0273-2289/05/120/0001/$30.00

Expression of Human β-Defensin-2 With Multiple Joined Genes in *Escherichia coli*

Zhinan Xu,*[,1] Fang Wang,[1] Li Peng,[1] Xiangming Fang,[2] and Peilin Cen[1]

[1]Institute of Bioengineering, Department of Chemical and Biochemical Engineering, Zhejiang University, Hangzhou 310027, The People's Republic of China, E-mail: znxu@zju.edu.cn; and [2]The Central Laboratory of Sir Run Run Shaw Hospital, School of Medical Science, Zhejiang University, Hangzhou 310016, The People's Republic of China

Received June 2, 2004; Revised August 2, 2004; Accepted August 9, 2004

Abstract

Human β-defensin (HBD)-2 is a small cationic peptide with a broad range of antimicrobial activity. In this study, multiple copies of the hBD2 gene were linked in tandem, and a number of different *Escherichia coli* expression vectors were evaluated, including pQE-30, pBV220, pET-28a(+), and pGEX-4T-2. No expression of multiple joined genes was detectable in the pQE-30 expression system, whereas in pBV220 with one or two joined hBD2 genes and in pET-28a(+) with one, two, or four copies, target proteins were expressed at a low level. Only when pGEX-4T-2 was applied as expression plasmid with one or two joined hBD2 genes were target proteins expressed in high level, and the expressed fusion proteins account for 26 and 16% of the total insoluble proteins, respectively. In the pGEX-4T-2 and pET-28a(+) expression systems, the effects of multiple joined genes on the growth of host strains and plasmid stability were examined. Host cells containing plasmid carrying fewer copies of hBD2 genes were faster in cell growth. Plasmid stability decreased with an increase in multiple joined genes, which was especially noticeable in the pET-28a(+) system. Furthermore, the presence of glucose in culture medium brought about a positive effect on plasmid stability when using pET28-nhBD2 as expression vectors.

Index Entries: Antimicrobial peptide; human β-defensin-2; tandem repeated genes; fusion expression; plasmid stability; T7 expression system.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Over the past decade, many natural peptide antibiotics, with a broad range of antimicrobial activity, have been discovered, and, in recent years, the production of small antimicrobial peptides by recombinant DNA techniques has become an interesting challenge *(1)*. However, large-scale production of these small cationic peptides using *Escherichia coli* as the host cell has been hampered by two barriers that resulted from their specific characteristics: the potential intrinsic antibacterial activity to the producing strains and the susceptibility to proteolytic degradation *(1)*. To overcome these barriers, a general applied strategy was developed that forms multiple copies of the gene encoding the small peptide, which are tandemly linked and expressed into a stable multidomain protein *(2,3)*. By this method, the small peptides can be synthesized in stable form and expressed in high yield.

First discovered in human skin in 1997, human β-defensin (HBD)-2 is a cysteine-rich cationic antimicrobial peptide with 41 amino acids *(4)*. It is mainly produced by a number of epithelial cells and exhibits potent antimicrobial activity against Gram-negative bacteria (e.g., *E. coli* and *Pseudomonas aeruginosa*) and the yeast *Candida albicans* without observed acquired resistance. Unlike human α-defensin, HBD-2 is the first β-defensin found to be inducibly produced following stimulation of epithelial cells by contact with microorganism or various cytokines *(5)*. It is also a crucial component of innate and adaptive immune responses by attracting both immature dendritic cells and memory T-cells *(6)*. Furthermore, it is intriguing to speculate that the inducibility of HBD-2 and no acquired resistance make it an ideal therapeutic agent. However, study in this field has been hindered by the absence of the techniques to produce HBD-2 at a high level.

Our group previously reported the cloning and expression of HBD-2 in *E. coli (7)*. However, the expression level was limited. To circumvent this problem, in the present work, multiple copies of the hBD2 gene were linked in tandem and the target protein was a fusion molecule with multiple joined HBD-2 repeats. The expression systems of the multiple joined genes using different vectors, including pQE-30, pBV220, pET-28a(+), and pGEX-4T-2, were examined. The effects of the number of joined genes on plasmid stability and the growth of host cells were evaluated.

Materials and Methods

Host Strains and Plasmids

E. coli TG1 was used as the host strain for cloning, and *E. coli* TG1, *E. coli* M15, as well as *E. coli* BL21(DE3) were applied as host strains for plasmids pBV220 (Qiagen, GmbH, Germany), pQE-30 (donated by Prof. Xun Xu, Third Institute of Oceanography, P.R. China), and two other plasmids as expression vectors, respectively.

Construction of the cloning vector pGEMT-hBD2 has been reported elsewhere *(7)*. The hBD2 gene concatemer was cloned in pQE-30, pBV220, pET-28a(+) (Novagen, Madison, WI), and pGEX-4T-2 (Amersham Pharmacia Biotech, Piscataway, NJ) to express the corresponding protein.

Enzymes and Antibodies

All the restriction endonucleases and T_4 DNA ligase were purchased from Takara Biotech (Dalian, P.R. China).

Penta·His antibody (mouse anti-$[His]_5$) is a product of Qiagen. Rabbit anti-HBD2 polyclonal antibody was kindly donated by Prof. Tomas Ganz (UCLA School of Medicine). Horseradish peroxidase (HRP)–conjugated goat anti–mouse IgG and HRP-conjugated goat anti–rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Construction of Cloning Vectors Containing Multiple Copies of hBD2 Gene

The construction of various cloning vectors is schematically presented in Fig. 1. Following digestion with *Bam*HI and *Sac*I, pGEMT-hBD2 was cleaved into two fragments, in which the larger one was recovered using a QIAquick™ Gel Extraction Kit (Qiagen). Then, pGEMT-hBD2 was cleaved into two other fragments after digestion with *Bgl*II and *Sac*I to recover the smaller fragment. The cloning vector containing two copies of the hBD2 gene, pGEMT-2hBD2, was constructed through ligation reaction between the two recovered fragments. This procedure was repeated successively to construct pGEMT-4hBD2 and pGEMT-8hBD2. To facilitate the detection and purification of the products using pGEX-4T-2 and pBV220 as expression vectors, a $(His)_6$ tag was linked to the 5' end of the nhBD2 coding sequence (n = 1, 2, 4, 8) to obtain pGEMT-6his-nhBD2 (n = 1, 2, 4, 8).

Construction of Expression Vectors

To construct pQE-30-nhBD2 (n = 1, 2, 4, 8) and pET28-nhBD2 (n = 2, 4, 8), the plasmid pGEMT-nhBD2 (n = 1, 2, 4, 8) was digested with *Bgl*II and *Hin*dIII, and the smaller fragment was recovered; both expression plasmids, pET-28a(+) and pQE-30, were cleaved by *Bam*HI and *Hin*dIII, respectively, to recover larger fragments. Then, the expression vectors pET28a-nhBD2 and pQE-30-nhBD2 were constructed successfully through a ligation reaction between the two corresponding recovered fragments.

The cloning vectors pGEMT-6his-nhBD2 (n = 1, 2, 4, 8) were treated by *Eco*RI and *Sal*I. Then, the smaller fragment was recovered and inserted between the same sites of pBV220 to obtain pBV220-nhBD2 (n = 1, 2, 4, 8). Following a similar procedure, plasmids pGEX-nhBD2 (n = 1, 2, 4, 8) were successfully constructed except that the restriction endonucleases applied were *Bam*HI and *Sal*I.

Except for pBV220, on which the foreign gene is induced by temperature (42°C), the heterologous genes on all expression vectors are induced by the presence of isopropyl-β-D-thiogalactoside (IPTG).

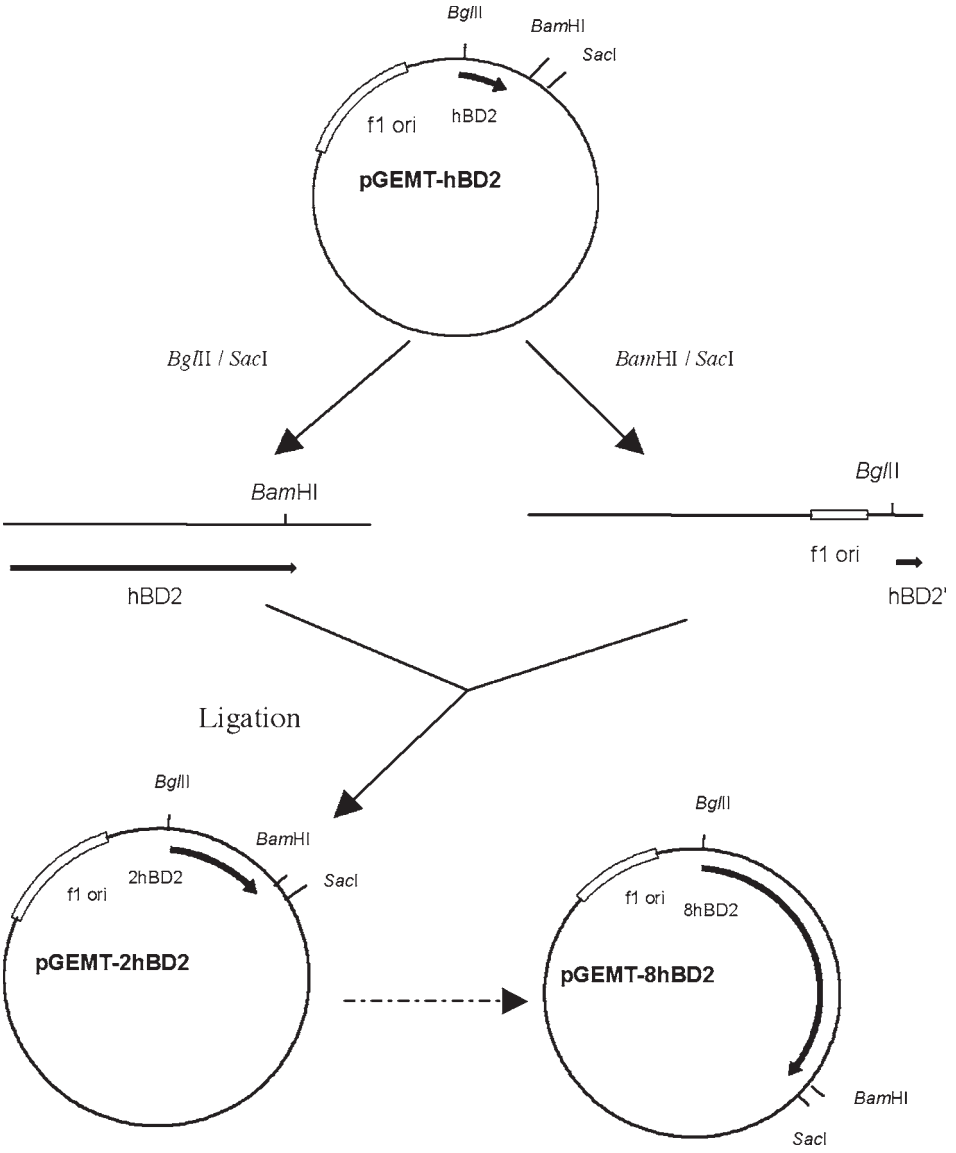

Fig. 1. Construction of cloning vectors containing multiple joined hBD2 genes.

Protein Expression and Cell Disruption

The protocols to express the multiple joined hBD-2 genes using plasmids pQE30-nhBD2, pET28a-nhBD2, and pGEX-nhBD2 (n = 1, 2, 4, 8) in *E. coli* and to disrupt the cells were the same as those described previously *(7)*. Expression was carried out in 30 mL of medium in a 250-mL flask unless otherwise specified. Using plasmids pBV220-nhBD2 as expression vectors, the growth temperature was maintained at 30°C before the cell density reached an optical density (OD_{600}) of 0.5. Then the cultivation temperature was raised immediately to 42°C to induce the expression of multiple joined

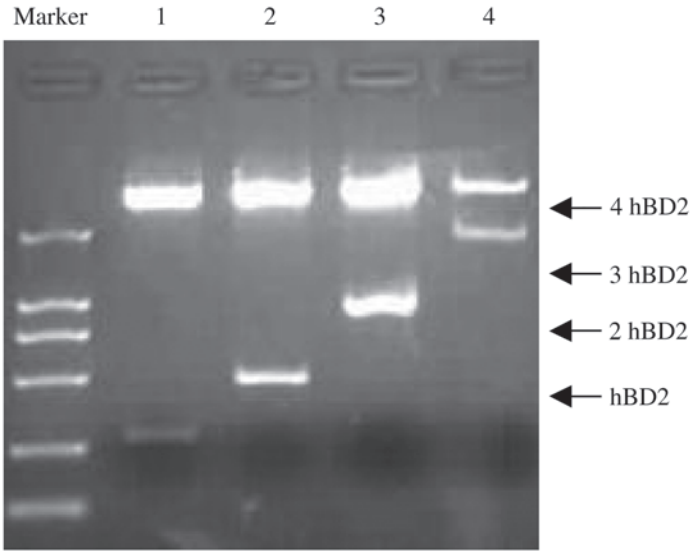

Fig. 2. Analysis of cloning vectors containing multiple joined hBD2 genes. Following digestion with *Eco*RI and *Bam*HI, the cleaved mixture of pGEMT-nhBD2 was analyzed in 1% agarose gel. Marker: from top to bottom, 2000, 1000, 750, 500, 250, and 100 bp. Lanes 1–4: pGEMT-nhBD2 bearing 1, 2, 4, and 8 copies of hBD2 coding sequence, respectively.

hBD-2 genes. The incubation temperature was maintained at 42°C for 5 h before the cells were harvested *(8)*.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Western Blot Analysis

The standard procedures of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses were adopted *(7,9)*.

Measurement of Plasmid Stability

Samples were withdrawn from cultivation broth at the end of fermentation and then diluted appropriately using aseptic techniques. A diluted sample was spread on a nonselective Luria-Bertani (LB) agar plate and on a selective LB agar plate containing 50 µg/mL of ampicillin. The plates were incubated at 37°C for about 12 h, and the colonies on each plate were counted. The ratio of the number of colonies on the selective plate to that on the nonselective plate was calculated to determine the percentage of plasmid-containing cells, which was regarded as an index of plasmid stability.

Results and Discussion

Construction of Cloning Vectors Containing polyhBD2 Gene

Following the procedure described in Materials and Methods, various cloning vectors containing one, two, four, and eight copies of the hBD2

coding sequence were successfully constructed, and the agarose electrophoresis results are shown in Fig. 2.

Expression of polyhBD2 Gene Using Different Plasmids

Four different types of expression plasmids (pQE30-nhBD2, pBV220-nhBD2, pET28-nhBD2, and pGEX-nhBD2) (n = 1, 2, 3, 4) were constructed. For each plasmid, a $(His)_6$ tag was linked with the upstream of polyhBD2 coding sequence to facilitate the detection of target protein expression, and an additional glutathione-*S*-transferase (GST) partner was fused with $(His)_6$-nhBD2 in pGEX-nhBD2, compared with three other types of plasmids.

Using pQE30-nhBD2 as expression vectors, there were no evident bands of target proteins either on the gel after staining with Coomassie brilliant blue G-250 or by Western blot (data not shown). A possible explanation for this phenomenon is the lack of a suitable fusion partner in the upstream of the hBD2 coding sequence. Either $(His)_6$ tag or multiple joined hBD2 in pQE30-nhBD2 did not bring about the substantial expression of hBD2.

As reported previously *(9)*, when the same nhBD2 coding sequences were inserted into pET-28a(+) expression plasmid, the fusion protein expressed from one, two, and four copies of the hBD-2 coding sequence could be detected by Western blot, although the expression level was still very limited because of no target bands detected with SDS-PAGE. These results suggest that the expression of hBD2 was not only affected by the fusion partner of the target gene but also the employed expression system.

Using pBV220-nhBD2 as expression vectors, it was observed that the target multicopy peptides appeared at approx the 8- and 16-kDa positions on the film, respectively (Fig. 3). Additional bands were also visualized at positions other than 8 and 16 kDa, which might be owing to the nonspecific binding of the polyclonal antibody since an anti-HBD2 polyclonal antibody was used. Although the target proteins from one and two copies of hBD2 genes were detected by Western blot, the expression level was also very limited. A possible reason is the special thermal induction mechanism of the pBV220 expression system. Before induction, host cells should be cultivated at 30°C, and the heterologous gene is triggered to express only when the cultivation temperature is raised to and maintained at 42°C, which is a barrier in large-scale production of hBD2 because high temperature (42°C) will reduce the growth of recombinant *E. coli* obviously.

As for pGEX-nhBD2 as expression vector, it was obvious that the additional fusion partner (GST) enhanced the expression of the fusion proteins GST-HBD2 (molecular mass = 33 kDa, theoretically) and GST-2HBD2 (molecular mass = 42 kDa, theoretically) significantly, which were detected by both SDS-PAGE and Western blot analyses. The results revealed that the GST fusion expression system improved the expression of the target genes significantly, and the fusion proteins derived from one and two fused cod-

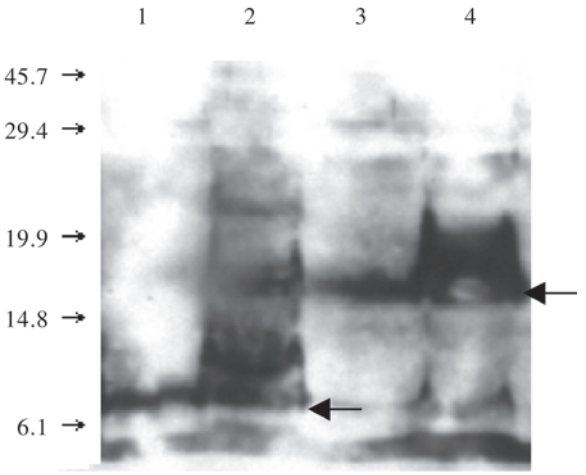

Fig. 3. Western blot analysis of target protein expression using pBV220 system with rabbit anti-HBD2 polyclonal antibody. Lanes 1 and 2: soluble and insoluble protein fractions of *E. coli* TG1/pBV220-hBD2, respectively; lanes 3 and 4: soluble and insoluble protein fractions of *E. coli* TG1/pBV220-2hBD2, respectively.

ing sequences accounted for approx 26 and 16% of the total insoluble cellular proteins, respectively.

These experimental results indicated that an appropriate fusion partner seemed necessary for high-level production of small cationic antimicrobial peptides such as HBD-2, and that the number of tandemly repeated genes that can be expressed was dependent on the plasmids as well as the host cells. The highly repeated gene linkage cannot guarantee high-level expression. It seems that the number of multiple joined genes, four for pET-28a(+) and two for both pBV220 and pGEX-4T-2, is suitable. Other researchers observed similar phenomena *(2,3)*. Possible explanations are that the expression of recombinant proteins can affect the normal growth of the host strains *(10)*; and that the growth of the plasmid-containing cells can be retarded greatly by the replication of the large plasmids, which results in plasmid instability and low-level expression of product.

To evaluate the effects of the number of tandemly repeated genes on host cell growth and plasmid stability, two expression systems, pET-28a(+) and pGEX-4T-2, were selected for further study.

Effect of Number of Multiple Joined Genes on Growth of Host Cells

Using pET-28a(+) Expression System

Figure 4 shows that no evident difference in growth rate and cell density was observed among four recombinant strains of *E. coli* BL21(DE3)/pET28-nhBD2 ($n = 1, 2, 4, 8$) before induction. However, after adding IPTG, only BL21(DE3)/pET28-hBD2 grew normally into the stationary phase,

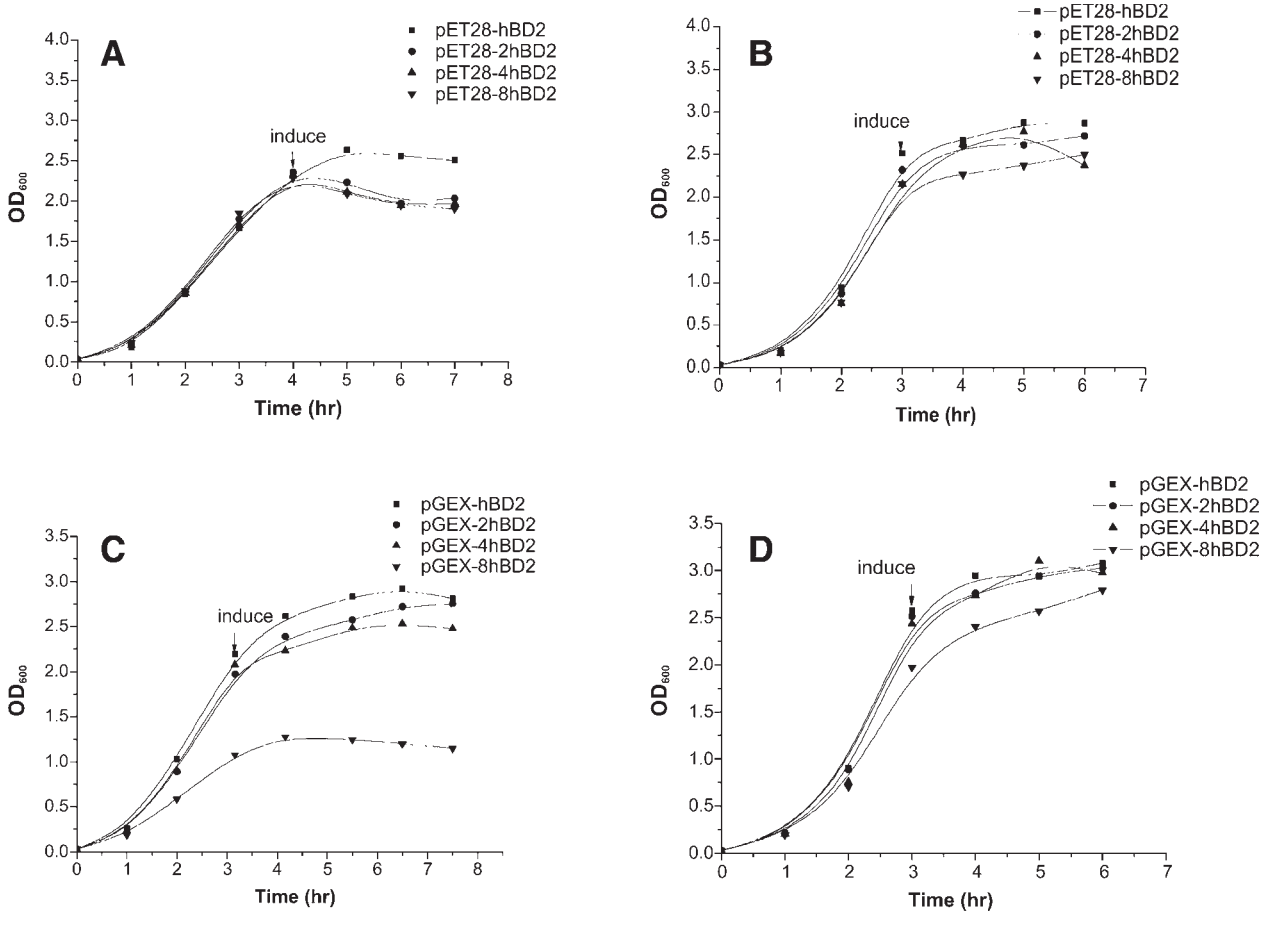
A
pET28-hBD2
pET28-2hBD2
pET28-4hBD2
pET28-8hBD2
induce
OD600
Time (hr)
B
pET28-hBD2
pET28-2hBD2
pET28-4hBD2
pET28-8hBD2
induce
OD600
Time (hr)
C
pGEX-hBD2
pGEX-2hBD2
pGEX-4hBD2
pGEX-8hBD2
induce
OD600
Time (hr)
D
pGEX-hBD2
pGEX-2hBD2
pGEX-4hBD2
pGEX-8hBD2
induce
OD600
Time (hr)

Fig. 4.

whereas the cell densities of the other three strains dropped slowly and were apparently lower than that of BL21(DE3)/pET28-hBD2.

As shown in Fig. 4, when LB + 0.5% glucose medium was applied, the growth of host cells with fewer multiple joined genes was better than that with higher number, whereas after induction the difference in cell density among the four strains was not as conspicuous compared with the results in Fig. 4. After IPTG induction, the cell density of BL21(DE3)/pET28-nhBD2 (n = 2, 4, 8) continued to increase into the stationary phase with prolonged expression time, instead of decreasing as in LB medium.

Using pGEX-4T-2 Expression System

As shown in Fig. 4, the growth rate of the host cells dropped gradually with an increase in the number of fused hBD2 coding sequence, especially after induction. BL21(DE3)/pGEX-nhBD2 (n = 1, 2, 4) showed similar growth patterns until IPTG was added to the medium. After induction, the difference in the growth rates of the three recombinant strains became more and more evident. Another interesting phenomenon is that after a brief lag phase, BL21(DE3)/pGEX-8hBD2 grew much more slowly compared with the other three strains, with a cell density of OD_{600} of only about 1.2 in the stationary phase, whereas for the other three strains the OD_{600} ranged from approx 2.5 to 3.0. The difference in the growth rates of the four strains in LB + 0.5% glucose was not as noticeable (Fig. 4).

In light of these experimental results, it is confirmed that host cells carrying fewer copies of heterologous genes have an obvious advantage in growth and that the presence of glucose in the media benefits the growth of host cells harboring more copies of heterologous genes. In comparison with *E. coli* without plasmids, recombinant *E. coli* always needs to consume additional energy to support cell growth and the expression of heterologous proteins. The suitable supplementation of 0.5% glucose in LB may enhance the supply of energy because no glucose was contained in the LB medium, thus significantly improving the growth rates of recombinant *E. coli* containing multiple copies of the hBD2 gene. Regarding the difference between the growth profiles of BL21(DE3)/pET28-nhBD2 and BL21(DE3)/pGEX-nhBD2, it is possibly owing to the difference between the two expression systems. Further work should be conducted to elucidate the cause of this difference.

Fig. 4. *(previous page)* Growth profiles of recombinant strains in different culture media: **(A)** *E. coli* BL21(DE3)/pET28-nhBD2 (n = 1, 2, 4, 8) in LB medium; **(B)** *E. coli* BL21(DE3)/pET28-nhBD2 (n = 1, 2, 4, 8) in LB + 0.5% glucose medium; **(C)** *E. coli* BL21(DE3)/pGEX-nhBD2 (n = 1, 2, 4, 8) in LB medium; **(D)** *E. coli* BL21(DE3)/pGEX-nhBD2 (n = 1, 2, 4, 8) in LB + 0.5% glucose medium. The recombinant strains were grown at 32°C in a 500-mL Erlenmeyer flask containing 75 mL of medium and maintained at 200 rpm for 0.8 m*M* IPTG induction as indicated. The tests were performed three times. Values are the average of three independent determinations, and all the standard deviations are <5%.

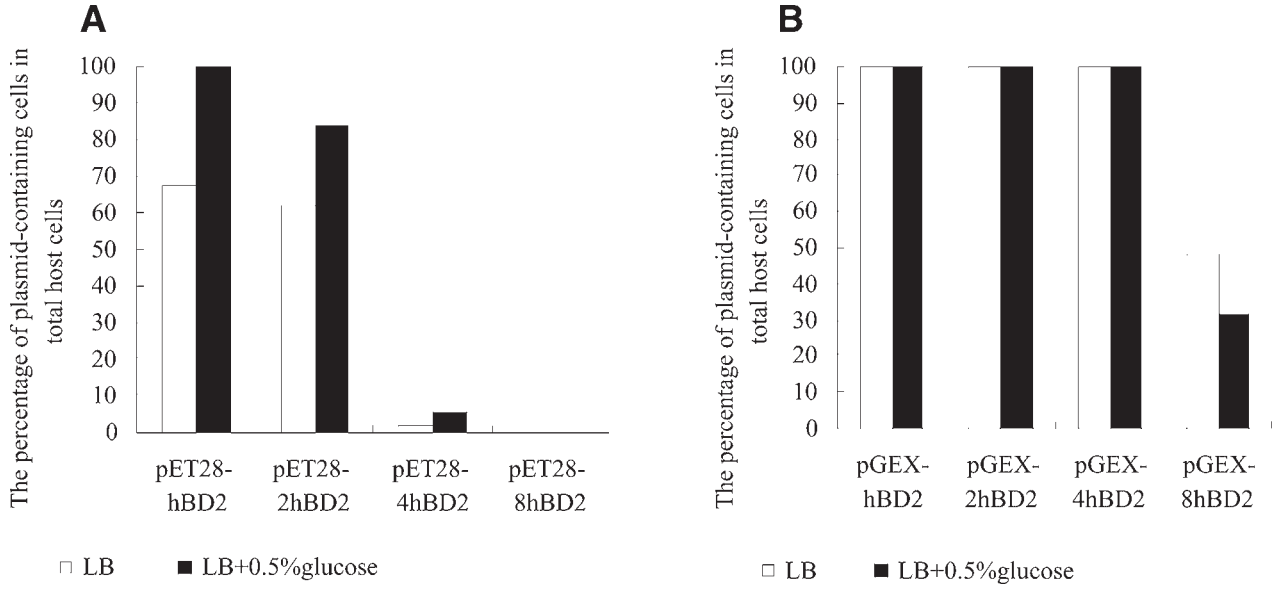

Fig. 5. Percentage of plasmid-containing cells in total host cells in different culture media: **(A)** *E. coli* BL21(DE3)/pET28-nhBD2 (n = 1, 2, 4, 8); **(B)** *E. coli* BL21(DE3)/pGEX-nhBD2 (n = 1, 2, 4, 8).

Effect of Number of Multiple Joined Genes on Plasmid Stability

On harvesting, samples were withdrawn from the fermentation broth of each recombinant strain, and the corresponding plasmid stability was evaluated. As shown in Fig. 5, with an increase in the number of multiple joined hBD2 genes, the ratio of plasmid-containing cells was reduced dramatically in the pET-28a(+) expression system, from 67.2% for BL21(DE3)/pET28-hBD2 to 0.053% for BL21(DE3)/pET28-8hBD2. Moreover, there is a noticeable difference in plasmid stability between the two kinds of culture media: LB and LB + 0.5% glucose. It appears that the existence of glucose in the medium enhanced plasmid stability. Compared to the results in the pGEX-4T-2 expression system, almost no loss of plasmid in both kinds of culture medium was observed, except while using pGEX-8hBD2 as expression vector. The ratio of plasmid-containing cells decreased to 31.58 and 48% in LB medium and LB + 0.5% glucose medium, respectively (Fig. 5B).

Higher plasmid stability always enhances recombinant protein expression. In the pET-28a(+) expression system, the improvement in expression level by the addition of glucose was observed using Western blot analyses, but the target bands still did not appear in SDS-PAGE gel after staining. As for the pGEX-4T-2 expression system, after the detection of hBD2 expression with SDS-PAGE, about 5–10% improvement in hBD-2 fusion was achieved by the addition of 0.5% glucose to the medium.

Plasmid stability is a main concern in recombinant strain fermentation. A number of factors, such as plasmid load, type of insert, and medium composition, may influence plasmid stability *(11)*. Plasmid-containing cells usually grow slower than plasmid-free cells. This tendency is especially serious when a recombinant strain is fully induced, which results in an increased burden coming from the synthesis of target protein, and when the plasmid is loaded with more heterologous inserts *(12)*. This might explain the decrease in plasmid stability with an increase in hBD2 gene copies in our expression systems.

Apart from the metabolic burden, the T7 expression system (comprising T7 promoter and T7 RNA polymerase) could be a serious factor causing plasmid instability *(13)*. The overactive T7 RNA polymerase can bring about negative effects on plasmid stability and protein production, especially for toxic protein expression *(12)*. This is also a possible explanation for the difference in plasmid retention between the pET-28a(+) and pGEX-4T-2 systems; the former belongs to the T7 expression system and the latter comprises a weaker promoter, P_{tac}. It was reported that plasmid stability could be improved by reducing the strength of promoter during construction of the upstream plasmid *(14)*.

It is interesting that the addition of glucose to the medium improved plasmid stability greatly in the pET28 expression system. Compared with no glucose supplementation in the LB medium, the addition of 0.5% glucose ameliorated the lack of carbon source in the LB medium and then

enhanced the growth of both plasmid-free and plasmid-containing cells. The tendency for enhancement might be more conspicuous in the latter kind of cells, which resulted in the improvement of plasmid stability. However, if the concentration of glucose continues to increase, the plasmid stability will decrease rapidly (data not shown), probably owing to the growth predominance of plasmid-free cells. Moreover, the catabolite repression might be caused by glucose on the lacUV5 promoter that drives the T7 RNA polymerase gene in BL21(DE3) *(15)*. Catabolite repression might inhibit the production of the T7 RNA polymerase in the cells, which then would reduce the basal expression and improve the ability of plasmid-containing cells to maintain plasmid stability *(13)*.

Conclusion

Different copies of hBD2 coding sequence were joined tandemly and cloned into four different expression plasmids, pQE-30, pBV220, pET-28a(+), and pGEX-4T-2. The expression of target proteins from multiple copies of hBD2 gene was detected except in the first system, though the expression level was different. Multiple joined hBD2 genes affect the normal growth of host strains and plasmid retention. With an increase in the number of tandemly repeated hBD2 genes, the growth of host strains was evidently retarded, and plasmid stability dropped dramatically, especially when using the pET-28a(+) expression system. Moreover, a study of plasmid stability showed that the existence of glucose in culture medium enhanced plasmid stability using the pET-28a(+) system, but not using the pGEX-4T-2 system.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (nos. 20276066 and A30070854) and the Department of Science and Technology (no. 413491030J30007001103241), Zhejiang Provincial People's Government, People's Republic of China.

References

1. Piers, K. L., Brown, M. H., and Hancock, R. E. W. (1993), *Gene* **134,** 7–13.
2. Lennick, M., Haynes, J. R., and Shen, S. H. (1987), *Gene* **61,** 103–112.
3. Raingeaud, J., Lavergne, F., Lelievre, V., Muller, J. M., Julien, R., and Cenatiempo, Y. (1996), *Biochimie* **78,** 14–25.
4. Harder, J., Bartels, J., Christophers, E., and Schröder, J. M. (1997), *Nature* **387,** 861.
5. Schröder, J. M. and Harder, J. (1999), *Int. J. Biochem. Cell Biol.* **31,** 645–651.
6. Ganz, T. (1999), *Science* **286,** 420–421.
7. Fang, X. M., Peng, L., Xu, Z. N., Wu, J. M., and Cen, P. L. (2002), *Protein Pept. Lett.* **9,** 31–37.
8. Zhang, Z. Q., Yao, L. H., and Hou, Y. D. (1990), *Chin. J. Virol.* **6,** 111–116.
9. Wang, F., Fang, X. M., Xu, Z. N., Peng, L., and Cen, P. L. (2004), *Prep. Biochem. Biotechnol.* **34,** 215–225.

10 Sivakesava, S., Xu, Z. N., Chen, Y. H., Hackett, J., Huang, R. C., Lam, E., Lam, T. L., Siu, K. L., Wong, R. S. C., and Wong, W. K. R. (1999), *Process Biochem.* **34,** 893–900.
11. Zhao, F. Y., Bai, Y. L., Zhang, X. M., and Gao, C. C. (2001), *Microbiology* **28,** 50–55.
12 Zhang, Y. K., Li, T. M., and Liu, J. J. (2003), *Protein Exp. Purif.* **29,** 132–139.
13 Chao, Y. P., Chiang, C. J., and Hung, W. B. (2002), *Biotechnol. Prog.* **18,** 394–400.
14 Xu, Z. N., Liu, G., Cen, P. L., and Wong, W. K. R. (2000), *Bioprocess Eng.* **23,** 669–674.
15. Spehr, V., Frahm, D., and Meyer, T. F. (2000), *Gene* **257,** 259–267.