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Screening of *Pseudomonas* sp. for Cephalosporin Acylase Activity

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Abstract: Medically useful semisynthetic cephalosporin antibiotics are made from precursor 7-aminocephalosporanic acid (7-ACA). Cephalosporin Acylase (CA), which catalyzes hydrolysis of both glutary l-7-aminocephalosporanic acid (GL-7ACA) and cephalosporin C (CPC) to 7-ACA, is thus a very important enzyme for producing semisynthetic beta-lactam antibiotics. The cephalosporin acylase can be found in several *Pseudomonas* sp. (such as *P. putida*, *P. cepacia* BY 21 *P. nitroreducens*, *P. syringae*, p. SE83, p. V22, p. SY-77, p. sp.130 ,...) and other bacteria. Therefore, screening of cephalosporin asylase positive pseudomonas is very important. We isolated 130 *Pseudomonas* sp. from clinical and environmental specimens (75 clinical samples, 40 hospital environment samples, 10 water samples, 5 soil samples). All of isolated *Pseudomonas* sp. were tested for cephalosporin acylase by P.C.R method and analysis of gel electrophoresis patterns. We found only 2 cephalosporin acylase pseudomonas positive from clinical samples. These strains were selected for sequencing and cloning in *E. coli* and assessment of gene expression and cephalosporin acylase production in others stages of our study.

Key words: *Pseudomonas* sp., cephalosporin acylase, gene cloning, semisynthetic cephalosporin

INTRODUCTION

The cephalosporins belong to the family of b-lactam antibiotics. These are named after the reactive moiety of the compounds, the b-lactam ring. In CPC, the four membered b-lactam ring is coupled to a 6-membered dihydrothiazine ring to form the nucleus, 7-aminocephalosporanic acid (7-ACA) and a side chain, α-aminoadipic acid, is coupled viaan amide bond to the nucleus. The semi-synthetic cephalosporins can be produced from the cephalosporin nucleus, either 7-ACA or 7-aminodesacetoxycephalosporanic acid (7-ADCA) (Linda *et al*., 2002; Zhu *et al*., 2003).

The cephalosporin acylases are b-lactam acylases, highly specialized peptidases that are capable of cleaving the amide bond between a b-lactam nucleus and a side chain without damaging the b-lactam ring. b-lactam acylases are divided into two groups: those that accept substrates with aromatic side chains, also known as the penicillin acylases (EC 3.5.1.11) and those that accept substrates with a charged, aliphatic side chain, also known as the cephalosporin acylases (no separate EC entry, usually classified as well as 3.5.1.11). However, these trivial names can be confusing, as a cephalosporin with an aromatic side chain, e.g. phenylacetyl-7-ADCA, will not be hydrolyzed by a cephalosporin acylase, but is a substrate for the penicillin acylases. A proposal for a new classification based on side chain substrate specificity, e.g. dividing the b-lactam acylases into aromatic acylases and dicarboxylic acid acylases has not been adopted by the other groups working in this area. The physiological role of the b-lactam acylases is unknown. Deacylation of b-lactam antibiotics does not destroy the active b-lactam ring, so this cannot be their natural function. Penicillin acylases are presumably involved in nutrient scavenging, but there are no indications yet of the role of cephalosporin acylases. The b-lactam acylases are used commercially for the hydrolysis of b-lactams into the intermediates for the production of semi-synthetic penicillins and cephalosporins, 6-APA, 7-ACA and 7-ADCA. However, they can also be used for a variety of other reactions, including the addition of alternative side chains to b-lactam nuclei to form the clinically used semisynthetic penicillins and cephalosporins and the kinetic resolution of stereoisomers out of racemic mixtures by stereoselective acylation and/or deacylation. Recent key publications on alternative uses are the cephalosporin acylase catalyzed addition of the glutaryl side chain to amines at low water concentrations and the use of

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cephalosporin acylase for resolution of stereoisomers (Nat, 2006; Lee *et al*., 2000; Fritz-Wolf *et al*., 2002).

Seven-Amino Cephalosporanic Acid (7-ACA) is the starting material for the industrial production of most cephalosporin antibiotics. It can be obtained by chemical deacylation of cephalosporin C (CPC) produced by fermentation. However, chemical methods such as those using iminoether and nitrosyl chloride involve many expensive steps with ecological problems. Therefore, enzymatic methods of deacylation are of great interest. Two types of cephalosporin acylases have been found: glutary l-7-ACA acylase (GL-7ACA acylase) has high activity on GL-7ACA but much lower activity on CPC and cephalosporin C acylase (CPC acylase) is active on both CPC and GL-7ACA. GL-7ACA acylase could be used in the production of 7-ACA from CPC which has first been oxidized to GL-7ACA by D-amino acid oxidase, while CPC acylase could be employed directly to convert CPC to 7-ACA (Li *et al*., 1999; Aramori *et al*., 1991; Matsuda and Komatsu, 1985; Ishiye and Niwa, 1992).

Although, the commercial and technological aspects of cephalosporin acylases have been studied extensively, their primary structures and enzymological properties have been reported only recently. Ten genes encoding cephalosporin acylases from different sources have been cloned. The gene encoding a cephalosporin acylase from *Pseudomonas* sp. 130 (CA-130) has been expressed in *Escherichia coli* and some properties of the enzyme studied. The DNA sequence and sequencing strategy, the detailed alignment of CA-130 with the cephalosporin acylases from Pseudomonas GK16 and C427 and penicillin G acylase from *E. coli* and the preliminary X-ray diffraction analysis of CA-130 will be described elsewhere (Wang and Zheng, 2002; Chen and Li, 1998).

The cephalosporin acylase can be found in several *Pseudomonas* sp. (such as *P. putida, P. cepacia* BY 21 *P. nitroreducens*, *P. syringae*, p. SE83, p. V22, p. SY-77, p. sp. 130 ,...) and other bacteria (Linda *et al*., 2002; Shi *et al*., 1991; Zha and Yang, 2003). Therefore isolation of cephalosporin acylase positive *Pseudomonas* sp. from clinical and environmental specimens is very important.

There are over 230 described species in the genus Pseudomonas. Approximately 50 species are divided into five major subgroups with the fluorescent group being the largest (roughly 24 species). Because there are so many strains within these subgroups that differ only in minor biochemical properties, these are further divided into biovars instead of an endless list of closely related species. For example, *P. fluorescens* contains 5 biovars. Widely distributed in nature, some species are pathogenic for humans, animals, or plants. The pseudomonads are the largest group of Gram negative aerobic heterotrophic bacteria found in soil, several species have been described as aquatic and many are associated with the rhizosphere of plants. They have been widely studied because of the amazing metabolic diversity within this genus. The *Pseudomonads* have been heavily used in biological disease control and bioremediation. The literature on degradation of organic compounds probably contains more citations on *Pseudomonas* than any other bacterial genus (Pollegioni *et al*., 2005)

Ten genes encoding cephalosporin acylases have been cloned. The acylases were classified into two types: GL-7ACA acylase and CPC acylase and then subdivided into several groups for industrial convenience. In enzymology, this classification is not reasonable because CPC acylase hydrolyzes GL-7ACA more efficiently than CPC. For example, the representative engineered CPC acylase from Pseudomonas N176 deacalyted CPC at a rate that was only one-fifteenth of that seen with GL-7 ACA. In addition, CA-130 showed higher activity on GL-7ADCA than GL-7ACA. The physiological role and true substrates of cephalosporin acylases *in vivo* have not yet been clarified, so it is difficult to classify the acylases according to their substrates (Yang, 1992; Lee, 1998).

Our Purposes in deferent stages of this research are:

- Screening of cephalosporin acylase positive *Pseudomonas* sp. from clinical and environmental specimens by PCR method.
- Isolation and sequencing of cephalosporin acylase gene from pseudomonas sp and cloning it in *E. coli*.
- Assessment of expression gene in *E. coli*.

MATERIALS AND METHODS

We tested many clinical (patient samples) and environmental (water, soil and hospital environment) samples (about 300 samples) for *Pseudomonas* sp. The samples were cultured in general and selective media and the routine biochemical laboratory tests (such as oxidase test, oxidation-fermentation test...) were used for diagnosis of *Pseudomonas* sp. In this research, we isolated 130 *Pseudomonas* sp. All of isolated *Pseudomonas* sp. were tested for cephalosporin acylase by P.C.R method. For extrication of DNA, 1.5 mL overnight culture of *Pseudomonas* in LB broth (with 0.8 OD) in eppendorf tube was centrifuge for 9000 rpm for 3 min and supernatant was discard and then, cell pellet was resuspended in 400 μL of TE buffer containing SDS (1%), protease K (10 μg mL^{-1}) and incubated overnight at 40 °C followed by RNase treatment, phenol-cholorphorm extraction and isopropanol precipitation We

designed the suitable forward and reverse primers for P.C.R and then, we used P.C.R and gel electrophoresis for screening and selection of cephalosporin acylase positive pseudomonas by analysis of gel electrophoresis patterns.

RESULTS AND DISCUSSION

In this research, we isolated 130 *pseudomonas sp* from clinical and environmental samples (75 clinical samples, 40 hospital environment samples, 10 water samples, 5 soil samples) Fig. 1.

We tested all of isolated *pseudomonas* for cephalosporin acylase gen by P.C.R method by analysis of electrophoresis pattern. We found only 2 cephalosporin acylase positive *pseudomonas* isolates from clinical samples. These strain were selected for sequencing and cloning in *E. coli* and assessment of gene expression in others stages of our study.

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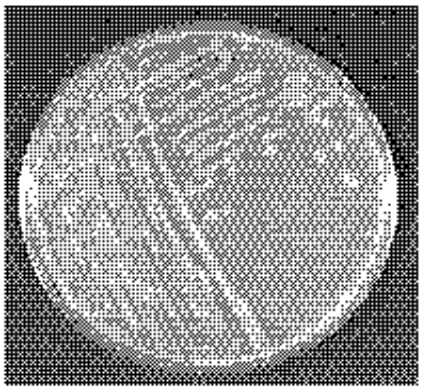

Fig. 1: *Pseudomonas* in tryptic soy agar

CONCLUSION

Two cephalosporin acylase positive pseudomonas were isolated in this research. These strains are new source of cephalosporin acylase gene for extraction, cloning in *E. coli* and assessment of gene expression.

REFERENCES

Aramori, I., M. Fukagawa, M. Tsumura, M. Iwami, H. Ono, H. Kojo, M. Kohsaka, Y. Ueda and H. Imanaka, 1991. Cloning and nucleotide sequencing of a novel 7 beta-(4-carboxybutanamido) cephalosporanic acid acylase gene of Bacillus laterosporus and its expression in *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol., 173 (24): 7848-7855.

Chen, J.F. and Y. Li, 1998. Seecretory Expression of GL-7-ACA acylase in *E. coli*, Sheng Wa Hua, 30 (4): 393-393.

Fritz-Wolf, K., K.P. Koller, G. Lange, A. Liesum, K. Sauber, H. Schreuder, W. Aretz and W. Kabsch, 2002. Structure-based prediction of modifications in glutarylamidase to allow single-step enzymatic production of 7-aminocephalosporanic acid from cephalosporin C. Protein Sci., 11: 92-103.

Ishiye, M and M Niwa, 1992. Nucleotide sequence and expression in E. coli of the cephalosporin acylase gene of a Pseudomonas strain. Biochim. Biophys. Acta, 20; 1132 (3): 233-239.

Lee, Y.S. and S.S. Park, 1998. Two- Step Autocatalytic Processing of the Glutaryl 7-Aminocephalosporanic Acid Acylase from *Pseudomonas* sp. Strain GK16. J. Bacteriol, 180 (17): 4576-4582.

Li, Y., W. Jaing and Y. Yang, 1998. Overproduction and purification of glutaryl 7-amino cephalosporanic acid acylase. Protein Expr Purify, 12 (2): 233-238.

Linda, G. Otten, Charle F. Sio, Johanna Vrielink, Robbert H. Cool and Wim J. Quax, 2002. Altering the Substrate Specificity of Cephalosporin Acylase by Directed Evolution of the-Subunit. J. Biol. Chem., 277 (44): 42121-42127.

Matsuda A. and K. Komatsu, 1985. Molecular cloning and structure of the Gene for 7B-(4-carboxybutanamido) cephalosporanic Acid Acylase from a *Pseudomonas* strain. J. Bacteriol., 163 (3): 1222-1228.

Natl, P., 2006. Insight into autoproteolytic activation from the structure of cephalosporin acylase: A protein with two proteolytic chemistries. Acad. Sci. U.S.A., 103 (6): 1732-1737.

Pollegioni, L., S. Lorenzi, E. Rosini, G.L. Marcone, G. Molla, R. Verga, W. Cabri and M.S. Pilone, 2005. Evolution of an acylase active on cephalosporin C. Protein Sci., 14 (12): 3064-3076.

Shi, L., W.X. Xu and Q.J. Zhang, 1991. 2-Nitro-5-(6-bromohexanoylamino) benzoic acid test paper method for detecting microorganisms capable of producing cephalosporin acylases. Anal. Biochem., 196 (2): 201-206.

Wang, E.D. and V.G. Zheng, 2002. Expression of gene encoding GL-7ACA acylase in *E. coli*, Sheng Wu Hua, 34 (4): 526-531.

Yang, Y., Yund, 1992. Restriction mapping and localization of GL-7-ACA acylase gene. Chin. J. Biotechnol., 8 (1): 15-22.

Yong, Li. and Jian Feng Chen, 1999. *In vivo* post-translational processing and subunit reconstitution of cephalosporin acylase from *Pseudomonas* sp. 130. Eur. J. Biochem., 262: 713-719.

Young Sik Lee, 2000. Hyung Wook Kim and Sung Soo Park, The Role of -Amino Group of the N-terminal Serine of Subunit for Enzyme Catalysis and Autoproteolytic Activation of Glutaryl 7-Aminocephalosporanic Acid Acylase, J. Biol. Chem., 275 (50): 39200-39206.

Zha, S. and Y. Yang, 2003. A rapid and specific method to screen environmental Microorganisms for cephalosporin acylase activity. J. Microbiol Methods., 54 (1): 131-135.

Zhang, W., Y. Liu, H. Zheng, S. Yang and W. Jiang, 2005. Improving the activity and stability of GL-7-ACA acylase CA130 by site-directed mutagenesis. Appl. Environ. Microbiol., 71 (9): 5290-5296.

Zhu, S., X. Huang, G. Zhao and W. Jiang, 2003. Selection of A strain producing beta-lactam antibiotics acylases. Wei Sheng Wu Xue Bao, 43 (1): 79-86.