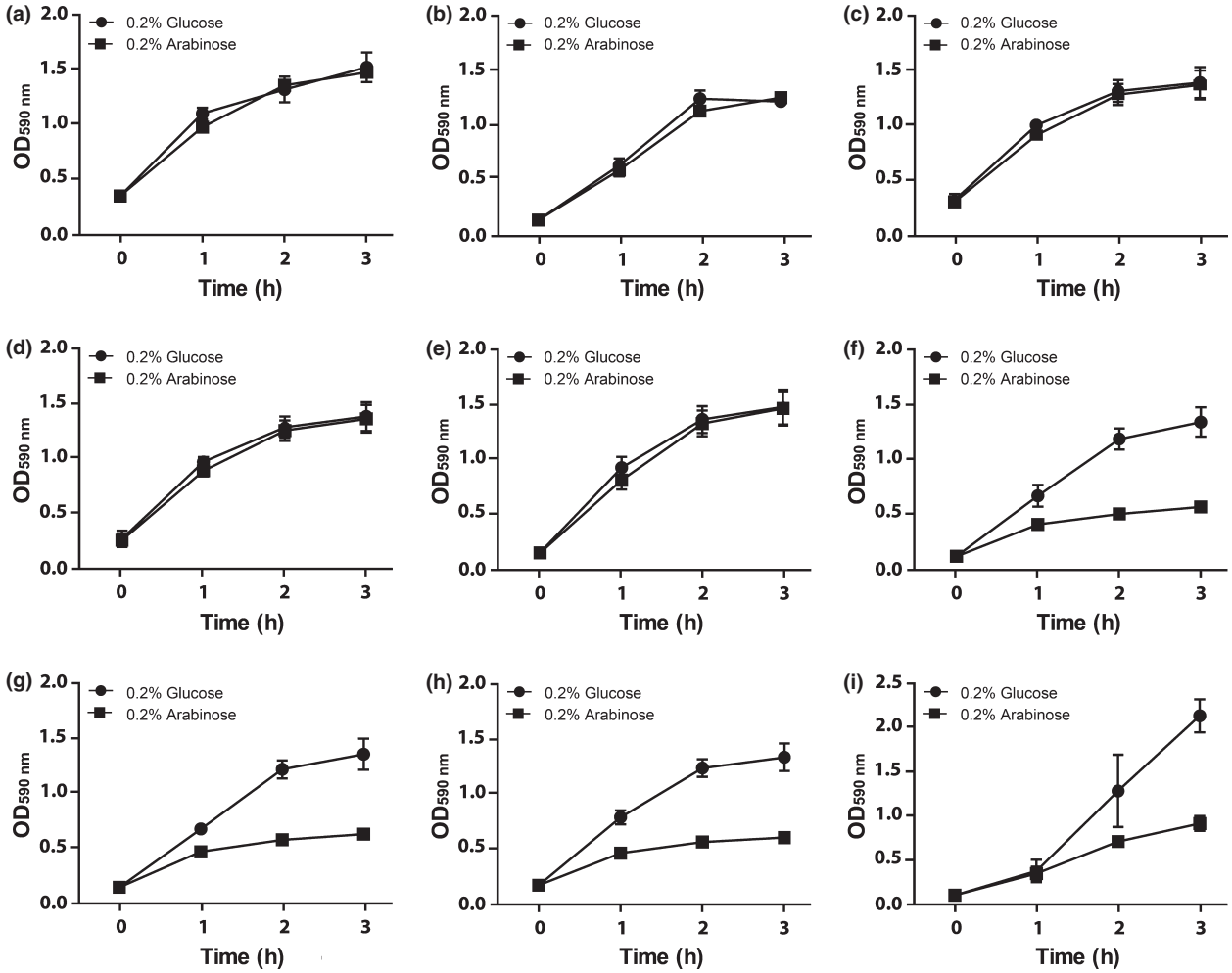

Fig. 1. Effect of putative toxin expression on optical density. The eight putative TA toxins were cloned into the pBAD vector and expressed in *Escherichia coli* MG1655 (a) BPSS1584 (b) BPSS0395 (c) BPSL0559 (d) BPSL2333 (e) BPSL3261 (f) BPSS1060 (g) BPSS0390 (h) BPSL0175 (i) *E. coli ΔhipBA* pBAD-BPSS1584. Cultures were grown to early log phase (OD_{590} ~0.2) before inducing expression with 0.2% (w/v) arabinose or repressing expression with 0.2% (w/v) glucose. The optical density was measured every hour for 3 h. The data shown are the average of three biological repeats. Error bars show SEM.

BPSS1584 (*hipA*) expression in this strain. Induction in this mutant resulted in a marked reduction in growth rate assessed by a change in $OD_{590\,nm}$ (Fig. 1i). We could not identify *E. coli* MG1655 genes encoding proteins with homology to BPSL0558-0559, BPSL2333-2334, BPSL3260-3261 or BPSS0394-0395.

relE1 or r*elE2* expression inhibits growth of *E. coli* MG1655, whereas *hicA* or *hipA* expression inhibits growth and reduces the number of culturable cells

The number of culturable bacteria following induction (arabinose) or repression (glucose) of toxin expression was next determined. One hour after the induction of *relE2* or *relE1* expression, the cultures had continued to grow (Fig. 2a and b). However, 2-h postinduction, the number of culturable bacteria no longer increased. In contrast, cultures to which glucose had been added continued to grow. After the induction of *hicA* expression, the number of culturable bacteria reduced over the next 3 h by approximately 10 000-fold (Fig. 2c). In contrast, the glucose-repressed culture grew to stationary phase. After the induction of *hipA* expression in *E. coli* Δ*hipBA* (Fig. 2d), the number of culturable bacteria reduced by approximately 10 000-fold, whereas glucose-repressed culture continued to grow.

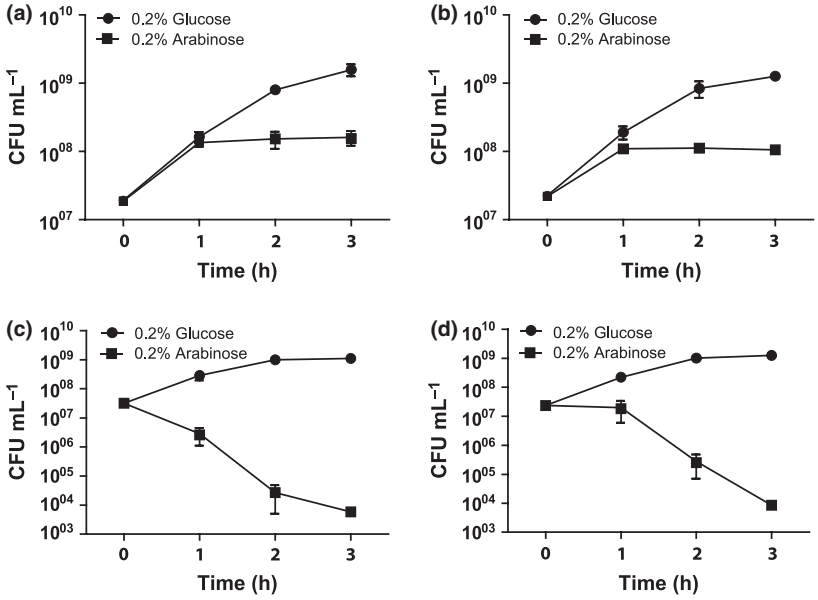

Fig. 2. Effect of toxin expression on culturability. Enumeration of *Escherichia coli* MG1655 harbouring plasmid-cloned *relE1* (a), *relE2* (b) or *hicA* (c) or *E. coli* MG1655 *ΔhipBA* harbouring *hipA* (d) monitoring culturable cells (CFU). Toxin expression was repressed or induced by adding 0.2% (w/v) glucose or arabinose, respectively, to cultures at t0. Data are the average of three biological repeats, and error bars show the SEM.

The reduction in number of culturable cells following *hicA* and *hipA* expression is not the result of cell death

To confirm that the expression of *hicA* does not cause cell death, LIVE/DEAD staining was carried out on bacteria from cultures of *E. coli* MG1655/pBAD-*hicA*. Over 90% of bacteria fluoresced green when taken from MG1655/pBAD-*hicA* cultures grown for 3 h in the presence of glucose or arabinose. Similar results were obtained when cells were imaged from cultures of MG1655 *ΔhipBA*/pBAD-*hipA* strain in which *hipA* expression was repressed or induced with glucose or arabinose, respectively.

Co-expression of the toxin with cognate antitoxin sequesters activity

We next identified the cognate antitoxins for *hicA*, *relE2* and *hipA* [*hicB* (BPSS0390), *relB2* (BPSL0174) and *hipB* (BPSS1583), respectively] and expressed these genes from an IPTG-inducible promoter in a plasmid, which could be co-maintained alongside pBAD in *E. coli* MG1655. As *relBE1* is paralogous to *relBE2*, only *relBE2* was further experimentally validated in this study.

As expected, 2 h after the induction of expression of *relE2*, there was a significant difference in the number of culturable *E. coli* MG1655 compared to the uninduced control (Fig. 3a). The addition of IPTG to cultures of *E. coli* MG1655 containing the plasmid-cloned *relB2* antitoxin had no effect on the number of culturable bacteria. When arabinose and IPTG were added to cultures of *E. coli* MG1655 containing both the plasmid-cloned *relB2* and the plasmid-cloned *relE2*, the number of culturable bacteria was similar to that in control cultures of *E. coli* MG1655.

A similar pattern was seen when *hicA* and *hicB* were expressed individually or co-expressed (Fig. 3b). The expression of *hicA* toxin resulted in a reduction in culturable bacteria in comparison with control cultures. This reduction could be blocked by co-expressing the *hicB* antitoxin. Co-expressing the cognate *hipB* antitoxin with *hipA* in *E. coli* Δ*hipBA* could only partially block the reduction in culturable bacteria compared to *hipA* expression alone but this difference was significant (Fig. 3c).

hicB, *relB2* and *hipB* expression can resuscitate growth after toxin expression

Next, we determined whether the cognate antitoxin could rescue growth after toxin-induced arrest. The expression of *hicA* toxin reduced the number of culturable bacteria by approximately 10 000-fold (Fig. 4a). However, 2 h after adding IPTG to induce expression of the *hicB* antitoxin, the number of culturable bacteria was restored to the level at the start of the experiment (approximately 10^7 CFU).

In contrast, expression of the *relB2* antitoxin was unable to fully reactivate growth over the 2-h time period (data not shown). Therefore, we next investigated whether washing the cells to remove arabinose before adding IPTG would improve recovery (Fig. 4b). Under these conditions, we saw recovery and an increase in cell numbers by 2-h postinduction of antitoxin.

Next, we investigated the potential for *hipB* to recover the culturability of cells in which *hipA* had been

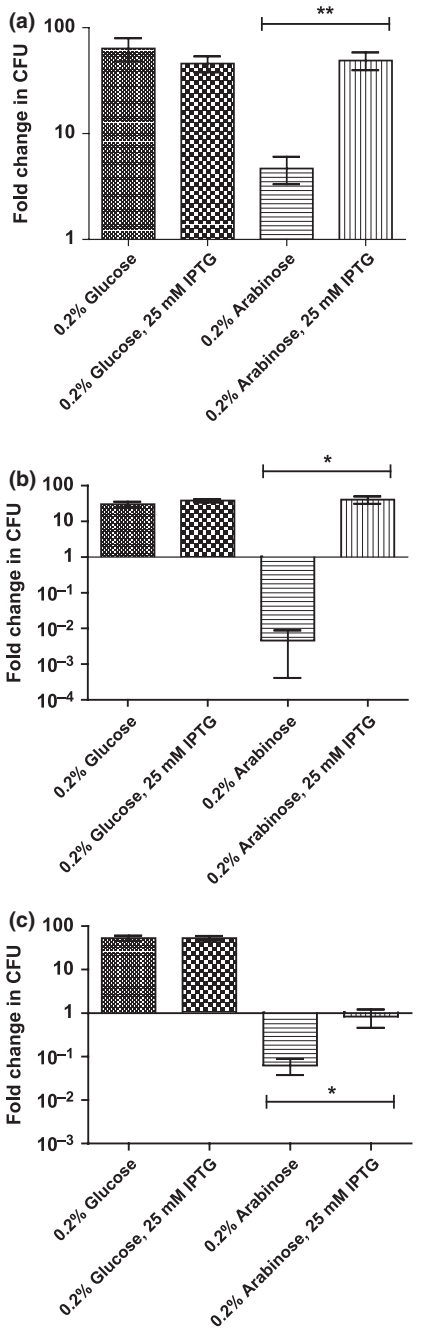

Fig. 3. Co-expression of cognate toxin and antitoxin pairs. Change in the number of culturable cells (CFU) of *Escherichia coli* MG1655 harbouring (a) plasmid-cloned toxin (*relE2*) and the cognate plasmid-cloned antitoxin (*relB2*); (b) plasmid-cloned toxin (*hicA*) and the cognate plasmid-cloned antitoxin (*hicB*); (c) *E. coli* MG1655 *ΔhipBA* harbouring plasmid-cloned toxin (*hipA*) and the cognate plasmid-cloned antitoxin (*hipB*). The results shown are the fold change in number of culturable bacteria 2 h after inducing expression of toxin, antitoxin or both with arabinose, IPTG or arabinose and IPTG, respectively. Glucose was added to control cultures. Data shown are the mean of three biological repeats, and error bars show SEM. $*P < 0.05$ for an unpaired two-tailed Student's *t*-test. $**P < 0.01$.

expressed. When cultures were washed to remove arabinose and then IPTG was added, the cells were able to re-grow to levels comparable to the number at the start of the experiment (Fig. 4c).

The reduction in cell numbers following *hipA* expression is cell density dependent

Previously, Tashiro *et al.* (2012) have shown that the over-expression of *relE* consequently reduced the number of culturable bacteria and that this effect was more pronounced at high cell densities. Therefore, we tested the effect of cell density on the reduction in culturability of MG1655Δ*hipBA* expressing *hipA*. Cells were grown to an $OD_{590\,nm}$ of 0.1 before inducing at different cell densities (Fig. 5). At a starting density of 10^7 CFU, cell numbers declined over the course of the experiment. At a starting density of 10^6 or 10^5 CFU, cell numbers increased for 2 h and then decreased after this time period.

Discussion

The role of TA systems has been widely debated in recent years, and there is increasing evidence that some TA systems are involved in persistence and cellular dormancy (Moyed & Bertrand, 1983; Dörr *et al.*, 2010; Rotem *et al.*, 2010; Maisonneuve *et al.*, 2011; Tashiro *et al.*, 2012). This has resulted in an increasing interest in this area due to the link with persistence and chronic and untreatable infections (Lewis, 2010; Fauvart *et al.*, 2011).

Candidate TA systems can be identified using programmes such as RASTA (Sevin & Barloy-Hubler, 2007; Goulard *et al.*, 2010). The confirmation that these TA systems are functional might be achieved by assessing the phenotype of TA system mutants. However, several workers have reported that the inactivation of individual TA systems in *E. coli* does not provide an obvious phenotype (Shah *et al.*, 2006; Tsilibaris *et al.*, 2007), possibly because of redundancy of TA systems (Tsilibaris *et al.*, 2007). Indeed, there is now evidence that the inactivation of multiple TA systems is required to reveal clear phenotypes (Maisonneuve *et al.*, 2011).

An alternative approach is to over-express candidate toxins. Over-expression in the native host may not reveal a phenotype unless the genes encoding cognate antitoxin (s) are also inactivated because they may mask the phenotype (Budde *et al.*, 2006). We chose to express the toxins in *E. coli*. Even in this host, our results indicate that a homologue of the cognate antitoxin can mask the phenotype.

Our results indicate that 4 of 8 candidate toxins possessed activity in *E. coli*. The remaining four genes may not encode toxins or expression of the toxins may not

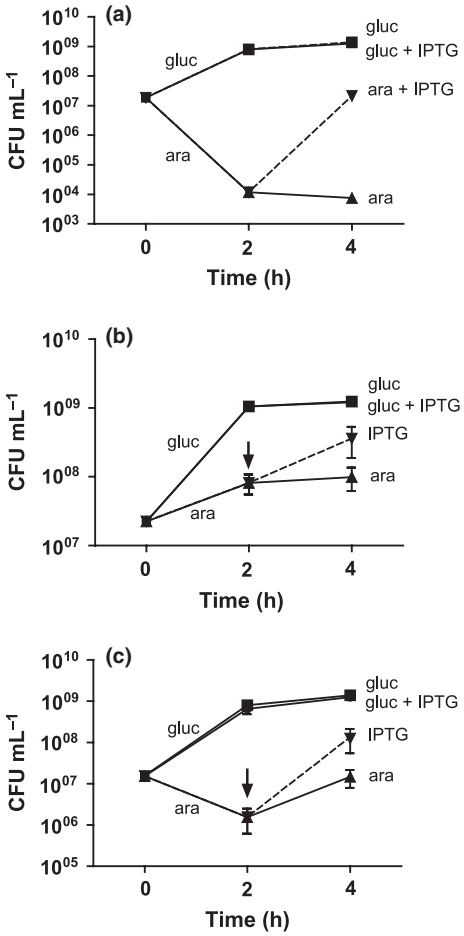

Fig. 4. Resuscitation by antitoxin. Culturable cells (CFU) were enumerated at the time shown. (a) *hicA* (toxin) expression was induced with arabinose, and 2 h later, *hicB* (antitoxin) expression was induced with IPTG. (b) *relE2* (toxin) expression was induced with arabinose, and 2 h later, the cells were washed, re-suspended in fresh medium and *relB2* (antitoxin) expression was induced with IPTG. (c) *hipA* (toxin) expression was induced with arabinose, and 2 h later, the cells were washed in fresh medium and *hipB* (antitoxin) expression was induced with IPTG. Arrows indicate wash steps. The data shown are the average of three biological repeats, and error bars show SEM.

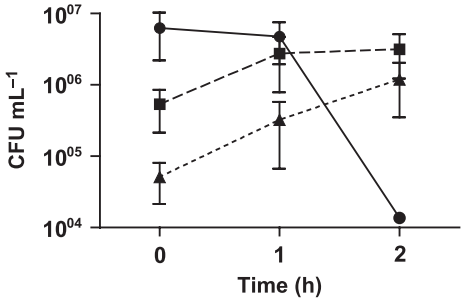

Fig. 5. Effect of the cell density on culturability after toxin induction. Change in the number of culturable *Escherichia coli* MG1655 *ΔhipBA* pBAD-*hipA* cells after the addition of arabinose. Cultures at three different starting densities were used. Expression was induced at t0, and cell numbers were monitored over a subsequent 2-h period. The data shown are averages of three biological repeats, and error bars show SEM.

have been induced under the condition tested. The active toxins elicited two distinct phenotypes in *E. coli*. Some (RelE1 and RelE2) caused the apparent cessation of growth, with no change in the number of culturable cells. Others (HicA and HipA) resulted in a rapid decline in the number of cells, which were culturable on agar and formed colonies. Of the eight putative TA systems identified, five were located on genome islands including HicAB, RelBE1 and RelBE2. Four of these GIs [2, 3, 13 and 15] encode bacteriophages or putative prophages. For example, GI 2 (RelBE2) encodes phiK96243. A homologous RelBE system is also located in the bacteriophage phiE12-2, isolated from *B. pseudomallei* E12-2. This suggests that TA systems could represent a mechanism for maintaining prophages that are integrated within the bacterial chromosome. Sim *et al.* (2008) have previously shown that HicAB and RelBE1 are on GIs associated with clinical strains of *B. pseudomallei*. This also suggests that these TA systems may play a role in human disease. The molecular targets of the toxins have not yet been determined. However, RelE1 and RelE2 show 54% sequence identity with RelE from *K. pneumonia*. The RelE toxin targets mRNA in a ribosome-dependent manner (Yamaguchi *et al.*, 2011). HicA shows 78% sequence identity with HicA from *A. baumannii*. The HicA toxin targets mRNA, independent of translation (Jorgensen *et al.*, 2008). HipA shows 40% sequence identity with *E. coli* HipA, which targets elongation factor thermal unstable (Schumacher *et al.*, 2009).

We have also shown that the toxin-induced stasis or nonculturability of bacteria could be reversed following expression of the cognate antitoxin. Our results are similar to those reported by Jorgensen *et al.* (2008) who showed similar reduction in the culturability of *E. coli* when HicA was over-expressed and the ability of HicB to rescue cells. This phenotype has also been observed with the RelBE system and with a MazEF homologue ChpAK/AI (Pedersen *et al.*, 2002) when cells in a state of dormancy could be rescued by antitoxin expression though the recovery of translation and/or replication. Amitai *et al.* (2004) also observed this resuscitation phenotype with the MazEF system. However, 8 h after MazF expression, MazE was no longer able to restore growth (Amitai *et al.*, 2004). Similar results were reported for HigBA: the longer HigA was expressed, the lower the ability of HigB to counteract toxicity (Budde *et al.*, 2006).

Cell density has a profound impact on the magnitude of the toxin-induced response. Tashiro *et al.* (2012) have

previously shown a population effect when expressing RelE in *E. coli*. They attribute this to an unidentified dormancy factor that is heat liable and accumulates during stationary phase. *Escherichia coli* MazF toxin has also been shown to have population-density regulated activity involving a small peptide named the extracellular death factor. This factor is at optimum levels during mid-log phase and is needed for the cell death activity via the MazF toxin (Kolodkin-Gal *et al.*, 2007; Kolodkin-Gal & Engelberg-Kulka, 2008). Leung and Levesque have shown that the competence-stimulating peptide pheromone is involved in producing persister cells in *Streptococcus mutans* (Leung & Lévesque, 2012). All of these studies reveal that cellular dormancy, persistence and the action of TA toxins involve communication between members of the bacterial population, perhaps as an insurance policy or act of altruism to protect the population as a whole from stress. Further investigation is needed into the molecular basis for the cell density-dependence effect on CFU reduction following *hipA* expression.

Acknowledgements

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