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Stationary phase protein overproduction is a fundamental capability of *Escherichia coli*☆

Jingxing Ou,[a,1] Lei Wang,[a,1] Xiaolan Ding,[b] Jianyang Du,[a] Yuan Zhang,[a] Huiping Chen,[a] and Anlong Xu[a,*]

[a] *Key Laboratory of Genetic Engineering of Ministry of Education, Department of Biochemistry, College of Life Sciences, Sun Yat-Sen (Zhongshan) University, Guangzhou, 510275, People's Republic of China*
[b] *State Key Laboratory of Biomembrane and Membrane Biotechnology, Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, People's Republic of China*

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Abstract

Although *Escherichia coli* is well studied and various recombinant *E. coli* protein expression systems have been developed, people usually consider the rapid growing (log phase) culture of *E. coli* as optimum for production of proteins. However, here we demonstrate that at stationary phase three *E. coli* systems, BL21 (DE3)(pET), DH5α (pGEX) induced with lactose, and TG1 (pBV220) induced with heat shock could overexpress diversified genes, including three whose products are deleterious to the host cells, more stably and profitably than following the log phase induction protocol. Physical and patch-clamp assays indicated that characteristics of target proteins prepared from cultures of the two different growth phases coincide. These results not only provide a better strategy for recombinant protein preparation in *E. coli*, but also reveal that rapid rehabilitation from stresses and stationary phase protein overproduction are fundamental characters of *E. coli*.

Keywords: Stationary phase; Protein overproduction; *Escherichia coli*

As a model organism, *Escherichia coli* has been the subject of elaborate studies for decades, which render us a more and more vivid biological portrait of *E. coli*; on the other hand, this Gram-negative bacterium can efficiently overexpress many heterologous proteins, prompting scientists to set up a lot of *E. coli*-based protein expression systems and strategies [1–3].

Among the numerous systems, BL21 (DE3)(pET) is such a system that the λDE3 lysogenic host has a chromosomal copy of bacteriophage T7 RNA polymerase gene under the control of IPTG-inducible *lac*UV5 promoter. Target genes are harbored into the expression plasmids, such as pETs, under the control of T7 specific Φ10 or T7*lac* promoter and cannot be transcribed unless by T7 RNA polymerase [4,5]. For DH5α (pGEX), target genes can be fused to the 3′-end of that of *E. coli* glutathione *S*-transferase (GST) under the control of IPTG-inducible *tac* promoter. For TG1 (pBV220), target genes are under the control of heat-inducible tandem promoters $P_{\lambda_R}P_{\lambda_L}$ from λ phage [6]. Although inductive mechanisms of *E. coli* systems are various, rapid growing (log phase) culture of *E. coli* is conventionally preferred for protein preparation [7]. Such a protocol may be based on the knowledge that rapid growing cells are physiologically the optimum.

It has long been acknowledged that nutriments deprivation could lead to RNA degradation [8] and gene expression regulation [9] in growth-stagnated *E. coli* cells. During the last decade many efforts have been made on researches concerning bacterial activities at stationary phase, and very recently Loewe et al. [10]

☆ *Abbreviations:* TRX, *Escherichia coli* thioredoxin; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI-TOF, matrix-assisted laser desorption–ionization time-of-flight (mass spectrometry); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. CD, circular dichroism.

* Corresponding author. Fax: +86-20-84038377.
E-mail address: ls36@zsu.edu.cn (A. Xu).

[1] These authors contribute equally to this work.

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reported that in prolonged stationary phase the genome mutation rate in *E. coli* can be deleteriously high.

Nonetheless, here we report that after charges of stresses at early stationary phase, *E. coli* cells are still fit for recombinant protein overexpression, and by using the mild lactose instead of IPTG, both cell viability and high productivity can be simultaneously achieved for strains induced to synthesize toxic target products. Yields of target products from stationary phase cultures could increase by 121% or more in contrast with those from optimized log phase cultures. Sea anemone neurotoxin Hk16a [11] was prepared on a larger scale and subject to physical and initial patch-clamp assays, which revealed that proteins synthesized by stationary phase *E. coli* cultures are characteristically the same as those produced by optimized log phase cultures. Our work not only suggests a better strategy for recombinant *E. coli* protein expression, but also presents some useful knowledge on *E. coli* activities during early stationary phase.

Materials and methods

Strains and plasmids. Escherichia coli strain BL21 (DE3) (F^- *ompT* $hsdS_B$ (r_B- m_B-) *gal dcm* λ(*DE3*) (*lacI lacUV5-T7 gene* 1 *ind*1 *Sam*7 *nin*5)), original plasmids pET21a and pET22b were obtained from Novagen, USA. Strains DH5α (*supE44* Δ*lac*U169 (Φ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1), TG1 (K12 Δ(*lac-proAB*), *supE*, *thi*, *hsd*Δ5/*F′* [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) and plasmid pGEX series were from Amersham Biosciences, USA. Plasmid pBV220 was a gift from Institute of Virology, Chinese Academy of Preventive Medicine, and described elsewhere [6].

Plasmid pTRX was reconstructed from pET22b so that the original *pel B* leader gene and multiple-cloning-site (MCS) are substituted sequentially with *E. coli trxA*, a short ORF coding for linker N-Gly-SerGlySerGly-C, 6-His tag and a new MCS. Details were described elsewhere [11].

Media and chemical reagents. Recipe of Luria–Bertani (LB) medium was adjusted (bacto-tryptone 12 g; bacto-yeast extract 6 g; and NaCl 7 g) and used throughout the work. Tryptone and yeast extract were from Oxoid, England. Tris base, SDS, and IPTG were from Shanghai Sangon, China. All other chemicals were of analytic grade and from local manufacturers. The 1 M IPTG stock solution was filter-sterilized and lactose (40% w/v) was autoclaved. Lactose should be freshly prepared prior to use.

Protein expression procedures. Preliminary experiments obtained the respective optimum conditions for the seven genes' overexpression, such as pH, temperature, IPTG concentration, etc. (Table 1). For uniqueness, the pH of the cultures was adjusted to 7.0 before inoculation. Single colony was inoculated to LB medium and shaken-incubated overnight at 37° (TG1 at 30°) (16 h); subsequently appropriate amount of the overnight seed culture was transferred to fresh medium to obtain an OD_{600} of 0.05. Log phase *E. coli* cultures were processed following the classical protocols described by Sambrook and Russell [7] except that glucose was added to a concentration of 0.2% (w/v) at the time of induction to provide additional carbon and energy resource. Stationary phase cultures of TG1 (pBV220-Src I) induced with heat shock were provided with glucose to a concentration of 0.2% at the time of induction, while stationary phase cultures of other strains were supplied with 0.2% glucose before inoculation and induced with lactose. *E. coli* culture entering stationary phase could be reflected by constant optical density of the broth at 600 nm (OD_{600}) and the

Table 1
Strains, target proteins, and production conditions

Target proteins	Brief description	Strains and plasmids	Optimized conditions
Hk16a [11]	Sea anemone neurotoxin. Target fusion proteins can be cleaved by 3C protease	BL21 (DE3)(pTRX)	Highly soluble; induced with 0.1 mM IPTG/0.4% (w/v) lactose; optimized production at 21 °C for 10 h
PLA_2 [17]	Sea snake phospholipase A2. Fusion protein can be cleaved by bovine enterokinase	BL21 (DE3)(pTRX)	Weakly soluble; induced with 0.1 mM IPTG/0.4% (w/v) lactose; optimized production at 25 °C for 8 h
IL-10	Human interleukine-10. Fusion protein can be cleaved by bovine enterokinase	BL21 (DE3)(pTRX)	Soluble; induced with 0.1 mM IPTG/0.4% (w/v) lactose; optimized production at 25 °C for 8 h
Src I [18]	Sea anemone cytolysin. Fusion protein can be cleaved by bovine enterokinase	TG1 (pBV220)	Inclusion body, induced with heat shock at 42 °C for 4 h.
J374[a]	Apoptosis-related protein from *Scatephagus argus*	BL21 (DE3)(pET21)	Inclusion body, induced with 0.5 mM IPTG/2% (w/v) lactose; optimized production at 37 °C for 4 h
Cystatin[a]	Cysteine protease inhibitor from *Pasyatis akajei*	BL21 (DE3)(pET21)	Soluble; induced with 0.5 mM IPTG/2% (w/v) lactose; optimized production at 21 °C for 10 h
3C [19]	Human rhinovirus 3C protease fused to *E. coli* GST protein	DH5α (pGEX)	Highly soluble; induced with 0.5 mM IPTG/2% (w/v) lactose; optimized production at 25 °C for 8 h

[a] Genes were discovered and harboured into expression plasmids by Huiping, Chen (unpublished work).

increasing pH of the culture [12]. By altering the amount of glucose added before inoculation, the pH of *E. coli* cultures could stay in the range fit for protein expression [13] after growth stagnation.

Plasmid stability assay. We tested the stability of expression plasmids of Hk16a and PLA_2. Aliquots of 150 μl glycerol stocks were, respectively, inoculated to 150 ml LB (ampicillin$^-$, 0.2% gluose) and another 150 ml LB (ampicillin$^-$, glucose$^-$). Cultures were shaken-incubated at 37 °C overnight (16 h). The two cultures were each transferred to 150 ml of the counterpart medium to obtain an OD_{600} of 0.01 and incubated likewise. The inoculation → incubation cycle were repeated five times. One milliliter of the final cultures was collected and diluted appropriately. One hundred microliters of the diluted cultures were spread on LB and LB (ampicillin$^+$, 100 μg/ml) agar plates and incubated at 37 °C for 10 h. Plasmid stability (%) was calculated using the formula $(N_{A+}/N) \times 100$, where N_{A+} represents the number of colonies formed on the LB (ampicillin$^+$) plate and N the number of colonies on LB plate.

Protein purification procedures. Recombinant sea anemone neurotoxin Hk16a was produced and processed from flask cultures on a 5-L scale. Details were described elsewhere [11].

Characteristics assays on Hk16a prepared from the two different cultures. The accurate molecular mass (MM) of recombinant sea anemone neurotoxin was determined by matrix-assisted laser desorption–ionization time-of-flight (MALDI-TOF) mass spectrometry (REFLEX III, Bruker, Germany). The lyophilized samples of Hk16a were dissolved in 0.1% trifluoroacetic acid. Sinapinic acid was used as the matrix. The acceleration voltage was 25 kV.

Lyophilized samples of Hk16a were dissolved in ddH_2O to 1 mg/ml and transferred to quartz cuvettes of 1 mm path-length. Circular dichroism (CD) spectra (J710 spectropolarimeter, Japan) of the samples at the far-UV range (190–250 nm) were obtained to calculate the ratio of α helixes, β sheets or turns of the proteins using Yang's algorithm [14].

In patch-clamp assays, dissociation of hippocampal neurons from neonatal SD rats (2–7 days) was carried out according to the methods of Gong et al. [15]; sodium currents were recorded in the cell-attached configuration of the patch-clamp methods described by Hamill et al. [16]. Data were collected by Fetchex software (pCLAMP 8.0 package) and mean open-time histograms were fitted with two-exponential function and simulated by Histogen (pCLAMP 8.0 package).

Results

Stationary phase cultures have higher yields

In our routine recombinant protein expression processes with BL21 (DE3)(pET) system and complex media, considerable amount of target proteins might accumulate in uninduced control cultures when grown to stationary phase (Fig. 2, lane 'C'), which is unreasonable if elder *E. coli* cells were unfit for protein expression. Subsequently, 7 genes from various origins were harbored onto different *E. coli* expression plasmids via routine molecular cloning techniques to obtain recombinant strains as investigation samples (Table 1).

A typical growth curve of BL21 (DE3) control culture nurtured by LB/0.2% glucose medium and change of the culture pH is shown in Fig. 1. In our work, induction of protein overexpression occurred after 9 hours' incubation, which belonged to the early stationary phase judging by the curve. OD and pH of the cultures were assessed to assure that cultures were growth stagnated (Materials and methods, Protein expression procedures).

As it turned out, for TG1 (pBV220-Src I), heat shock significantly inhibited cell growth so that during foreign protein production phase the average growth rate for stationary and log phase cultures of the strain was only 0.05 and 0.17 h^{-1}, respectively. The stationary one surpassed the log one in both ultimate biomass and target protein productivity (Table 2 and Fig. 2, lane 'Src I'). Similar results were retrieved if TG1 was substituted by BL21 (DE3) or DH5α (data not shown).

For BL21 (DE3)(pET) and DH5α (pGEX), when induced with lactose, the average growth rate for stationary phase cultures varied from 0.04 to 0.11 h^{-1} (Table 2), indicating that in cases where complex media are preferred, growth arrest is due to carbon source exhaustion. In contrast, growth rate for log phase cultures supplied with IPTG and glucose could be up to 0.61 h^{-1}, but the ultimate cell density of the cultures was significantly lower than the former (Table 2). Stationary phase cells of the 6 strains had similar or slightly lower

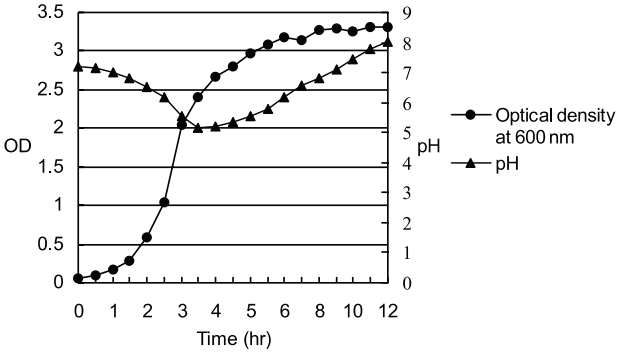

Fig. 1. Growth and pH curves of BL21 (DE3)(pTRX-Hk16a) cultivated in LB/0.2% glucose medium. The initial OD_{600} and pH values of the culture were 0.050 and 7.20, respectively; 150 ml of the culture was shaken-incubated (240 rpm) in 500 ml flask at 37 °C.

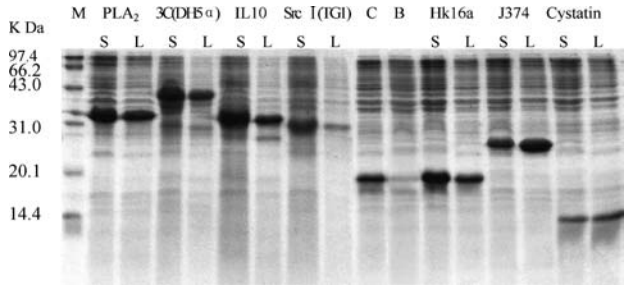

Fig. 2. Recombinant protein overexpression in stationary and log phase *E. coli* cultures. Total cell extract was presented. Lane 'C' for control culture of Hk16a allowed to grow to stationary phase, showing the level of 'leaky expression'; 'B' for glucose-added stationary phase cultures of Hk16a before induction, showing the alleviation of 'leaky expression'; 'S' and 'L' mean stationary and log phase cultures of the respective strains; and 'M,' protein marker comprises: rabbit phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; rabbit actin, 43.0 kDa; bovine carbonic anhydrase, 31.0 kDa; trypsin inhibitor, 20.1 kDa; and hen egg white lysozyme, 14.4 kDa.

Table 2
Change of cell density after protein production in stationary and log phase cultures

Cultures[a]	OD_{600} (stationary, before induction)	OD_{600} (stationary, end of production)	Average growth rate[b]	OD_{600} (log, before induction)	OD_{600} (log, end of production)	Average growth rate[b]
Hk16a	3.08	4.61	0.05	0.93	3.59	0.29
J374	3.37	4.82	0.11	0.65	2.24	0.61
Cystatin	3.30	5.44	0.06	0.78	1.00	0.03
IL-10	3.02	5.10	0.09	0.57	2.71	0.47
Src (TG1)	2.85	3.41	0.05	0.92	1.54	0.17
PLA_2	3.47	6.00	0.09	0.84	1.08	0.04
3C (DH5α)	1.86	3.19	0.09	0.58	1.12	0.12

[a] All cultures were of BL21 (DE3) cells unless specifically denoted.
[b] $(OD_{\text{end of induction}} - OD_{\text{before induction}})/(OD_{\text{before induction}} \times \text{production hours})$.

productivities of target proteins to those of log phase cells (Fig. 1).

Cystatin, PLA_2, and 3C protease are deleterious to the host. Log phase cultures producing these products had lower cell-densities and growth rates extremely stagnated to $0.03\,h^{-1}$ (Table 2). In cases where more than 0.5 mM IPTG was added to these cultures, severe cell lysis occurred (not shown). However, even when supplied with 2% lactose, the counterpart stationary phase cultures of the three strains had similar growth and biomass yield to those of other strains (Table 2).

Supplied with IPTG and glucose, stationary phase cells of the six strains could similarly overexpress target genes and cells producing the toxic proteins mentioned above would lyse when IPTG was added over 0.5 mM (not shown).

Sea anemone neurotoxin Hk16a (Table 1) was prepared from 5-L flask cultures of the two growth phases, cleaved by protease 3C, purified, and lyophilized. As much as 33.2 mg/L lyophilized toxins could be acquired from stationary phase cultures in contrast with that of up to 15.0 mg/L from log phase ones, which means an increase of 121% in specific volumetric yield, suggesting that our approach is much more efficient.

Plasmid stability was improved if glucose was added

One negative consequence of *E. coli* cultures entering stationary phase is that famine and the stress of synthesizing toxic products lead to plasmid instability [20]. However, by adding sufficient glucose to the medium (2 g/L LB in our work) prior to inoculation, BL21 (DE3) cells bearing the expression plasmids of Hk16a or PLA_2, respectively, comprised $(87 \pm 5)\%$ and $(83 \pm 5)\%$ of the cultures' overall population after five successive growth-saturating cultivations without antibiotic selection, in contrast to those of $(59 \pm 5)\%$ and $(23 \pm 5)\%$ where glucose was not added. This result is consistent with the previous report of Pan and Bruce [21].

Hk16a products from cultures of two growth phases were identical

Another worry may be whether characteristics of proteins synthesized by growth-arrested and stress-stimulated *E. coli* cells are the same as those produced by younger cells, as on the former occasion cells may be recovering from extensive repression of gene expression and degradation of mRNAs and rRNAs. In our case, before induction, *E. coli* cells were not only out of energy source but some also were subjected to heat or cold shock, which would further deteriorate the living circumstances.

Patch-clamp techniques have been successfully utilized in studies of interactions between sea anemone toxins and voltage-sensitive sodium channels in excitable cell membrane [22,23]. Sea anemone neurotoxin Hk16a has over 70% and 90% of its residues homologous to the well-identified anthopleurin B and C, respectively [11]. Thus, it is a good sample to study whether the characteristics of proteins synthesized by exponentially growing and stress-rehabilitating (carbon source exhaustion and temperature shifts in our work) *E. coli* cells are identical.

Purification results are presented in Fig. 3, which indicates identical behaviors of recombinant Hk16a prepared from stationary and log phase cultures.

The accurate molecular mass of Hk16a was determined by MALDI-TOF mass spectrometry. Products prepared from stationary and log phase cultures got results of 5011.6 and 5010.5 Da, respectively, both moderately deviating from 4999.6 Da computed by ProtParam on the website of ExPASy [24]. Thus, the errors were 0.24% and 0.22%, respectively.

The CD spectra of Hk16a prepared from two kinds of cultures were similar to each other (Fig. 4) and to that of anthopleurin B [22,23]. Calculated secondary structures of both products were as follows: 0% helix and turn; 71.1% β sheets and 28.9% random coils for product prepared from stationary phase culture, and 64.4% β sheets and 35.6% random coils for product from log phase culture. It is still unclear which kind of product

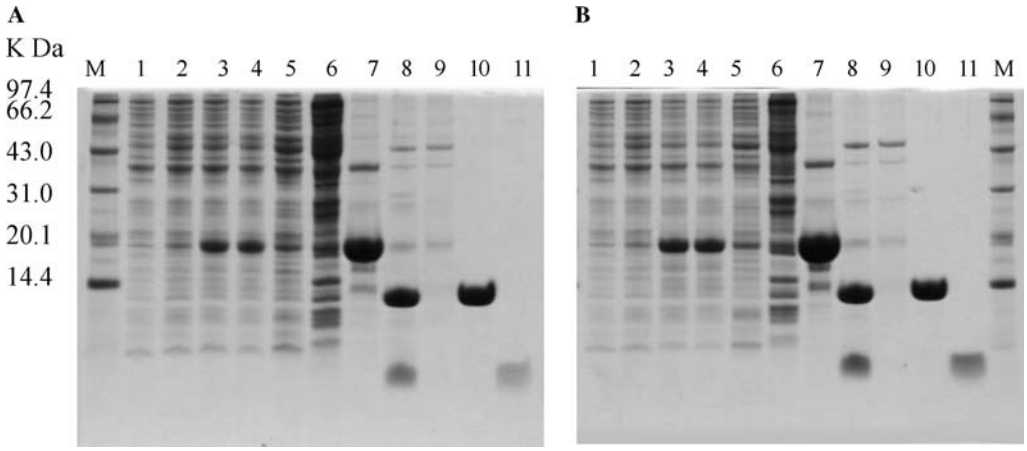

Fig. 3. SDS–PAGE presentation of purification results of sea anemone neurotoxin Hk16a. (A) Proteins produced by log phase cultures induced with IPTG; (B) proteins produced by stationary phase cultures induced with lactose. Lane 1, total cell extract of uninduced stationary phase culture; 2, total cell extract of culture before induction; 3, total cell extract of the culture after induction; 4, supernatant of the total cell extract; 5, flow-through fraction in Ni^{2+}-chelating chromatography; 6, fraction washed out by 100 mM imidazole in Ni^{2+}-chelating chromatography; 7, fraction washed out by 300 mM imidazole; 8, cleavage of the fusion proteins; 9, the first fraction eluted in gel-filtration chromatography; 10, the second fraction; and 11, target fraction (the third) pooled in gel-filtration chromatography, lyophilized, and re-dissolved in ddH_2O or other solvents to proper concentration; M, marker.

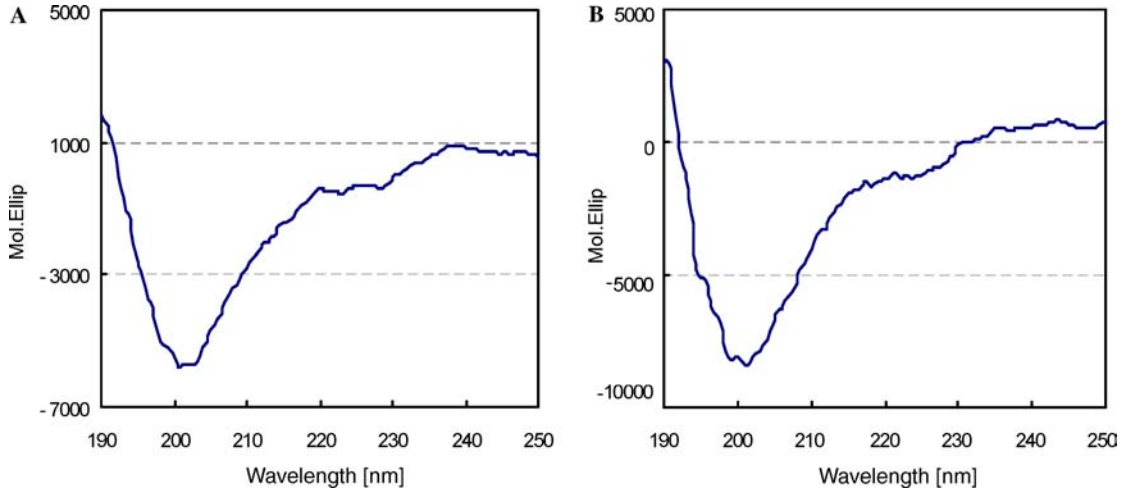

Fig. 4. Circular dichroism spectra of recombinant sea anemone neurotoxin Hk16a. (A) Hk16a prepared by stationary phase lactose induction and (B) Hk16a prepared by log phase IPTG induction.

has the conformation more similar to that of the natural entity.

Initial patch-clamp assays turned out that Hk16a prepared from stationary and log phase cultures could similarly and significantly prolong the open dwell-time of sodium channels in hippocampal neurons from neonatal SD rats. Results are presented in Fig. 5.

Discussion

Successful overexpression of seven diversified genes in three *E. coli* systems with different induction mechanisms at stationary phase implied that the protein synthesis machinery of *E. coli* cells suffered from carbon source deprivation and temperature shifts is as efficient as that of log phase ones at all tested temperatures. More than 0.5 mM IPTG caused cell lysis of strains overexpressing PLA_2, cystatin, and protease 3C, yet up to 2% lactose was safe, indicating that lysis was mainly due to IPTG's instant intensive derepression effect on expression of the toxic target genes and the high efficiency of protein assembly within a broad range of temperatures. Together with the results that the specific volumetric yield of Hk16a could increase by 121%, the biomass yields of the cultures were higher, and plasmid stability was significantly upgraded, lactose induction after stationary phase proved to be a better strategy for preparation of recombinant proteins at temperatures from above 20 to 37 °C.

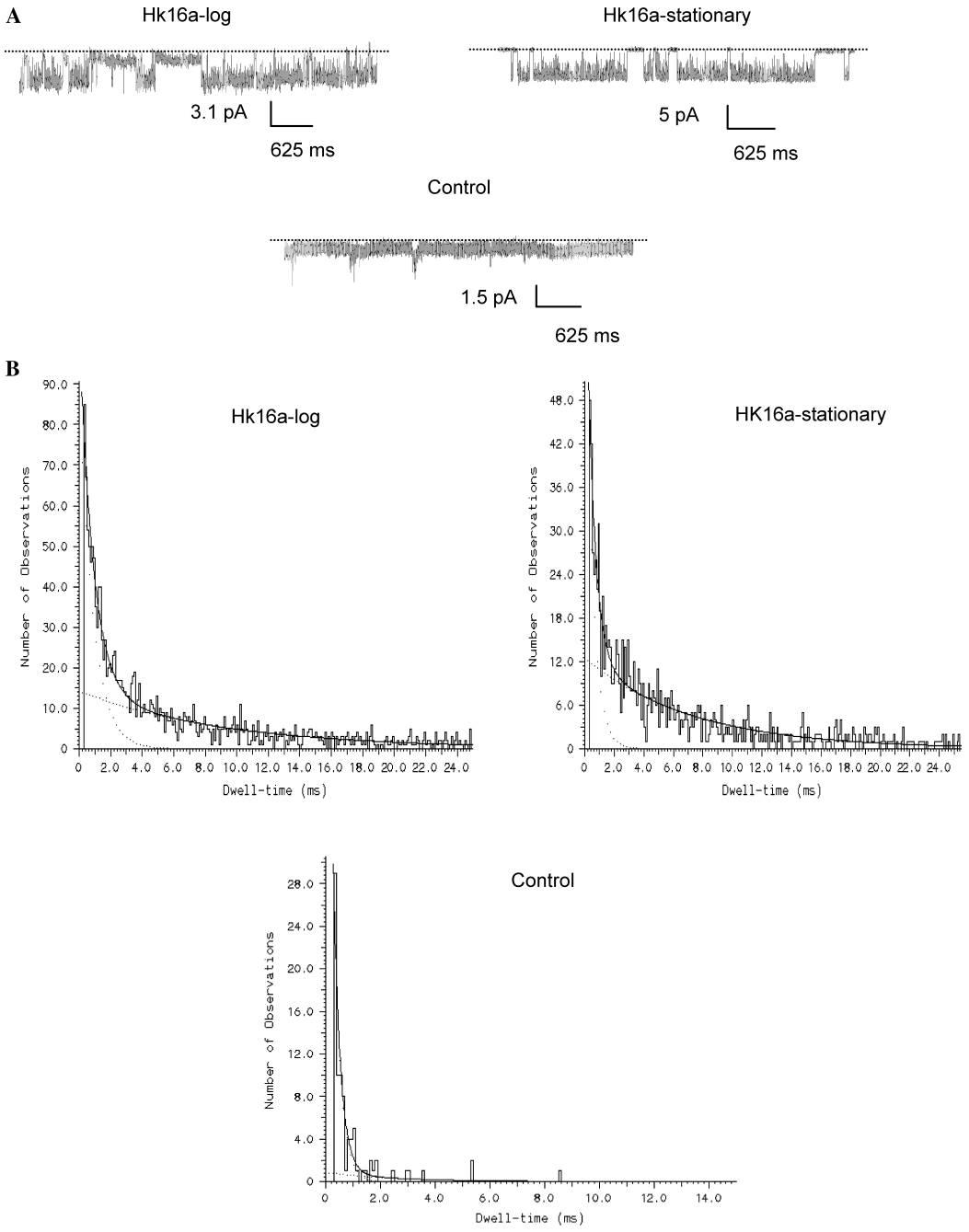

Fig. 5. Patch-clamp assays comparing the physiological activity of recombinant sea anemone neurotoxin Hk16a prepared from stationary and log phase cultures. (A) Traces of single sodium channel currents showing neurotoxins' positive action on the opening of sodium channel; membrane voltage was held at −20 mV and concentration of the proteins was 2×10^{-6} mol/L; the dotted lines indicate the current level at which the channel was closed and downward current indicates its opening. (B) Dwell-time histograms of single sodium channel fitted with two-exponential function; the mean dwell-time and standard deviation (mean ± SD) for control, Hk16a from stationary and log phase cultures are 1.060 ± 1.875, 8.235 ± 11.651, and 8.604 ± 12.593 ms, respectively.

Rowe and Summers [25] developed a quiescent-cell expression system so that the *hns* mutant cells' growth and native protein synthesis could be induced to cease while expression of plasmid-based foreign genes was not inhibited. Martin and colleagues used starvation-related promoter to control heterologous gene expression once the culture entered stationary phase [9]. The former case could accumulate target products to more than 40% of total cell proteins but must be based on individual mutant strains, and the latter must be challenged by deficiency of energy to support protein overexpression. Around the same time of our process, Galloway et al. and Chae et al. [26,27] reported their attempts of inducing protein overexpression at late log or stationary

phase. In their cases soluble fraction of target proteins was increased and cytoplasmic proteolytic events were decreased. Their works are useful supports of ours. Nevertheless, besides introducing the advantage of lactose induction at stationary phase, we attempted to further elucidate that stationary phase protein overexpression is not a specific issue which happened only for *lac* promoter-based systems, like BL21 (DE3)(pET) and DH5α (pGEX), but also works for TG1/BL21(DE3)/DH5α (pBV220) induced with heat shock, as long as energy source was recruited. Thioredoxin plays a protective role in cellular responses against stringent environments [28], yet similar results could be achieved in strains not overexpressing thioredoxin. Physical and initial patch-clamp assays on recombinant Hk16a further indicated that stationary phase *E. coli* cells are as competent in gene transcription, mRNA translation, and polypeptide folding as cells in physiologically optimum status (log phase). These evidences indicate that the capabilities of rapid adaptation to starvation and temperature shifts as well as protein overexpression after entry of stagnated growth phase are universal characters of *E. coli* but not issues of individual strains or systems.

Acknowledgments

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