Characterization of the BolA Homolog IbaG: A New Gene Involved in Acid Resistance

Guinote, Inês Batista¹², Ricardo Neves Moreira¹, Patrick Freire¹², and Cecília Maria Arraiano¹*

¹ Instituto de Tecnologia Química e Biológica/Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal
² Laboratório Nacional de Investigação Veterinária - INRB, Estrada de Benfica 701, 1549-011 Lisboa, Portugal

Received: July 18, 2011 / Revised: November 7, 2011 / Accepted: November 30, 2011

BolA protein homologs are widely distributed in nature. In this report, we have studied for the first time YrbA, the only BolA homolog present in Escherichia coli, which we have renamed ibaG. We have constructed single and multiple ibaG mutants, and overexpressed ibaG in wild-type strains, in order to characterize this gene. The ibaG phenotypes are different from the bolA-associated round morphologies or growth profiles. Interestingly, ibaG and bolA single- and double-deletion mutants grow faster and have higher viabilities in rich media, whereas the overexpressed strains are significantly growth impaired. However, the mutant strains have lower viabilities than the wild type in the late stationary phase, indicating that both bolA and ibaG are important for survival in difficult growth conditions. bolA, as a transcription factor, binds to some promoters, but ibaG does not interact with the same DNA regions. We have determined that ibaG is transcribed in an operon with the murA gene, involved in the synthesis of peptidoglycan precursors. ibaG was also seen to change its mRNA expression pattern in response to acidic stress. ibaG may thus represent a new gene involved in cell resistance against acid stress.

Keywords: yrbA, BolA, acid stress

BolA protein homologs are widely distributed in nature, with the exception of Gram-positive bacteria and some organisms that have several genome copies. These proteins seem to be involved in protection from stress and cell proliferation, or cell-cycle regulation [18]. The overall topology of a mouse BolA-like protein is similar to the class II KH fold [1]. Interestingly, all the conserved residues in BolA-like proteins are assembled on one side of the protein [17]. E. coli BolA is included in the list of transcriptional factors [23]. It exhibits a helix-turn-helix motif that may correspond to a DNA-binding domain, through which it can eventually interact and transcriptionally regulate different genes [1]. In agreement with this hypothesis, BolA has been shown to repress the actin-like E. coli protein MreB [12] and to induce the DD-carboxypeptidases PBP5 and PBP6 [15, 33]. When bolA is overexpressed, the cells reduce in size and present a spherical morphology [3, 32, 33]. Rod to sphere shape modulation occurs in the range of exponentially growing to stationary phase cells, in an FtsZ-dependent manner [3, 19]. BolA overexpression induces biofilm formation [34], and alters outer membrane properties, namely accessibility and sensitivity towards detergents and antibiotics [13]. These biochemical and physiological alterations may depend on the role of BolA regulation over inner membrane proteins [1].

The bolA gene is preceded by two promoters: bolA2p and bolA1p. The upstream promoter bolA2p is weak, constitutive, and dependent on σ70. The bolA1p expression is driven by σ5 and is a gearbox promoter, showing an activity inversely dependent on the growth rate [1–3]. BolA transcription increases substantially in the transition to the stationary phase [1]. The bolA1p promoter is also induced in the exponential phase in response to several stresses, such as heat, acid, oxidative, osmotic, and glucose depletion [3, 32]. At the post-transcriptional level, BolA is indirectly modulated by ribonuclease III, which increases rpoS mRNA and σ6 protein levels [11, 30]. Polyadenylation has been shown to reduce σ5 proteolysis, and consequently affects bolA [31]. Since the expression of bolA is considerably increased under stress, and quite promptly repressed when growth conditions become favorable, it is therefore suggested that this gene is involved in promoting general resistance mechanisms. In agreement, the bolA yeast homolog is a UV-inducible gene that accelerates spore formation, decreases the proliferation rate, enhances cell size, and confers UV resistance [18].

*Corresponding author
Phone: +351 214469547; Fax: +351 214469549;
E-mail: cecilia@itqb.unl.pt

# Supplementary data for this paper are available on-line only at http://jmb.or.kr.
Given the importance of BolA, we decided to look for homologs. In silico analyses (protein BLAST) were performed and a BolA homolog protein (YrbA) was found in E. coli. YrbA has an overall 23% amino acid identity, with a 58% similarity at the BolA/YrbA domain. In addition, over 70% of the amino acid residues of both the BolA and YrbA proteins can be aligned. In common with BolA, YrbA has a helix-turn-helix motif, usually responsible for protein–DNA interaction.

In this work, we have constructed a single isogenic yrbA mutant and double bolA/yrbA mutant and compared them with the wild type. The yrbA overexpression and characterization under several growth conditions were also performed, and the results have shown that this gene is responsive to acid stress. Therefore, we have proposed to rename yrbA as ibaG (influenced by acid gene), since this gene may represent a new factor involved in cell resistance against stress.

 MATERIALS AND METHODS

Bacterial Strains and Plasmids

The strains and plasmids used in this study are described in Table 1. MG1693 chromosomal DNA was used as template for ibaG, whereas the surrounding regions PCR utilized Pfu DNA polymerase (Fermentas, Vilnius, Lithuania) and the primer pair pCLON1/pCLON2 (Table S1). DNA templates were prepared using the genomic DNA purification kit from PUREGENE and DNA Cell & Tissue Kit Purification System from Gentra Systems (Minneapolis, USA). Both Pfu and the portion of genome amplified contained the AatI and PstI restriction sites that were separately digested. The 5319 bp plasmid fragment and the PCR fragment digestion were purified with the illustra GFX PCR DNA and Gel Band Purification Kit from GE Healthcare (Little Chalfont, UK). Overnight ligation was performed with T4 DNA ligase (Roche, Basel, Switzerland) to produce the pBGA01 plasmid.

The bolA and ibaG deletions were transferred from the Keio collection deletant strains to the MG1693 strain. The Keio collection deletant strains were kindly provided by Keio University [4]. All deletion constructs from Keio were performed inserting a kanamycin resistance cassette while removing the genes, with the exception of the first and last few base pairs. This prevented frameshift and downstream genes expression, to be affected in the case of operon co-expressed genes. In this case, the ibaG (previously designated yrbA) gene was removed, leaving the upstream promoter region and the downstream essential gene expression unperturbed [4]. Gene transfer was achieved by P1-mediated transduction according to previously described methods [26]. For construction of the double-deletion mutant, the kanamycin resistance cassette (introduced to delete the bolA gene) was eliminated before the second transduction. The FRT (FLP recombination target) sites flanking the antibiotic resistance cassette were eliminated by recombination by the FLP recombinase encoded in the pCP20 plasmid that was transformed and then temperature cured, following the published protocol [9].

The plasmid pRM12 was constructed encoding the gfp (green fluorescent protein) gene (for green fluorescent protein) under the control of ibaG promoters, using the vector p536 [24]. To do so, the ibaG promoter was PCR amplified using the primers yrFw and yrRev (see Table S1). The resulting fragment was digested with CiaI (Fermentas, Vilnius, Lithuania) and ligated to the p363 fragment digested with the same restriction enzyme. When necessary, strains were transformed with

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)</td>
<td>DH5α + pCP20 (thermosensitive plasmid expressing recA, to RFFP excise the antibiotic cassette inserted in the genome)</td>
<td>Novagen commercial strain</td>
</tr>
<tr>
<td>BT340</td>
<td>MG1693 + pMAK580 (strain with plasmid overexpressing bolA)</td>
<td>Cherepanov and Wackernagel [8]</td>
</tr>
<tr>
<td>CMA40</td>
<td>MG1693 + pMAK580 (strain with plasmid overexpressing bolA)</td>
<td>Santos et al. [28]</td>
</tr>
<tr>
<td>CMA50</td>
<td>BL21 (DE3) + pPFA02 (plasmid overexpressing (His)6–BolA)</td>
<td>Freire et al. [9]</td>
</tr>
<tr>
<td>CMA63</td>
<td>BL21+p363 (plasmid encoding for GFP after multi cloning site to evaluate transcription levels of the genes after the promoters inserted)</td>
<td>Freire et al. [9]; Miksch et al. [25]</td>
</tr>
<tr>
<td>CMA83</td>
<td>MG1693 + pBr325 (Gibco Brl commercial plasmid)</td>
<td>This study</td>
</tr>
<tr>
<td>CMA84</td>
<td>MG1693 + pBGA01 (plasmid overexpressing yrbA[ibaG])</td>
<td>This study</td>
</tr>
<tr>
<td>CMA85</td>
<td>MG1693 ΔβhaG::Kan′ (yrbA[ibaG] deletion mutant; Keio derived)</td>
<td>This study</td>
</tr>
<tr>
<td>CMA86</td>
<td>MG1693 ΔbolA::Kan′ (bolA deletion mutant; Keio derived)</td>
<td>This study</td>
</tr>
<tr>
<td>CMA87</td>
<td>MG1693 ΔbolA (bolA deletion mutant without the kanamycin resistance cassette)</td>
<td>This study</td>
</tr>
<tr>
<td>CMA88</td>
<td>MG1693 ΔbolA::βhaG::Kan′ (double bolA &amp; yrbA[ibaG] deletion mutant)</td>
<td>This study</td>
</tr>
<tr>
<td>CMA89</td>
<td>MG1693+pRMA02 (plasmid expressing GFP downstream the yrbA[ibaG] promoter)</td>
<td>This study</td>
</tr>
<tr>
<td>CMA90</td>
<td>MG1693ΔbolA::Kan′ + pRMA02</td>
<td>This study</td>
</tr>
<tr>
<td>CMA91</td>
<td>BL21 (DE3) + pPFA02+pRMA02 (CMA50 with plasmid expressing GFP downstream the yrbA[ibaG] promoter)</td>
<td>This study</td>
</tr>
<tr>
<td>JW3157</td>
<td>MG1655::βhaG::Kan′ (Keio collection mutant for yrbA[ibaG])</td>
<td>Baba et al. [4]</td>
</tr>
<tr>
<td>JW5060</td>
<td>MG1655::bolA::Kan′ (Keio collection mutant for bolA)</td>
<td>Baba et al. [4]</td>
</tr>
<tr>
<td>MG1693</td>
<td>thyA715 (background strain)</td>
<td>Bachmann and Low [5]</td>
</tr>
</tbody>
</table>
plasmid pCP20 (commercial plasmid), pBR325 (commercial plasmid), pBGAl (this study), pMAK580 [3], or pRMA02 (this study). Transformations were carried out as previously described [29]. All plasmids and deletion mutants were confirmed by DNA sequencing at STAB Vida, Portugal.

Media, Growth Conditions, and Viability Evaluation
Luria broth (LB), M9, and Luria agar (LA) compositions were prepared as has been previously described [26]. When required, the media were supplemented with 0.4 mM thymine, 50 mg/ml chloramphenicol, 50 mg/ml kanamycin, 0.04% glucose (w/v) (all from Sigma-Aldrich, St. Louis, USA), and 1 mM IPTG (Apollo Scientific, Stockport, UK). For acid and osmotic challenge experiments, strains were grown in LB. Overnight growth cultures were diluted to an OD$_{620}$ of 0.08 and allowed to grow until an OD of about 0.5. At such a time all cultures were centrifuged at 5,500 × $g$ for 15 min at 4°C, to change the media into a buffered LB at a different pH or ionic strength. Tests were performed at pH 3, 4, and 5 in LB buffered with sodium citrate and LB plus citric acid, or LB with 85 mM, 175 mM, 350 mM, and 1,000 mM NaCl, according to what has been previously described [20]. Optical densities were measured in an Amersham Biosciences Ultrospec 500/1100pro spectrophotometer at 620 nm, using 10-mm-light-path cuvettes (GE Healthcare, Little Chalfont, UK). The ODs were determined according to the Lambert–Beer law’s limits of direct proportionality between OD and sample concentrations (dilutions were made in LB so that the density values would be read between 0.02 and 0.6); the phases of growth analyzed were determined according to the growth curves. Batch cultures were either launched from 16 h overnight inoculi at 30°C (for mild growth) or 37°C and 100 rpm, which were diluted to an optical density of 0.08 measured at 620 nm (OD$_{620}$). Cultures were grown aerobically at 37°C and 120 rpm. For nutritional stress evaluation, cells were grown in M9 supplemented with glucose until an OD$_{620}$ of 0.35 was reached, corresponding to the exponential phase (M9 Exp), and then washed twice in M9 without glucose with the growth resumed in the same glucose-depleted media for 60 min, corresponding to starvation (Starv 1 h). After 1 h of glucose starvation, cultures were replenished with glucose for some additional 15 min, corresponding to reversal (Rev 15 min). For evaluation of viability, the samples were processed in LB serial dilutions, and plated in LA. The number of colony-forming units (CFU) was counted and viability was determined according to the following equation:

$$\text{Number of dividing cells per ml} = \text{CFU} \times 10^{\text{dilution}} \times 1,000/100$$

Microscope Preparations
To observe the effect of IbaG (YrbA) on cell morphology, planktonic cells were harvested from cultures growing in LB, at the time points corresponding to the log, early exponential, late exponential, early stationary and late stationary phases, or from cultures grown in M9 at the exponential phase (M9 Exp), after 1 h of starvation (Starv 1 h), and after 15 min of reversal (Rev 15 min). Cells were fixed with 0.75% (v/v) formaldehyde and stored at 4°C. For the Differential Interference Contrast (DIC) microscopy photographs, 20 µl of the samples was observed in slides coated with a thin 1.5% (w/v) agarose film, and enclosed with nr.1 cover glass. Images were obtained using a DMRA microscope (Leica, Wetzlar, Germany) under Nomarski optics coupled to a CCD camera, with Metamorph software.

Overexpression and Purification of BolA Protein
BoLA overexpression using the pPFA02 plasmid and sequential purification was performed according to methods previously illustrated [15]. The plasmid used for the expression of BolA was a pET22a-derived pPFA02 [12] transformed into a Novagen E. coli BL21 (DE3) strain (Table 1). Purification of BolA was performed by histidine affinity chromatography using His Trap Chelating HP columns and an AKTA Fast protein liquid chromatography system (GE Healthcare, Little Chalfont, UK). Proteins were eluted with a continuous imidazol gradient (until 100 mM) and the buffer was exchanged to a 20 mM sodium phosphate (pH 7.4) and 50 mM NaCl buffer. The protein concentration was determined by spectrophotometry using a Nanodrop device measuring the OD at 280 nm. Ten µl of purified protein fractions was applied to a 15% SDS-PAGE and visualized by Coomassie blue staining to assess protein purity (data not shown).

Surface Plasmon Resonance (SPR) Analyses
SPR analysis was performed in a BIACORE 2000 instrument. Purified BolA protein was immobilized in a CM5 sensor chip by the amine coupling immobilization method according to the manufacturer’s instructions (GE Healthcare, Little Chalfont, UK). The same immobilization procedure was performed with the same molarity of BSA control protein in a reference flow cell, used to correct for refractive index changes and nonspecific binding [15]. The ibaG promoter and open reading frame were amplified by PCR using primers yrfw/YrRev and 3/5 primers, respectively (see Table S1). To create a fragment containing the ORF exclusively, the second PCR product was digested with NcoI, and purified with a Nucleic Acid and Protein Purification kit: NucleoSpin Extract II, from Macherey-Nagel (Düren, Germany). The promoter sequence of the mreBCD operon was used as a positive control, and the bolA open reading frame DNA-encoding fragment as a negative control, as has been previously described [12]. The assays were run at 25°C in 20 mM sodium phosphate, at a pH of 7.4, 1 mM dithiothreitol, and a 500 mM NaCl buffer, a method detailed previously [15]. Equilibrium constants were determined using the BIA Evaluation 3.0 software package, according to the fitting model 1:1 Langmuir binding, and χ$^2$ statistics were used to evaluate the fitness of the model to the data.

RNA Extraction and Probe Preparation
Culture samples were taken at the desired time points along the growth curve (OD$_{620}$ = 0.4, 1.7, and 5) or after imposition of osmotic or acidic stresses: 350 mM NaCl [27] or 30% HCl, lowering the pH at 7.2 to 4.4 [6] both at time 0 and 60 min. Total RNA was extracted as has been previously described [30]. In all experiments, 1 µl of the RNA samples was quantified by UV spectrometry with NANODROP and integrity verified. The probes used for bolA and ibaG in Northern blot experiments, spanned the entire transcriptional units and were obtained by PCR using Taq polymerase (Fermentas, Vilnius, Lithuania) with the primers at P2/X9 and 3/5, respectively, in the case of DNA probes, or P2/X9_T7 and 3/5_T7 in the case of RNA probes (see Table S1). Three µl of pUC Mix Marker 8 (Fermentas, Vilnius, Lithuania) was labeled with [$\gamma$-32P]ATP using PNK and 100-times diluted into the RNA loading buffer. Then 7.5 µl of labeled pUC 8 was run simultaneously with the samples to determine their molecular weight. All radioactive labels were cleaned in G-50 columns from GE Healthcare (Little Chalfont, UK).

746 Guinote et al.
Reverse Transcription PCRs (RT-PCR)

Reverse transcription PCRs (RT-PCRs) were carried out with 50 ng of total RNA, with a OneStep RT-PCR kit (Qiagen, Hilden, Germany), according to the supplier’s instructions, using the oligonucleotides RNM017 and 5 (see Table S1). As an independent control, the 16S rRNA-specific primers 16sF and 16sR were used. Prior to RT-PCR, all RNA samples were treated with a TURBO DNA-free Kit (Ambion, Life Technologies, Carlsbad, USA). Control experiments, run in the absence of reverse transcriptase reactions, yielded no product.

Northern Blot and Hybridization

Samples containing 15 µg of total RNA were dissolved in 90% formamide, 0.01 M EDTA (pH 7.0), 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue buffer [29], heated for 5 min at 100°C for denaturation, and incubated for 10 min on ice. Total RNA samples were electrophoresed run on a 6% denaturing polyacrylamide gel and transferred to a nylon membrane (Amersham Hybond-N+ nitrocellulose; GE Healthcare, Little Chalfont, UK) according to the procedure described by Fitzwater et al. [10]. The RNA was then fixed to the membrane by UV light and hybridized with the PCR probe radiolabeled with [α-32P]-dCTP, using the Multiprime DNA labeling system from Amersham (GE Healthcare, Little Chalfont, UK) or with [α-32P]-rUTP, using the Promega labeling system for riboprobes (Fitchburg, USA). Probe hybridization with a PerfectHyb Plus Hybridization Buffer 1×, was carried out at 42°C for DNA probes and 68°C for RNA probes. Amersham Hybond-N+ nitrocellulose membranes optimized for nucleic acid transfer from GE Healthcare (Little Chalfont, UK) were hybridized and washed as described by Sambrook et al. [29]. The results were visualized using the PhosphoImager System from Molecular Dynamics (Sunnyvale, USA).

Transcription Evaluation Using GFP as a Reporter

Transcription evaluation was analyzed with gfp as the reporter gene using the p363-derived vector pRMA02 [24]. BL21 + pPFA02 + pRMA02 was grown at 37°C, 120 rpm, until OD620 = 0.5 when the culture was split in two. Half the culture was added to 1 mM IPTG (to induce bolA expression) and the other to 0.04% glucose (to repress bolA expression). In a parallel experiment, MG1693 and the isogenic bolA deletant were grown until OD 1.7 (the mid-exponential transcripts evaluation time point). Total protein was extracted using Bugbuster reagent (Novagen, Merck, Darmstadt, Germany) and GFP fluorescence was quantified in a Varian-Eclipse Spectrofluorimeter. SDS-PAGE gels and Western blots were performed by methods detailed elsewhere [12]. Results were normalized and are shown in percentages (%) as the ratio of fluorescence/EF-Tu quantified in the Western blots. Final data represent the average plus standard deviation of fluorescence per cell, from at least three independent experiments.

Primer Extension Analyses

Total RNA was extracted as described above. The primer PExtYrbA was end-labeled using T4 polynucleotide kinase (Fermentas, Vilnius, Lithuania) and [γ-32P] ATP. Unincorporated [γ-32P] ATP was discarded using a MicroSpin G-25 column (GE Healthcare, Little Chalfont, UK). A total of 2 pmol of primer was annealed to 10 mg of RNA, and cDNA was synthesized using 200 U of Superscript III RT from Invitrogen (Life Technologies, Carlsbad, USA). M13 sequencing reaction was performed with a Sequenase Version 2.0 sequencing kit, according to the instructions manual. The primer extension products were separated in parallel with the M13 sequencing reaction on a 6% polyacrylamide sequencing gel containing 8M urea. The gel was exposed and signals were visualized in a PhosphorImager (Storm Gel and Blot Imaging System; Amersham Bioscience, GE Healthcare, Little Chalfont, UK).

Results and Discussion

IbaG is the Only BolA Homolog in E. coli

NCBI public resources were used to search for potential homologs of bolA and an uncharacterized gene was found to have a strong protein similarity with BolA, particularly...
considering the shared bolA/yrbA domain. The yrbA gene, renamed ibaG in this report, is located at 71.87 min of the E. coli genome, downstream of an operon with five genes co-directionally expressed (Fig. 1A). Nevertheless, ibaG is not predicted to be co-expressed with the upstream operon, but from its own s^0 single promoter region, as evaluated by the REGULON DB 6.7: Gene Form [14]. The upstream genes mlaBCDEF (plus mlaA) compose the Mla pathway, an ABC transport system whose function seems to prevent phospholipidic accumulation in the outer leaflet of Gram-negative bacteria, thus contributing to the preservation of outer membrane lipid asymmetry [21]. The essential murA gene is encoded downstream to ibaG. This vital gene encodes for the UDP-N-acetylglucosamine enolpyruvyl transferase, which synthesizes peptidoglycan precursors after N-acetylglucosamine acid and phosphoenolpyruvate [7, 16, 22]. All of these seven proteins are predicted to occur or be function-related to the outer membrane and are either essential, or significantly affect, the ability to resist against external injuries. The genes that occur in the proximity of ibaG, as well as the sequence homology that this gene has with bolA, prompted us to characterize this gene.

In order to study the ibaG transcriptional unit, Neural Network Promoter Prediction [28] software was also used to search putative promoters upstream of the ibaG ORF. One putative promoter region with a 0.7 score was found to match the ATG start codon already described in different databases. Primer extension reaction was performed in order to map the transcription start site of ibaG mRNA. The +1 nucleotide was determined and identified at 15 bp before the start codon of the ibaG mRNA (Fig. 2). A putative terminator has been described in REGULON DB 6.7: Gene Form [14]. Interestingly, this transcription terminator overlaps with the downstream murA gene. As a result, reverse transcription PCR (RT-PCR) was used to confirm if ibaG is present exclusively as a single transcriptional unit or if it is co-transcribed with the murA gene. We observed that ibaG is in fact transcribed in an operon with the murA essential gene (Fig. 1B). This relates ibaG to the metabolism of the murein precursors and might suggest a role for this gene in that process.

**ibaG is Not an Essential Gene and Both Single and Double bolA/ibaG Deletions Improve E. coli Growth**

In similarity with what occurs with bolA, the single deletion of ibaG is not lethal [4]. Moreover, if ibaG can complement any essential bolA functions or vice-versa, the double-deletion ΔbolAΔibaG would be expected to be lethal. However, the double-mutant strain is also viable. Growth, viability, and morphological analyses were performed to check the phenotypical effects due to the absence of these proteins. In the optimal growth conditions used, and contrarily to what had been anticipated, the deletion mutants grew similarly or better than the wild-type (wt) strain MG1693. Both ΔibaG and ΔbolAΔibaG deletion mutants grew approximately 20% times faster (as evaluated by the exponential phase rate of growth) and reached a higher OD than the wild type (Fig. 3A). These results were confirmed by the number of colony forming units obtained for the lag, early exponential, mid exponential, and late exponential/early stationary phases of growth (Fig. 3B).

The wt MG1693 strain formed about half, or even less, colonies than any of the deletion mutants, with the exception of the late stationary phase. In the beginning of the growth curve, and until the early stationary phase, all cultures revealed a viability increment. However, there was a transitory decrease in the viabilities of the ΔbolA strain at the mid exponential phase, perhaps when the stimulus for bolA expression was occurring. It is also possible to distinguish the deletion strains based on the colony forming units (CFU). The CFU were seen to be increasingly higher in the ΔbolA and ΔibaG mutants. Moreover, the ΔbolAΔibaG cell counts were even higher than the single mutants. The wt viabilities were quite stable along the entire growth curve. In contrast, the deletion strains largely reduced their cell counts in the late stationary phase, strengthening the idea that these genes may be important for survival in difficult growth conditions. Given that all deletion strains significantly increased their cell counts until the late stationary phase, the survival or tolerance that these genes provide at such a phase seems to have occurred at the expense of previous growth, as occurs in several other stress response genes. This seems to reflect the trade-off between growth and resistance related to the s^0 versus s^5 expression pattern. Eventually, the presence of both genes in the wild type exponential phase may reduce the exhaustion of important resources or prevent some catabolites from being produced and released to the media, favoring population maintenance in the late stationary phase (Fig. 3B).

Morphology assessments were made for wt and all deletion strains at the same time points for which viability had been evaluated. There were no noticeable changes in

---

**Fig. 2.** 5'-End mapping of ibaG transcriptional unit.

Reverse transcription with gene-specific primer was performed to extend the 5' region of ibaG mRNA. The first nucleotide of the transcript (+1) was identified with the aid of an M13 sequencing reaction and is represented in the above sequence.
BolA Homolog IbaG: A New Gene in Acid Resistance

489

morbidity (Fig. 4A). Given that bolA and ibaG may be involved in cell protection against stresses, morphologies were also analyzed in poor or nutritional stress conditions: M9 minimal media growth, 1 h of glucose starvation at the exponential phase, and 15 min of nutritional stress reversion by the re-addition of glucose. The results did not diverge according to strains, since the growth behavior of the deletion strains was very similar (Fig. 4B).

Morphologies Are Not Altered by an Increase in ibaG Copy Number

The deletion of bolA does not significantly affect the morphology of the cells during growth in LB. Nevertheless, when it is overexpressed, bolA does change the cell’s shape from rods to spheres [3]. To further pursue the characterization of ibaG, we cloned it into a pBR325-derived plasmid preceded by the respective promoter (pBGA01). Growth curves, viability assessment, and morphological studies were performed. The MG1693 strain transformed with pBR325 was used as an additional control or wt strain for all the other time points analyzed. In tandem to what was observed in the growth curves, viability results showed that the overexpression of ibaG by pBGA01 plasmid reduces colony counts and therefore this plasmid is in general detrimental, with the sole exception of the transition to stationary phase. Moreover, in the pMAK580 transformed strain, the viabilities were always quite low (Fig. 5B).

In E. coli, when the bolA morphogene is overexpressed, not only is cell division affected but the cells become rounder. Therefore, we evaluated the morphological phenotype caused by the presence of the plasmid pBGA01. Conversely to what occurs with bolA, no morphological changes were observed in the presence of ibaG overexpression (data not shown). Carbon depletion stress was also tested, but, only the pMAK580-transformed strain showed spherical morphologies; the pBGA01-transformed strain behaved similarly to the wild-type MG1693. Therefore, increased copy number of ibaG does not reduce the viabilities through a mechanism that involves morphological changes and thus cannot be monitored in that way; unlike bolA, ibaG is not a morphogene.

IbaG is Not Regulated by BolA but Seems to Require its Presence for Regular Transcription

According to the previous results, ibaG and bolA seem to concur in distinct pathways. Since BolA was shown to interact with the promoter regions of mreB, dacA, and dacC, by surface plasmon resonance (SPR), the same methodology was used to test the ability of BolA to recognize and interact with the ibaG promoter and the open reading frame regions. The results indicate that BolA has weak binding affinities to these nucleic sequences, and under these conditions BolA does not act as a direct transcriptional regulator of ibaG (Table 2). Nevertheless,
any indirect influence could not be detected by this experiment. Therefore, the dynamics of $ibaG$ transcription were evaluated using a GFP reporter gene fused with the $ibaG$ promoter region. The pRMA02 plasmid was constructed with $gfp$ being expressed according to $ibaG$ upstream promoter activity. In this methodology, the transcription activity of the cloned promoter(s) is measured by determination of GFP fluorescence per cell. Cells were transformed with pRMA02 and pPFA02 [12] ($bolA$ overexpression plasmid) and GFP fluorescence was measured. Transcription from the $ibaG$ promoter did not reveal significant variations due to increased levels of BolA. However, transcription levels of $ibaG$, measured by fluorescent GFP, were halved when $bolA$ was not induced (Fig. 6A). Hence, BolA seems to be required for the correct transcriptional activity of the $ibaG$ promoter. In order to confirm this hypothesis, wt and $bolA$ deletion isogenic strains were also transformed with pRMA02 plasmid and the transcription activity of the $ibaG$ promoter was evaluated in both backgrounds (Fig. 6B). The transcription activity of the $ibaG$ promoter in the $bolA$

![Fig. 4.](image)

**Fig. 4.** Differential interference contrast microscopy photographs, obtained using a DMRA microscope (Leica) for the strains MG1693, MG1693 $\Delta$bolA, MG1693 $\Delta$ibaG, and MG1693 $\Delta$bolA$\Delta$ibaG. (A) Evaluation at time points 210 and 1,420 min of the LB growth; (B) Evaluation at exponential phase, 1 h after induced starvation, and 15 min after reversion, in M9 minimal medium. The dark bar represents 5 µm.

<table>
<thead>
<tr>
<th>$\Delta k_{\text{prom}}$ (1/Ms)</th>
<th>$k_d$ (1/s)</th>
<th>$K_D$ (nM)</th>
<th>$K_A$ (1/nM)</th>
<th>$\chi^2$</th>
<th>$\Delta G^\circ$ (KJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$ibaG$ prom</td>
<td>285</td>
<td>1.00E-05</td>
<td>0.028</td>
<td>35.20</td>
<td>0.99</td>
</tr>
<tr>
<td>$ibaG$ ORF</td>
<td>616</td>
<td>1.04E-05</td>
<td>0.059</td>
<td>16.90</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Equilibrium constants ($K_D$) were determined by surface plasmon resonance using BIACORE2000 and according to the 1:1 Langmuir Binding Model. $k_a$ is the association rate constant, $k_d$ the dissociation rate constant, and $K_D$ the equilibrium dissociation constant of the reaction. $\chi^2$ was the statistics used to measure the fitness of the model to the data. $\Delta G^\circ$ values were determined according to the van’t Hoff equation: $\Delta G^\circ = RT \ln K_D$, where R and T are the universal gas constant and absolute temperature.
mutant decreased 30% in the mid exponential phase and 70% in the stationary phase. When \( \text{bolA} \) is physiologically expressed at lower levels, the difference of \( \text{ibaG} \) transcription between the wild type and the \( \text{bolA} \) deletion strain is much lower than in the stationary phase, when the expression of \( \text{bolA} \) is physiologically more significant. \( \text{bolA} \) is therefore shown to be necessary to maintain regular \( \text{ibaG} \) transcriptional levels, with a strong emphasis in the stationary phase of growth, when \( \text{bolA} \) is normally present in increased concentrations.

\( \text{ibaG} \) mRNA Expression Responds to Acid Stress

The \( \text{bolA} \) and \( \text{ibaG} \) patterns of expression along the growth curve and upon the imposition of stresses were evaluated by Northern blot. When the \( \text{bolA} \) probe was used, we could observe that the \( \text{bolA} \) mRNA levels only increased in the strain transformed with pMAK580, the \( \text{bolA} \) overexpression plasmid, and the transcript was absent in the deletion strain, as expected. The wild-type strain, the single \( \text{ibaG} \) deletant mutant, the strain with the control vector pBR325, and the strain with the \( \text{ibaG} \) overexpressing pBGA01 plasmid showed approximately the same levels of \( \text{bolA} \) mRNA. We can conclude that increased \( \text{ibaG} \) levels do not influence the regulation of the \( \text{bolA} \) gene (Fig. 7A).

When we used an \( \text{ibaG} \) probe for equivalent membranes, we could not detect a signal (data not shown). Nevertheless, expression of the \( \text{ibaG} \) gene was possible to evaluate in the strain transformed with the \( \text{pBGA01} \) plasmid, where the gene was still controlled by its own promoter but in a higher copy number (Fig. 7B). This plasmid was constructed with the \( \text{ibaG} \) gene plus the 305 bp region. The \( \text{ibaG} \) gene should be of about 320 bp in size. The presence of an additional band of about 625 bp in the \( \text{pBGA01} \) plasmid-
transformed strain further confirmed the co-transcription of *ibaG* and *murA*. Since pBGA01 is derived from pBR325, its copies were seen to be about five times higher in the stationary phase. However, the levels of *ibaG* transcripts were nevertheless the lowest in the stationary phase, which means that the gene is almost absent at this growth phase. The highest expression of *ibaG* occurs at the mid exponential phase, when *bolA* mRNA levels start increasing.

Two different stresses were checked, osmotic and acid stress. When osmotic stress is imposed, *ibaG* expression is not shut down, but mRNA seems to be present in significantly lower levels as compared with standard growth or acidic conditions. When cells are challenged with hydrochloric acid stress, *ibaG* levels increase and a different mRNA pattern can be observed (Fig. 7B). Since *ibaG* mRNA expression responds so strongly to acid stress, we decided to rename the formerly titled *yrbA*, as *ibaG* for “*influenced by acid gene*.”

**IbaG** Favors Growth in Acidic Conditions

Since *ibaG* was postulated to be involved in survival or growth enhancement in acid conditions, we monitored the growth of the strains with different backgrounds upon acid stress and compared it with osmotic stress. No differences were notable between genotypes when different osmotic pressures were applied (data not shown). The evaluation for acid challenges also did not differ for pH 3 and 4; in these extremely acidic conditions, all the *E. coli* strains stopped growing (data not shown). However, when neutral cultures were switched to LB at pH 5, the strain overexpressing *ibaG* grew better than the wild type and, conversely, the deletion strain was more sensitive to acid stress (Fig. 8). Between 180 and 240 min after the stress challenge, the ODs gradually diverged from that time point in an inverted pattern to that which occurred at a neutral pH in LB. The strain overexpressing *ibaG* was observed to grow significantly better than the mutant or even wild-type strains. Therefore, *ibaG* is shown to be involved in tolerance against mild acid environments (pH 5). *IbaG* mRNA increased in the exponential phase, upon acid stress imposition, and was shown to contribute to *E. coli* tolerance against acid stress.

**Final Remarks**

In this report, we have shown that *IbaG* (formerly *YrbA*) is an *E. coli* BolA homolog with significant amino acid sequence similarity. However, this homolog has been shown to possess different characteristics. Both the *bolA*, *ibaG* and the *bolA/ibaG* mutants were noted as being viable. The single *ibaG* (as the double) deletion mutant grew better than the wild type, but strains with increased levels of *ibaG* did not grow well and had a decreased viability. Most of the *bolA* known cell phenotypes are not reproduced by *ibaG*. *IbaG* levels do not affect *bolA* transcript levels. Conversely, *ibaG* is only properly transcribed when BolA is present. We have determined that *ibaG* is transcribed in an operon with the *murA* gene. Interestingly *ibaG* is responsive to acid stress, and was thus named *ibaG*, “*induced by acid gene*.” Upon pH 5 acid challenge, the *ibaG* overexpression strain grew better than the wt and the *ibaG* deletion strain, indicating that this gene is involved in resistance and survival against acid stress. Further studies of the *ibaG* role are of major interest since this gene may represent a new important factor involved in cell resistance against stress.

**Acknowledgments**

We thank Keio University [4] for all their kindness in providing us the deletion strains for *bolA* and *ibaG*. I.B.G. and R.N.M. were recipients of Doctoral fellowships from FCT (Fundação para a Ciência e Tecnologia - Portugal). We would also like to thank Andreia Aires for her technical support in the laboratory. Work at ITQB was supported by grants from Fundação para a Ciência e Tecnologia and grant Pest-OE/EQB/LA0004/2011, also from FCT.

**References**


