An Approach for Enhancing Heterologous Production of *Providencia rettgeri* Penicillin Acylase in *Escherichia coli*

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Heterologous production of *Providencia rettgeri* penicillin acylase (PAC) was optimized in *Escherichia coli*. Several factors, including carbon, temperature, and host effects, were identified to be critical for the enzyme overproduction. The optimum culture conditions for the enzyme production vary for different host/vector systems. With the optimization, both volumetric and specific PAC activities could be significantly improved by more than 50-fold compared to the native expression in *P. rettgeri*. The heterologous production could be possibly limited by translation or posttranslational steps, depending on the culture temperature and host/vector system. To our knowledge, this is the first evidence demonstrating the limiting step for the production of *P. rettgeri* PAC and the existence of the *P. rettgeri* PAC precursor.

Introduction

Penicillin acylase is an industrial enzyme primarily dedicated to penicillin hydrolysis for the production of 6-aminopenicillanic acid (6-APA), which is a critical starting compound for making several β-lactam antibiotics (Shewale et al., 1990; Shewale and Sivaraman, 1989). Bacterial strains, such as *Arthrobacter viscosus* (ATCC15294) (Konstantinovic et al., 1994), *Bacillus megaterium* (ATCC14954) (Martin et al., 1995), *Escherichia coli* (ATCC11105) (Oh et al., 1987), *Kluyvera cryocrescens* (ATCC21285) (Barbero et al., 1986), and *Providencia rettgeri* (ATCC31052) (Ljubijankic et al., 1992), are sources of type II PAC whose substrate is penicillin G. These PACs share the same heterodimer protein structure, and their amino acid sequences reveal high homology, suggesting that they might originate from the same ancestral gene. Although *E. coli* PAC is the most common source for industrial application, the applicability of other bacterial PACs has been constantly evaluated with promising results. For example, *B. megaterium* PAC exhibits broad substrate specificity on various cephalosporins, such as cephalexin and cefamandole (Kang et al., 1991). PAC from *A. viscosus* (Ohashi et al., 1989) or *B. megaterium* (Kang et al., 1991) could be produced in a large scale since most of the enzyme natively expressed or heterologously expressed in *Bacillus subtilis* could be extracellularly secreted. *K. cryocrescens* PAC has attracted interest because of many features suitable for industrial application, such as ease of immobilization and endurable stability against heat, pH, and organic solvents (Alvaro et al., 1992). Attempts based on genetic and protein engineering techniques were made to manipulate *P. rettgeri* PAC for acquiring cephalosporin C acylase activity, but with little success so far (Daumy et al., 1985b; Klei et al., 1995).

We are interested in developing strategies for overproducing *P. rettgeri* PAC through the application of recombinant DNA technology. Both the heterodimer protein structure (Daumy et al., 1985a) and the fact that the amino acid sequence of *P. rettgeri* PAC (Ljubijankic et al., 1992) is highly homologous to that of *E. coli* PAC lead to the hypothesis that the enzyme formation mechanism of *P. rettgeri* PAC is similar to that of *E. coli* PAC (Sizmann et al., 1990). It was recently reported that, depending on the host/vector system, the production of *E. coli* PAC could be limited by transcription, translation, or posttranslational steps (Chou et al., 1999b). In addition, the expression of the *E. coli pac* gene is usually subjected to regulation by several environmental conditions, such as temperature (Keilmann et al., 1993) and carbon source (Merino et al., 1992). It is also possible that the above limitations could occur upon the production of *P. rettgeri* PAC. In this study, the environmental (specifically, temperature and carbon) and host effects on the heterologous expression of *P. rettgeri* PAC in *E. coli* were investigated. A mutant strain of MDΔP7, derived from HB101 by a novel screening method, appeared to be quite suitable for the expression of the *E. coli pac* gene (Chou et al., 1999a). The screening operation was demonstrated to be useful in terms of deriving mutants with an enhanced PAC producing ability (Chou et al., 1999a). The feasibility of using the mutant strain for the heterologous production of *P. rettgeri* PAC was evaluated. Optimization of the enzyme production was conducted by taking the above culture parameters into account. With the optimization, PAC activity was more than 50-fold that of the native expression in *P. rettgeri*. In addition, the steps limiting the enzyme formation were identified and discussed.

Materials and Methods

HB101 (Boyer and Roulland-Dussoix, 1969), MDΔP7 (Chou et al., 1999a), a mutant derived from HB101 by screening under high-concentration penicillin, and JM109 (Yanisch-Perron et al., 1985), which overproduces LacI repressors, were used as the hosts for the production of *P. rettgeri* PAC. The plasmid, pUTKnPAC2601, carries the *P. rettgeri pac* gene, whose expression is controlled by the *tac* promoter (Figure 1). To construct the plasmid, the *pac* gene from *P. rettgeri* ATCC31052 genomic DNA was first amplified by polymerase chain reaction (PCR).

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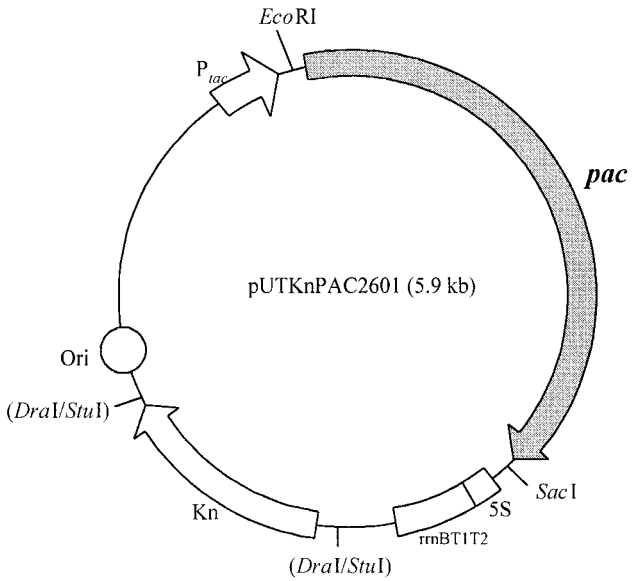

Figure 1. Plasmid restriction map for pUTKnPAC2601. Expression of the *pac* gene is regulated by the *tac* promoter. Arrows represent the direction of transcription for each gene.

The PCR product at about 2.7 kb was inserted into the pCR-Script Cam SK(+) cloning vector (Stratagene) to form pCL2601. Because pUT18 (Omori et al., 1994) contains the *bla* gene, whose gene product of β-lactamase tends to attack the β-lactam bond of penicillin and 6-APA and, hence, affect PAC enzyme assay, the *bla* gene was replaced with a Kn-resistance cassette to form pUTKn18 according to the procedures for derivation of pTrcKn99A from pTrc99A (Chou et al., 1999b). The 2.7-kb *Eco*RI/*Sac*I fragment from pCL2601 containing the *pac* gene was subcloned into pUTKn18 to form pUTKnPAC2601, and the expression of the *pac* gene was regulated by the *tac* promoter-operator system. Cultivation, preparation of cell extract, PAC enzyme assay, and SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE) were conducted according to the previous protocols (Chou et al., 1999b).

Results and Discussion

Effects of Culture Environment and Host. Using JM109 harboring pUTKnPAC2601 as the expression system, the effect of a selection of carbons, including fructose, glucose, glycerol, sorbitol, and sucrose, on the heterologous production of *P. rettgeri* PAC in *E. coli* was investigated; the results are summarized in Table 1. The heterologous expression was severely repressed by several carbons, including fructose, glucose, sorbitol, and sucrose. Since no CRP-binding site was found upon analyzing the DNA sequence of the *P. rettgeri pac* regulatory region (Ljubijankic et al., 1992), it is possible that synthesis of certain protein(s), which is critically involved in the mechanism of *P. rettgeri* PAC production, rather than acylase itself, in *E. coli* was catabolically repressed by these carbons. It appeared that glycerol was an appropriate carbon source for the production of *P. rettgeri* PAC in *E. coli*. Cell density was increased without affecting the specific PAC activity by adding glycerol up to 5 g/L, resulting in a significant improvement in the volumetric PAC activity. The above carbons posed minimum effects on the native expression in *P. rettgeri*, and the PAC expression level was extremely low compared to that of the heterologous expression (data not shown).

E. coli PAC is optimally synthesized at a low temperature (i.e., below 30 °C), and the enzyme production is minimal at 33 or 37 °C (Keilmann et al., 1993). This is also the case for the native expression of *P. rettgeri* PAC in ATCC31052 (data not shown). Such a low production temperature (e.g., 28 °C) is unfavorable since the optimum growth temperature for most of the *E. coli* strains is approximately 37 °C. The heterologous expression of the *P. rettgeri pac* gene in *E. coli* was optimal at a temperature as high as 33 °C for several pUTKnPAC2601-harbored strains (Table 2). Moreover, a large portion of PAC activity was retained even at 37 °C (e.g., JM109 harboring pUTKnPAC2601). The host/vector systems offer an opportunity for the high-temperature-oriented PAC production as well as for shedding light on temperature regulation with respect to the *pac* gene expression.

The glycerol and temperature effects were also investigated by using HB101 or its derivative, MDΔP7, as the expression host; the results are summarized in Table 2. The specific PAC activity for HB101 harboring pUTKnPAC2601 was approximately the same as that for JM109 harboring pUTKnPAC2601 when glycerol was not added. However, assimilation of culture medium appeared to be rather efficient for HB101 harboring pUTKnPAC2601 or MDΔP7 harboring pUTKnPAC2601, therefore the biomass concentrations and volumetric PAC activities were significantly higher than those of JM109 harboring pUTKnPAC2601 at 28 or 33 °C. Unlike JM109 harboring pUTKnPAC2601, the expression was severely repressed upon adding glycerol for HB101 harboring pUTKnPAC2601 or MDΔP7 harboring pUTKnPAC2601 at 28 or 33 °C, indicating that assimilation of glycerol was not favored for the host/vector systems. The heterologous production for the expression systems was also optimal at a temperature as high as 33 °C without glycerol supplementation.

Limiting Step for PAC Formation. Note that the mutant strain of MDΔP7 generally exhibited a higher enzyme production ability compared to the parent strain of HB101. As previously indicated (Chou et al., 1999a), the efficiency for translation of the *pac* gene in MDΔP7 could be higher than that in HB101. Transcription of the *pac* gene for pUTKnPAC2601 was expected to be efficient since it was regulated by the strong *tac* promoter. Hence, translation became a possible limiting step for the enzyme production. The translation efficiency could be enhanced upon employing MDΔP7 as the host, and the enzyme production was greatly improved. The difference in the translation efficiency between the two strains is

Table 1. Effects of Carbon on the Heterologous Production of *P. rettgeri* PAC in *E. coli* for Host/Vector JM109/pUTKnPAC2601[a]

	carbon					
	none	fructose	glucose	glycerol	sorbitol	sucrose
CD[b]	3.2 ± 0.1	6.5 ± 1.4	3.9 ± 0.3	8.0 ± 0.1	1.5 ± 0.2	4.7 ± 0.1
SA[c]	93.0 ± 6.5	49.8 ± 17.1	11.8 ± 0.8	103.2 ± 0.2	33.2 ± 9.3	63.6 ± 1.3
VA[d]	298 ± 8	324 ± 16	46 ± 4	826 ± 12	50 ± 12	299 ± 15

[a] LB supplemented with each sugar at 5 g/L was used for cultivation at 28 °C. For all cultures, IPTG at 0.1 mM was added for induction [b] Cell density in OD_{600}. [c] Specific PAC activity in U/L/OD_{600}. [d] Volumetric PAC activity in U/L.

Table 2. Effects of Host, Temperature, and Glycerol on the Heterologous Production of *P. rettgeri* PAC in *E. coli*[a]

		host/vector					
		JM109/pUTKnPAC2601		HB101/pUTKnPAC2601		MDΔP7/pUTKnPAC2601	
		no added glycerol	+ glycerol (5 g/L)	no added glycerol	+ glycerol (5 g/L)	no added glycerol	+ glycerol (5 g/L)
28 °C	CD[b]	3.2 ± 0.1	8.0 ± 0.1	4.6 ± 0.0	6.8 ± 0.1	5.0 ± 0.5	6.4 ± 0.1
	SA[c]	93.0 ± 6.5	103.2 ± 0.2	109.2 ± 2.2	23.1 ± 0.5	169.3 ± 7.3	120.0 ± 6.8
	VA[d]	298 ± 8	826 ± 12	505 ± 16	157 ± 1	808 ± 114	773 ± 46
33 °C	CD[b]	3.4 ± 0.4	3.4 ± 0.5	4.7 ± 0.1	6.2 ± 0.3	4.3 ± 0.0	5.2 ± 0.9
	SA[c]	107.3 ± 0.4	91.1 ± 5.8	177.4 ± 5.7	37.5 ± 3.9	190 ± 14	85 ± 14
	VA[d]	364 ± 3	311 ± 63	838 ± 37	234 ± 35	809 ± 62	442 ± 102
37 °C	CD[b]	2.7 ± 0.0	6.7 ± 0.1	2.2 ± 0.3	8.8 ± 0.2	3.9 ± 0.2	7.9 ± 0.4
	SA[c]	40.2 ± 1.3	19.2 ± 1.3	37.7 ± 4.4	41.8 ± 3.4	21.8 ± 8.1	105.9 ± 3.9
	VA[d]	109 ± 2	129 ± 10	82 ± 11	369 ± 35	85 ± 14	837 ± 25

[a] LB was the base medium for cultivation. For all cultures, IPTG at 0.1 mM was added for induction. [b] Cell density in OD_{600}. [c] Specific PAC activity in U/L/OD_{600}. [d] Volumetric PAC activity in U/L.

particularly significant when cultured at 37 °C. A protein band near 95 kD, which is the estimated size of the PAC precursor, was observed in the insoluble fraction of the cell extract for MDΔP7 harboring pUTKnPAC2601 at 37 °C (Figure 2). The band was not clear for HB101 harboring pUTKnPAC2601 at 37 °C and was not even observed for both strains when cultured at 33 °C or below. The results suggest that the heterologous production in the current expression systems was limited by translation at 28 or 33 °C, and even at 37 °C for HB101 harboring pUTKnPAC2601. The problem of translation limitation was, to some extent, resolved by employing MDΔP7 as the expression host, and the enzyme production was improved at 28 or 33 °C. Translation of the *pac* gene for MDΔP7 harboring pUTKnPAC2601 could be very efficient at 37 °C, and the enzyme production was limited by the posttranslational steps. PAC activity was, however, low since most of the synthesized PAC precursors formed insoluble protein aggregates without enzyme activity. To our knowledge, this is the first report demonstrating the limiting step for *P. rettgeri* PAC formation and the existence of the *P. rettgeri* PAC precursors. The observations support a common speculation that the enzyme formation mechanism of *P. rettgeri* PAC is similar to that of *E. coli* PAC (Sizmann et al., 1990).

Optimization of PAC Production. Since the effects of carbon, temperature, and host on the heterologous production are relevant, optimization of the culture performance was conducted by taking these factors into account. For JM109 harboring pUTKnPAC2601 cultured at 28 °C, the specific PAC activity was relatively high (93 U/L/OD_{600}), but cell growth was, to some extent, inhibited (3.2 OD_{600}). The specific PAC activity could be maintained without gaining biomass with increase in temperature to 33 °C (107 U/L/OD_{600}). The growth inhibition was avoided by adding glycerol or using other host strains, such as HB101 or MDΔP7. Cell density was increased to 8.0 OD_{600} and the specific PAC activity was well maintained (103 U/L/OD_{600}) for JM109 harboring pUTKnPAC2601 at 28 °C with glycerol supplementation, indicating that glycerol was an appropriate carbon for the host/vector system. On the other hand, the specific PAC activity was also high for HB101 harboring pUTKnPAC2601 cultured at 28 °C (109 U/L/OD_{600}), whereas it was even higher for MDΔP7 harboring pUTKnPAC2601 (169 U/L/OD_{600}). The specific PAC activity was significantly improved by increasing temperature to 33 °C for HB101 harboring pUTKnPAC2601 or MDΔP7 harboring pUTKnPAC2601 (177 or 190 U/L/OD_{600}). Supplementation of glycerol, however, did not improve the culture performance under the three temperatures investigated for HB101 or MDΔP7 harboring pUTKnPAC2601 except for MDΔP7 harboring pUTKnPAC2601 at 37 °C.

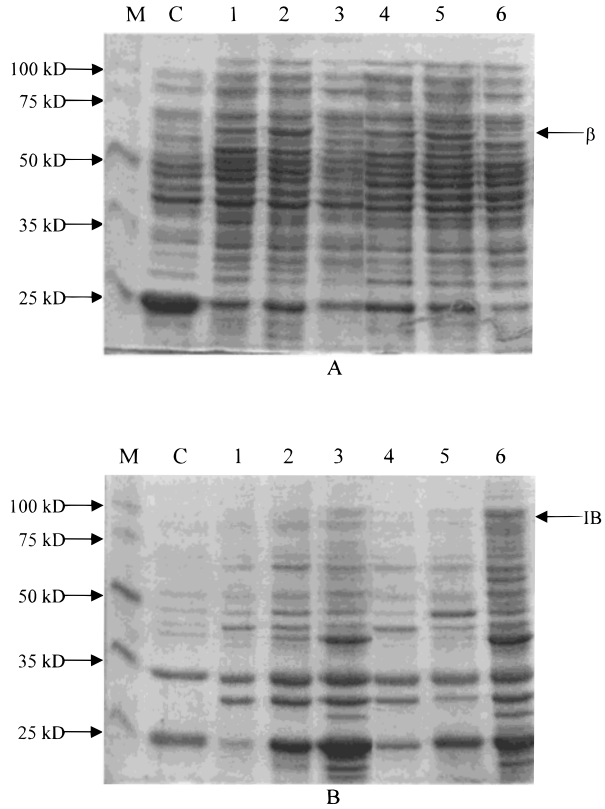

Figure 2. Results of SDS−PAGE analysis on protein contents for soluble (A) and insoluble (B) fractions of cells cultured in LB (without glycerol) at different temperatures. Lane M, markers (numbers are band sizes in kD); lane C, HB101 harboring pUTKn18 as the control experiment; lanes 1−3, HB101 harboring pUTKnPAC2601 at 28, 33, and 37 °C; lanes 4−6, MDΔP7 harboring pUTKnPAC2601 at 28, 33, and 37 °C; IB, inclusion body; β, β subunit. Note that the concentrations of the β bands for various samples in panel A are in agreement with the corresponding PAC activities in Table 2. The protein aggregates of PAC precursors appeared only for MDΔP7 harboring pUTKnPAC2601 at 37 °C.

In summary, the environmental and host effects were critical for the overproduction of heterologous *P. rettgeri* PAC in *E. coli*. The optimum culture conditions vary for different host/vector systems. The expression systems offer an opportunity for high-temperature-oriented production of PAC that is usually expressed at a low temperature. The heterologous production was repressed by several carbons, including fructose, glucose, sorbitol,

and sucrose, whereas it could be potentially enhanced by glycerol. However, the effects of glycerol supplementation were highly dependent on the expression system and culture temperature. The enzyme was optimally produced at 28 °C with glycerol supplement for JM109 pUTKnPAC2601 and at 33 °C without glycerol supplement for HB101 or MDΔP7 harboring pUTKnPAC2601. It should be emphasized that the optimum volumetric PAC activity for the heterologous production in this work (approximately 800 U/L) was more than 50-fold that of the native expression in *P. rettgeri* (approximately 15 U/L).

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