Novel Strategy for Efficient Screening and Construction of Host/Vector Systems to Overproduce Penicillin Acylase in *Escherichia coli*

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Abstract: A novel and simple method of using penicillin for screening of mutant strains with a high penicillin acylase (PAC) activity was developed. Random mutagenesis was conducted using a PAC-producing strain resistant to 6-aminopenicillanic acid (6-APA) as the parent strain and mutants were screened with penicillin at a high concentration. Results suggest that mutants with a high minimum inhibitory concentration for penicillin (MIC_{penG}) usually overproduce PAC. Both volumetric and specific PAC activities of a mutant, MD7, were significantly higher than those of the parent strain, HBPAC101 harboring pCLL2902. The mutation(s) resulting in the enhanced expression was mapped on the host chromosome rather than the plasmid. In addition, the mutant strain of MDΔP7, derived by elimination of the harbored plasmid in MD7, was demonstrated to be efficient in production of PAC by using the expression plasmids for which expression of the *pac* gene is limited by translation. An extremely high specific PAC activity of more than 350 U/L/OD_{600} was reached upon cultivation of MDΔP7 harboring pTrcKnPAC2902 in a bioreactor. As such, the strategy is effective in terms of constructing PAC overproducers and improving the process yield for production of PAC. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **65:** 219–226, 1999.

Keywords: *Escherichia coli;* minimum inhibitory concentration; penicillin acylase; random mutagenesis; recombinant protein; screening

INTRODUCTION

Penicillin acylase (PAC) is an important industrial enzyme for production of many semisynthetic antibiotics (Shewale et al., 1990). Among various enzyme sources (Vandamme and Voets, 1974), PAC from *Escherichia coli* is the most common one for industrial application and, therefore, developing technology for production of *E. coli* PAC has been constantly emphasized. Similar to other recombinant proteins, strategies for overproduction of PAC primarily focus on two aspects. The first one is to design an appropriate host/vector system for enhancing the *pac* gene expression level; the second is to develop technology for high-cell-density cultivation. Performance of the enzyme production process could be optimized when the above two goals are simultaneously attained.

While increasing cell density could be easily performed using a fed-batch culture, enhancing the *pac* gene expression level is particularly difficult for several reasons. First, to form mature PAC in the periplasm, the immediate translational product of the *pac* gene (i.e., preproPAC in Fig. 1) has to be appropriately exported and processed (Sizmann et al., 1990). It was recently reported that, depending on the host/vector system, production of PAC could be limited by transcription, translation, or posttranslational steps (i.e., translocation and periplasmic processing) (Chou et al., 1999a,c). On the other hand, expression of the *pac* gene in *E. coli* is usually regulated by several environmental conditions, such as temperature (Keilmann et al., 1993) and carbon sources (Merino et al., 1992). The regulatory mechanisms still remain unclear at a genetic level in many aspects (Valle et al., 1986). Due to limited knowledge of the mechanisms for the posttranslational steps as well as regulation of the gene expression, it has become challenging to systematically develop an optimal expression strategy for overproduction of PAC. Screening of PAC-overproducing mutants, therefore, turns out to be an easy and feasible approach for improving production of PAC (del Rio et al., 1995; Erarslan et al., 1991; Sobotkova et al., 1995). The operation is useful, though characterization of the mutation(s) resulting in the improved PAC production is usually difficult but not absolutely required for industrial application. Production of PAC could be further enhanced by expression of the mutant *pac* gene cloned in a multicopy plasmid (Sobotkova et al., 1996). In these approaches, mutants were usually derived through random mutagenesis since there were no general rules based on which a mutation could be designed toward increasing PAC activity. The mutagenesis was then followed by screening of PAC overproducers. The most com-

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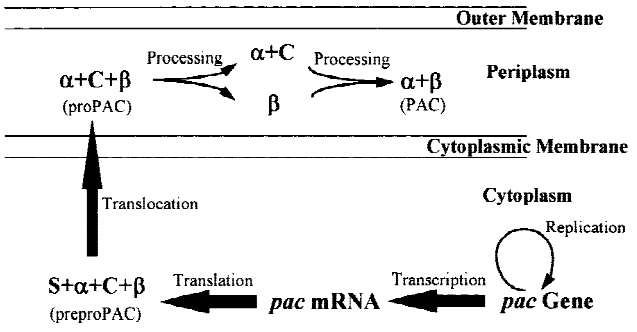

Figure 1. Synthesis and maturation of PAC in *E. coli*. The structural *pac* gene from *E. coli* ATCC11105 encodes a polypeptide precursor (preproPAC) which has a molecular weight of ~95 kD and is composed of, in the direction of N-terminus to C-terminus, a signal peptide (S), α subunit (α), connecting peptide (C), and β subunit (β). The signal peptide directs the export of preproPAC into the periplasm and is removed to form another type of PAC precursor (proPAC) after the translocation. Periplasmic proteolysis is followed to remove the connecting peptide and the two subunits of α and β are available for assembling mature PAC.

mon protocol for screening of PAC-producing strains is an overlaying test using a penicillin-resistant but 6-aminopenicillanic-acid (6-APA)-sensitive strain, *Serratia marcensscens* ATCC27117 (Meevootisom et al., 1983). However, there are several limitations with this microbiological test. First, the screening itself is a laborious process, provided the number of strains for the test is large. Second, although in principle PAC activity of the strain under the test increases with the size of the corresponding growth inhibition zone, the method can hardly be applied for a quantitative evaluation of the strain's PAC activity due to generic experimental errors. Therefore, it becomes imperative to develop a rational strategy for prescreening of mutants so that the probability of finding appropriate PAC overproducers among a subpopulation of mutants could be increased.

In the current study, a novel strategy of using penicillin for screening of mutant strains with a high PAC activity was developed. The idea is based on the fact that PAC expressed in cells could hydrolyze penicillin G to form the products of phenyl acetic acid (PAA) and 6-APA (Fig. 2). PAA could be assimilated by cells as a carbon source. Strains overproducing PAC are likely resistant to penicillin at a high concentration. A subpopulation of mutants with a high minimum inhibitory concentration for penicillin (MIC_{penG}) were constructed for evaluation of their PAC-producing capability. Mutagenesis was conducted using a PAC-producing strain resistant to 6-APA (i.e., HBPAC101 harboring pCLL2902) as the parent strain and mutants were screened with penicillin G at a high concentration. We observed that the mutant strains with a high MIC_{penG} usually overproduce PAC. We also conducted the experiments for localization of the mutation(s) resulting in enhanced PAC production for one of the mutants, MD7, which gave the highest volumetric PAC activity and might be of interest for industrial application. Moreover, the feasibility of employing the mutant strain, MDΔP7, derived by elimination of the harbored plasmid in MD7, as a host for production of PAC was evaluated using a selection of expression plasmids constructed in this laboratory.

MATERIALS AND METHODS

Bacterial Strains, DNAs, and Recombinant DNA Technology

HBPAC101 is a 6-APA-resistant mutant strain derived from HB101 (*F⁻ hsdS20 leuB6 recA13 ara-14 proA2 lacY1 thi-1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ⁻) (Boyer and Roulland-Dussoix, 1969). All expression plasmids in this study are derived from pBR322 and contain the *pac* operon from *E. coli* ATCC11105. pCLL3201 contains the whole *pac* gene, whereas a portion of the *pac* regulatory region was truncated in pCLL2902 (Chou et al., 1999c). pTrcKnPAC3201 and pTrcKnPAC2902 were made by subcloning the *pac* operon from pCLL3201 and pCLL2902, respectively, into pTrcKn99A, a vector derived from pTrc99A (Amann et al., 1988) by replacing the ampicillin (Ap)-resistance gene with a kanamycin (Kn)-resistance gene, and expression of the *pac* gene was, therefore, controlled by the *trc* promoter (Chou et al., 1999c). MD7, a mutant with a high MIC_{penG} and a high PAC activity, was derived from HBPAC101 harboring pCLL2902 (see Results and Discussion). MDΔP7 was derived from MD7 by eliminating the harbored plasmid. To do this, MD7 was subcultured in LB (5 g/L NaCl, 5 g/L Bacto yeast extract, and 10 g/L Bacto tryptone) medium supplemented with 0.1% SDS at 40°C at least three times. The stationary-phase culture was appropriately diluted with sterilized saline solution and plated on a tetracycline (Tc)-free agar. Single colonies were tested for Tc-resistance. A colony sensitive to Tc was selected and named MDΔP7. *E. coli* cells were cultivated in MPAC (5 g/L NaCl, 5 g/L Bacto beef extract, and 10 g/L Bacto yeast extract) or LB medium. Unless otherwise specified, the medium was supplemented with antibiotics or chemicals at the

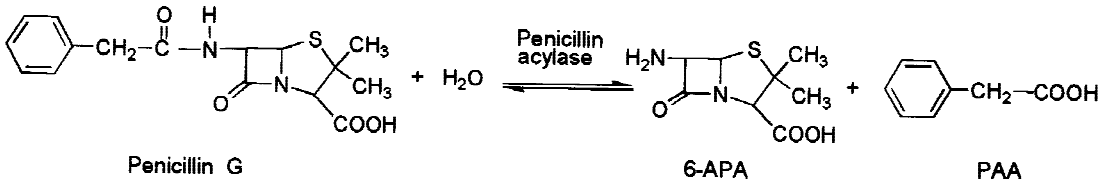

Figure 2. The reaction of penicillin G hydrolysis catalyzed by PAC.

following concentrations when necessary: Ap, 25 μg/mL, Tc, 10 μg/mL; Kn, 25 μg/mL; isopropyl β-D-thiogalactopyranoside (IPTG), 0.05 mM; PAA, 0.5 g/L. Purification of plasmid DNAs was performed using a spin-column kit purchased from Clontech (Palo Alto, CA, USA). Transformation was performed according to a standard protocol (Chung and Miller, 1993) or using an electroporator (*E. coli* Pulser, Bio-Rad, Hercules, CA, USA).

Random Mutagenesis (Miller, 1992)

E. coli cells at the mid-to-late exponential phase were mutagenized with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) at 50 μg/mL in citrate buffer (0.1 M, pH 5.5) for 40 min. The MNNG-treated cells were centrifuged, resuspended with phosphate buffer (0.1 M, pH 7.0), and spread onto a selecting agar plate. The plate was incubated at an appropriate temperature up to 50 h.

Microbiological Screening of PAC-Producing Strains

PAC-producing strains can be identified with a microbiological screening protocol, which is an overlaying test using a bacterial strain, *S. marcenscens* ATCC27117, resistant to penicillin but sensitive to 6-APA (Meevootisom et al., 1983).

Batch Cultivation in a Shake-Flask

Cells were initiated by streaking the −80°C glycerol-containing stock culture on an LB agar plate (LB with 15 g/L Bacto agar). The plate was incubated at 37°C for ~15 h. An isolated single colony was picked to inoculate a 125-mL Erlenmeyer flask containing 25 mL of MPAC medium. The flask was then shaken at 220 rpm and 37°C in a rotary shaker for about 15 h. Four mL of the seed culture was used to inoculate a 250-mL Erlenmeyer flask containing 50 mL of MPAC medium. Batch cultivation was conducted at 200 rpm and 28°C in a reciprocating shaker for ~24 h.

Batch Cultivation in a Bioreactor

Cells were initiated and the seed culture was prepared as described above. Ten mL of the seed culture was used to inoculate a lab-top bioreactor (Omni-Culture, VirTis, Gardiner, NY, USA) containing 1 L of MPAC medium. The medium was supplemented with Antifoam 289 (Sigma, St. Louis, MO, USA) at 10 μL/L to avoid excessive foaming. Filter-sterilized air at 1.5 L/min was purged into the culture for aeration. The culture pH was controlled at 7.0 ± 0.1 by adding 3 N NaOH and 3 N HCl using a combined pH electrode (Mettler-Toledo, Switzerland), a pH controller (PC310, Suntex, Taipei, Taiwan), and two peristaltic pumps (101U/R, Watson Marlow, Falmouth, UK). The bioreactor was operated at 28°C and 500 rpm for ~35 h.

Treatment of Samples

The culture sample was appropriately diluted with saline solution for measuring cell density (in OD_{600}) using a spectrophotometer (V-530, Jasco, Tokyo, Japan). For preparation of the cell extract, the culture sample at 10 mL was centrifuged at 2°C and 6,000*g* for 5 min. The cell pellet was resuspended in 2 mL of sodium phosphate buffer (0.05 M, pH 7.5). The cell suspension was sonicated for 2 min using an ultrasonic processor (Sonics and Materials, Danbury, CT, USA) and then centrifuged at 2°C and 16,000*g* for 15 min. The supernatant containing soluble proteins was used for determining PAC activity.

PAC Enzyme Assay

PAC was assayed at 37°C using penicillin G as a substrate (Gang and Shaikh, 1976). The amount of the enzymatic reaction product, 6-APA, was quantified using a colorimetric method developed previously (Balasingham et al., 1972). One unit is defined as the amount of enzyme hydrolyzing 1.0 μmole of penicillin G per min at 37°C. All assays were performed in duplicate. Volumetric PAC activity (in U/L) is the product of specific PAC activity (in U/L/OD_{600}) and cell density (in OD_{600}).

Measurement of MIC

Strains under the test were streaked on LB agar plates containing penicillin G (or 6-APA) at various concentrations from 20–250 μg/mL. The plates were incubated at 28°C for ~24 h. The MIC_{penG} (or $MIC_{6\text{-}APA}$) was determined as the lowest penicillin (or 6-APA) concentration under which cell growth was inhibited.

RESULTS AND DISCUSSION

Construction of Penicillin-Sensitive but 6-APA-Resistant Mutant, HBPAC101

E. coli HB101 was subjected to random mutagenesis by MNNG. The MNNG-treated cells were spread onto an LB agar plate containing 50 μg/mL 6-APA. Mutant colonies were restreaked on two LB agar plates containing 50 μg/mL 6-APA and 50 μg/mL penicillin G. Several mutants resistant to both antibiotics may have acquired an increased level of β-lactamase activity during the mutagenesis. The $MIC_{6\text{-}APA}$ for ~20 6-APA-resistant but penicillin-sensitive mutants was determined and one of them, HBPAC101, with the highest $MIC_{6\text{-}APA}$ of above 100 μg/mL, was selected for further studies. The reason for selecting the strain with the highest $MIC_{6\text{-}APA}$ to conduct the experiments is that, for the mutant strain to survive in an environment with penicillin at a high concentration, any raise of resistance to penicillin due to an increase in PAC-mediated hydrolysis should be accompanied with an increased resistance to the corresponding level of 6-APA. Hence, growth of the plasmid-bearing

mutant strain will not be inhibited by 6-APA, the product of PAC-mediated hydrolysis of penicillin, at an increased level.

To ensure that the mutant strain HBPAC101 could be employed for screening of the *pac* gene, two plasmids of pCLL2902 and pCLL3201 were used for the transformation test with penicillin as a selecting antibiotic. Both plasmids contain the Tc-resistance gene. pCLL3201 contains the whole *pac* gene from *E. coli* ATCC11105, whereas a portion of the *pac* regulatory region is truncated in pCLL2902. Due to the difference in the *pac* regulatory region between the two plasmids, production of PAC is PAA-inducible for pCLL3201, whereas constitutive for pCLL2902 (Chou et al., 1999b). In addition, PAC activity expressed from pCLL2902 is significantly higher than that from pCLL3201 due to more efficient transcription of the *pac* gene (Chou et al., 1999c). Using HBPAC101 as the host and pCLL2902 as the vector, we performed the transformation protocol (Chung and Miller, 1993). The transformants were selected on an LB agar plate containing 50 μg/mL penicillin. The plate was incubated at 28°C since mature PAC is optimally synthesized at a low temperature (Keilmann et al., 1993). Almost all colonies on the transformation plate were verified as Tc-resistant and having PAC activity, indicating that pCLL2902 had been transformed into HBPAC101 by the operation. Notice that no colony appeared when the transformation plate was incubated at 37°C. In addition, pCLL3201 could not be transformed into HBPAC101 with the above operation, possibly because PAC activity was too low. The results suggest that the 6-APA-resistant mutant HBPAC101 could be employed for an easy screening of the plasmid containing the *pac* gene if the PAC expression level is high enough. In other words, resistance to penicillin could become a phenotype of HBPAC101 harboring a *pac*-operon-containing plasmid.

Screening of PAC-Overproducing Strains

Several expression plasmids for production of PAC, including pCLL2902, pCLL3201, pTrcKnPAC2902, and pTrcKnPAC3201, were transformed into HBPAC101 using an appropriate antibiotic for selection of transformants. The MIC_{penG} and specific PAC activity of each host/vector system at an induced or noninduced state were determined. It was observed that the specific PAC activity usually increases with MIC_{penG} (Fig. 3). The results suggest that the strain's MIC_{penG}, which indicates the capability of hydrolyzing penicillin G, could possibly reflect its PAC expression level. On the basis of the assumption, strategies for constructing PAC overproducers could be directed toward isolating mutants with a high MIC_{penG}. Screening of such mutants could be easily performed on an agar plate containing penicillin at a high concentration. To demonstrate the idea, HBPAC101 harboring pCLL2902 was subjected to MNNG-mutagenesis. The MNNG-treated cells were spread onto an LB agar plate containing penicillin G at a concentration significantly higher than the MIC_{penG} for

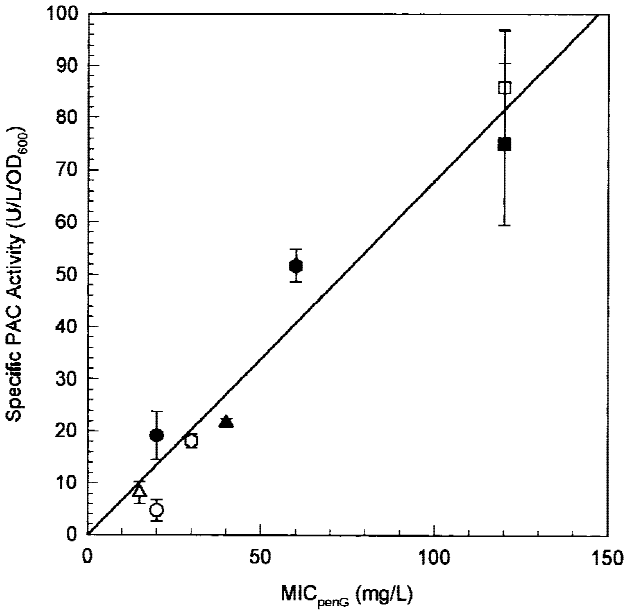

Figure 3. Relationship between the strain's MIC_{penG} and specific PAC activity. HBPAC101 was the host for harboring a selection of expression plasmids (squares, pCLL2902; circles, pCLL3201; hexagons, pTrcKnPAC2902; triangles, pTrcKnPAC3201). Open symbols represent the experiments in which culture medium or agar was not supplemented with an inducer (PAA for pCLL2902 and pCLL3201; IPTG for pTrcKnPAC2902 and pTrcKnPAC3201). Closed symbols represent the experiments in which culture medium or agar was supplemented with an inducer. Results are averages of three identical cultivations.

HBPAC101 harboring pCLL2902 (i.e., 120~200 μg/mL). About 150 mutant strains appeared and those with an MIC_{penG} higher than 200 μg/mL were considered as possible PAC-overproducing mutants and were stored for further investigation. The mutant strains were first identified for the PAC-producing capability by the microbiological test using *S. marcenscens* ATCC27117. Then the MIC_{penG} and specific PAC activity of each mutant were determined. As shown in Table I, production of PAC for several mutant strains was significantly improved compared to that for the parent strain of HBPAC101 harboring pCLL2902. The PAC-overproducing mutants (e.g., MA8, MC1, MC10, MD7, or ME5) had possibly acquired a mutation(s) on the gene(s) critically involved in the mechanism(s) related to the *pac* gene expression. The increased PAC activity for such mutant strains was reflected by an improved capability of hydrolyzing penicillin. The other mutants with a high MIC_{penG} but without a significant increase in PAC activity (e.g., MC3) might have acquired a mutation(s) to the cell wall structure or that resulting in enhanced non-PAC-mediated degradation of penicillin. All the above PAC-overproducing mutants were verified as having no β-lactamase activity using an LB agar plate containing 25 μg/mL ampicillin, indicating that the increased level of penicillin-resistance was not caused by β-lactamase. Notice that the probability of finding PAC-overproducing strains among the subpopulation of mutants, generated through the above penicillin-prescreening, was rather high (i.e., at least 5/11).

Table I. MIC_{penG} and specific PAC activity of the mutant strains constructed in this work.

Strain	Control	MA8	MB9	MC1	MC3	MC4
MIC_{penG} (μg/mL)	120	220	200	160	240	240
Cell density (OD_{600})	6.4 ± 0.1	6.5 ± 0.2	7.0 ± 0.3	6.1 ± 0.2	7.5 ± 0.1	6.3 ± 0.0
Specific PAC activity ($U/L/OD_{600}$)	48.3 ± 1.2	94.5 ± 3.3	51.7 ± 8.4	74.9 ± 23.8	34.7 ± 1.2	56.7 ± 1.9
Strain	MC10	MD1	MD7	ME2	ME4	MF1
MIC_{penG} (μg/mL)	230	200	260	240	200	280
Cell density (OD_{600})	6.5 ± 0.1	4.7 ± 0.1	6.8 ± 1.7	5.2 ± 0.4	7.8 ± 0.3	3.4 ± 0.1
Specific PAC activity ($U/L/OD_{600}$)	71.0 ± 3.3	63.8 ± 2.3	96.7 ± 12.2	106.5 ± 30.1	69.7 ± 3.6	147.5 ± 9.9

Cultivation was conducted in a shake-flask. Results are averages of at least three identical cultivations. The control experiment was conducted using the parent strain, HBPAC101 harboring pCLL2902.

It should be mentioned that the mutation(s) acquired by the PAC-overproducing strains might not be unique, though precise mapping of the mutation(s) is usually laborious.

Localization of the Mutation in MD7

MD7, which has the highest volumetric PAC activity and a relatively high specific PAC activity among the above PAC-overproducing mutant strains, was selected for localization of the mutation(s) resulting in enhanced PAC production. The MIC_{penG} of MD7 was 240 μg/mL and the strain's volumetric and specific PAC activities were significantly higher than those of HB101 (or HBPAC101) harboring pCLL2902. The plasmid harbored in MD7 (named as pCLL2902-D7) was purified and retransformed into HBPAC101. On the other hand, the host (named MDΔP7) was cured by elimination of the harbored plasmid in MD7 and then pCLL2902 was retransformed into MDΔP7. Culture performances in a bioreactor using four host/vector systems of HBPAC101 harboring pCLL2902, HBPAC101 harboring pCLL2902-D7, MDΔP7 harboring pCLL2902, and MD7 (i.e., MDΔP7 harboring pCLL2902-D7) are compared in Figure 4. Time profiles of cell growth, specific PAC activity, and volumetric PAC activity for HBPAC101 harboring pCLL2902 were about the same as those for HBPAC101 harboring pCLL2902-D7, whereas the time profiles for MDΔP7 harboring pCLL2902 were about the same as those for MD7. While the cell growth rate of MDΔP7 harboring pCLL2902 (or MD7) was much slower than that of HBPAC101 harboring pCLL2902 (or HBPAC101 harboring pCLL2902-D7), the final PAC activity (both specific and volumetric) for the culture of MDΔP7 harboring pCLL2902 (or MD7) was about twofold that for HBPAC101 harboring pCLL2902 (or HBPAC101 harboring pCLL2902-D7). The results suggest that the mutation(s) resulting in the improvement of culture performance occurred on the host chromosome rather than the plasmid. In other words, a host (i.e., MDΔP7) more compatible to the PAC expression vector (i.e., pCLL2902) was constructed by a chromosomal mutation(s) for improving production of PAC. Notice that production of PAC was slightly enhanced by the mutation(s) resulting in the 6-APA-resistance and the improvement in PAC production for MD7 was primarily caused by the mutation(s) resulting in the increase of MIC_{penG} (Fig. 4). Since production of PAC for HB101 harboring pCLL2902 is limited by translation (Chou et al., 1999c), the mutation(s) occurring in MDΔP7 might somehow enhance translation efficiency for the *pac* gene expression. Given the fact that the exact mutation point(s) in MDΔP7 is probably difficult to map, the current results still demonstrate the industrial feasibility of using penicillin for screening of PAC-overproducing strains and improving recombinant PAC production process.

Enhanced PAC-Producing Capability for MDΔP7

To further investigate whether MDΔP7 has a generic feature of enhanced PAC-producing capability, two other expression plasmids, pCLL3201 and pTrcKnPAC2902, were transformed into HBPAC101 and MDΔP7, respectively, for making various host/vector systems. Production of PAC in a bioreactor for recombinant MDΔP7 was compared with that for recombinant HBPAC101 (Fig. 5). It was found that PAC activity was not improved for MDΔP7 harboring pCLL3201, whereas production of PAC for MDΔP7 harboring pTrcKnPAC2902 was, similar to MDΔP7 harboring pCLL2902, significantly enhanced when compared to HBPAC101 harboring the corresponding expression plasmid. While the cell growth rate of MDΔP7 harboring pTrcKnPAC2902 was much slower than that of HBPAC101 harboring pTrcKnPAC2902, the final PAC activity (both specific and volumetric) for the culture of MDΔP7 harboring pTrcKnPAC2902 was more than twofold that for HBPAC101 harboring pTrcKnPAC2902. It should be emphasized that the final PAC expression level of more than 350 $U/L/OD_{600}$ for MDΔP7 harboring pTrcKnPAC2902 was extremely high. The results suggest that the enhanced PAC-producing capability for MDΔP7 is not generic and the use of an appropriate expression plasmid compatible to the host would be necessary for optimal production of PAC. As previously reported (Chou et al., 1999c), expression of

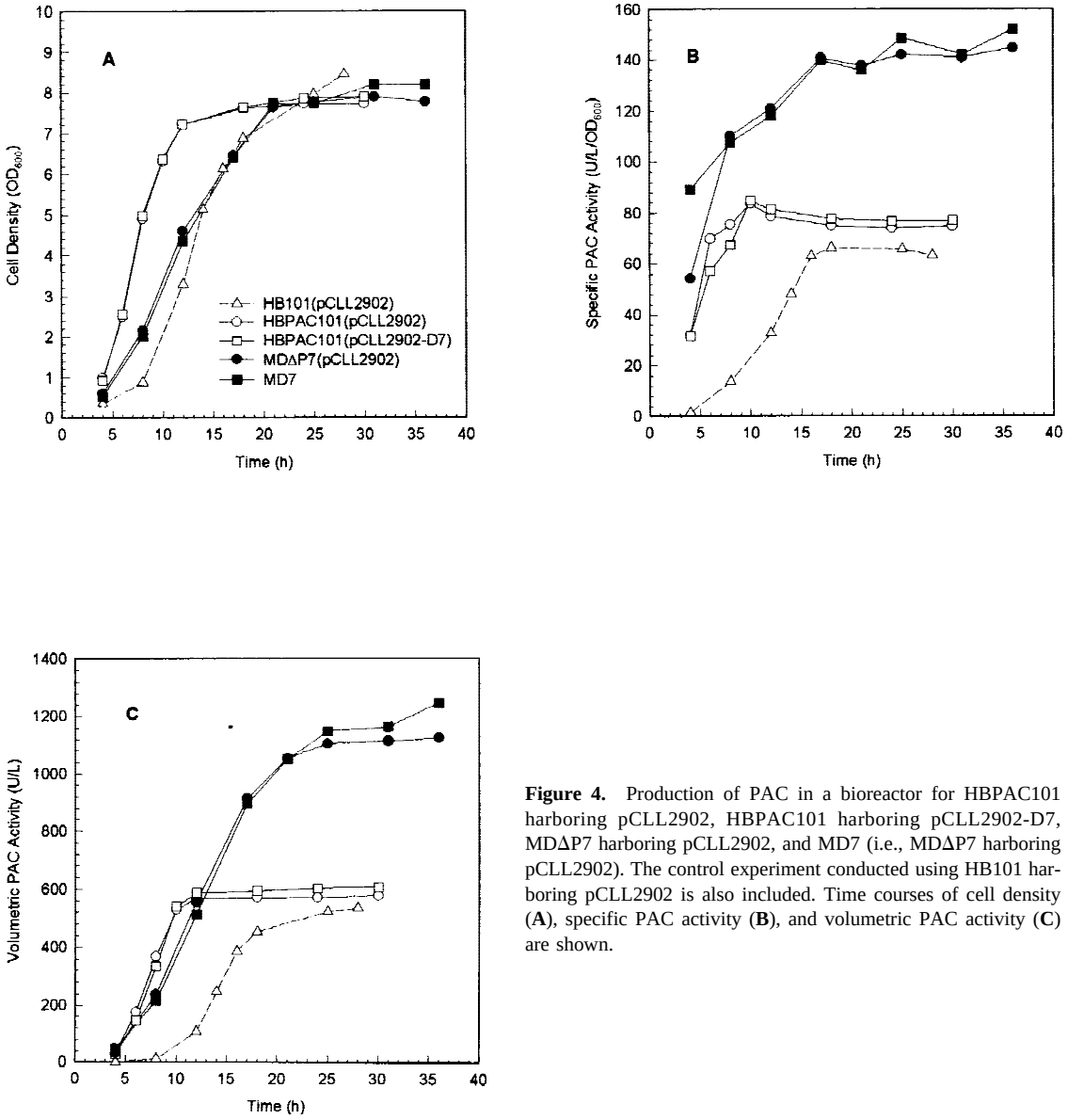

Figure 4. Production of PAC in a bioreactor for HBPAC101 harboring pCLL2902, HBPAC101 harboring pCLL2902-D7, MDΔP7 harboring pCLL2902, and MD7 (i.e., MDΔP7 harboring pCLL2902). The control experiment conducted using HB101 harboring pCLL2902 is also included. Time courses of cell density (**A**), specific PAC activity (**B**), and volumetric PAC activity (**C**) are shown.

the *pac* gene for pCLL3201 is limited by transcription, whereas that for pCLL2902 and pTrcKnPAC2902 is limited by translation, since transcription efficiency was significantly improved by manipulating the *pac* regulatory region and using the strong promoter (*trc*) system, respectively. Because improving production of PAC in MDΔP7 was effective only upon the use of pCLL2902 or pTrcKnPAC2902, the results support the above argument that the mutation(s) occurred in the mutant strain could be somehow involved in the enhancement of translation efficiency for the *pac* gene expression.

CONCLUSIONS

A novel and simple screening method on the basis of using penicillin for constructing PAC overproducers was developed. It was observed that the mutant strains with a high MIC_{penG} usually overproduce PAC. The strategy has several advantages over the application for improving PAC production. First, it provides a logical method for an efficient screening of PAC-overproducing strains. Second, it could save the screening cost by generating a subpopulation of mutants for investigation and the number of "false" mutants could be, therefore, reduced. Third, it could complement several drawbacks of the microbiological test using *S. marcenscens* ATCC27117 and the screening efficiency could be significantly enhanced by combining the two methods. Fourth, the screening chemical, penicillin G, is common and inexpensive. Finally, the screening protocol is simple and easy to follow. The same strategy could be applied to derive mutants with an improved PAC activity from the wild-type PAC-producing strains (e.g., ATCC11105 or

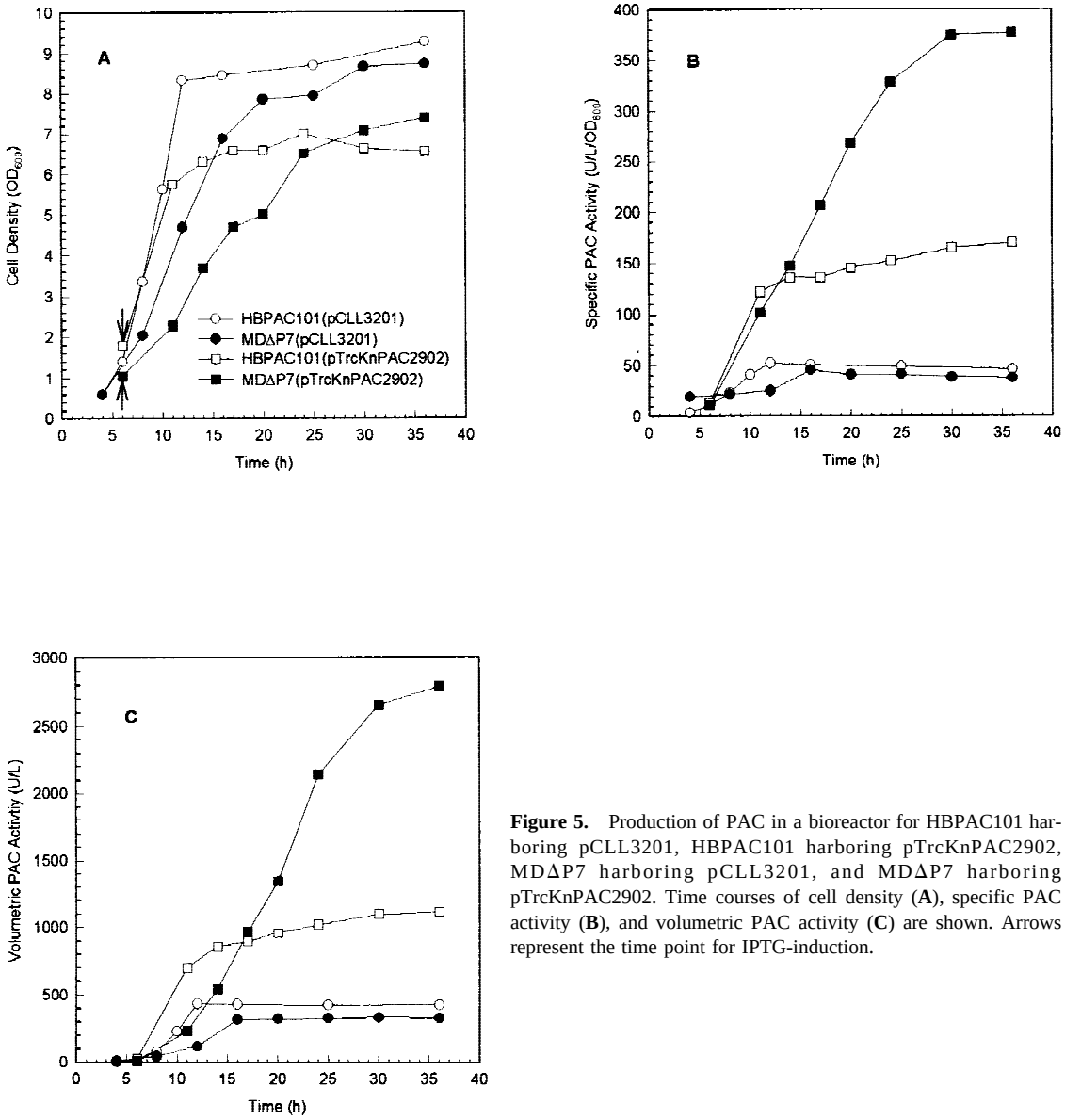

Figure 5. Production of PAC in a bioreactor for HBPAC101 harboring pCLL3201, HBPAC101 harboring pTrcKnPAC2902, MDΔP7 harboring pCLL3201, and MDΔP7 harboring pTrcKnPAC2902. Time courses of cell density (**A**), specific PAC activity (**B**), and volumetric PAC activity (**C**) are shown. Arrows represent the time point for IPTG-induction.

bacteria other than *E. coli*). One of the mutants constructed in this work, MDΔP7, was shown to be effective in production of recombinant PAC using several expression plasmids for which expression of the *pac* gene is limited by translation. The PAC expression level of more than 350 U/L/OD_{600} for one of our clones, MDΔP7 harboring pTrcKnPAC2902, was extremely high and the strain, therefore, might be of interest for industrial application. Results of the study also demonstrate an important strategy (i.e., developing an optimal host/vector system) that is widely addressed for improving recombinant protein production.

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