Discovery of a Novel (+)- γ -Lactamase from *Bradyrhizobium japonicum* USDA 6 by Rational Genome Mining

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A novel (+)- γ -lactamase used for the resolution of racemic γ -lactam from *Bradyrhizobium japonicum* USDA 6 was found as a result of sequence-structure guided genome mining. It consists of 409 amino acids, only 49% of which are identical to the amino acid sequences of the known (+)- γ -lactamase from *Sulfolobus solfataricus*. This is only the third (+)- γ -lactamase gene to be reported.

Over the past few decades, in the search for new antiviral drugs such as abacavir and peramivir, the synthesis and bioactivity screening of carbocyclic nucleosides have received increased attention. Abacavir is a carbocyclic 2'-deoxyguanosine nucleoside analogue that competitively inhibits HIV reverse transcriptase and terminates proviral DNA chain extension (5, 8). It is an important chemotherapeutic agent in the treatment of human HIV and hepatitis B viruses (3, 21). Traditionally, these nucleoside compounds are synthesized by chemical methods, but enzymatic synthesis of nucleosides can be simpler and quicker (7, 17, 19). In the biosynthesis of abacavir, the resolution of the bicyclic synthon (rac)- γ -lactam (2-azabicyclo [2.2.1] hept-5-en-3-one) is the most important step. A biocatalytic process was developed for the resolution of racemic γ -lactam using (+)- γ -lactamase-containing organisms, as shown in Fig. 1 (14).

Amidases (EC 3.5.1.4) are an interesting member of the nitrilase superfamily; they are used to cleave carbon-nitrogen bonds by transferring an acyl group to water to form free acids and ammonia (26). In recent years, amidases have turned out to be versatile biocatalysts in white and green chemistry. They have been the subject of extensive research due to their enantioselective and stereoselective properties (20). (+)- γ -Lactamase is a type of amidase that can cleave the [1(S), 4(R)]-(+)-enantiomers of γ -lactam. After the enzymatic reaction, the desired [1(R), 4(S)]-(-)-enantiomer can further be used to synthesize abacavir. There are six organisms with (+)- γ -lactamase in the resolution of (rac)- γ -lactam that have been reported: *Microbacterium hydrocarbonoxydans* (18), *Sulfolobus solfataricus* (9, 25, 26), *Comamonas acidovorans* (12, 22), *Pseudomonas cepacia* (10, 22), *Pseudomonas solanacearum* (23, 24), and *Pseudomonas fluorescens* (1, 23).

Traditionally, when there are no commercial (+)- γ -lactamases available for the biosynthesis of optically pure lactam, researchers usually try to find them from the environment, such as from a microbial source. All six previously mentioned organisms have been found and used as whole-cell biocatalysts. In this process, novel screening methods play a key role in finding the successful biocatalysts (22). But the disadvantage of using whole cells is that wild strains usually have a small amount of protein and are unstable during the reaction. A combination of classical molecular biology techniques, e.g., isolating the enzyme from the wild strain and then cloning the enzyme for overexpression, is a better strategy. The recombinant cloned enzyme with high (+)- γ -lactamase activity would be the best biocatalyst for optically pure lactam in the industrial processes because of its high efficiency and low cost

FIG 1 Enzymatic preparation of (-)- γ -lactam for the synthesis of abacavir.

(10). Moreover, when we obtain the gene of (+)- γ -lactamase, it will be easy to obtain the crystal structure of the recombinant enzyme that will give us more information to better understand the mechanism of the catalytic process.

Until now, only two (+)- γ -lactamases have been sequenced, expressed in *Escherichia coli*, and used in the industry process for (-)- γ -lactam: those from *C. acidovorans* and *S. solfataricus* (2, 10, 15, 22, 25, 26). Both enzymes have been crystallized, but structure determination by multiple isomorphous replacement is still in progress (6, 15). It is expected that structural information from these amidases will increase our knowledge of molecular catalytic mechanisms. The sequence of the (+)- γ -lactamase from *C. acidovorans* showed 80% identity to a formamidase from *Bradyrhizobium japonicum* USDA 6 and 63% identity to a formamidase from *Methylophilus methylotrophus* (27). The sequence of the (+)- γ -lactamase from *S. solfataricus* showed about 40% identity to an

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FIG 2 Multiple alignment of (+)- γ -lactamase sequence and homologous enzymes. Abbreviations: *S. solfataricus*, amidase from *Sulfolobus solfataricus* (accession number NP_343511); *R. spN771*, amidase from *Rhodococcus* sp. N771 (Protein Data Bank [PDB] 3A1K_A); *P. fluorescens*, amidase from *Pseudomonas fluorescens* (accession number ZP_07774754); *B. japonicum*, amidase from *Bradyrhizobium japonicum* (accession number YP_005605100). Helices are represented by large dark-gray cylinders, β -strands by arrows, and coils by lines. A dashed line represents gaps in the amino acid sequence alignment.

amidase from *Rhodococcus rhodochrous* J1 (11) and *Rhodococcus* sp. N-771 (16). It is interesting that no identity was found between the (+)- γ -lactamases from *C. acidovorans* and *S. solfataricus*, which means that two different kinds of amidases showed similar (+)- γ -lactamase activity. Novel (+)- γ -lactamases need to be identified and examined to better understand this useful industrial catalyst.

Although novel screening methods for finding new organisms with (+)- γ -lactamase activity have been developed, it is widely believed that biotechnology has missed up to 99% of existing microbial resources by using traditional screening techniques (13). The isolation of enzymes from wild strains is usually time-consuming and requires a heavy workload. During purification, different methods such as ammonium sulfate fractionation, ion exchange, and hydrophobic and gel filtration chromatography are used, and the protein loses activity during purification and storage (1). This means that it is difficult to obtain the gene of (+)- γ -lactamase using these methods. Current advances in genome sequencing have revolutionized research in the field of biotechnology, not only giving us a glimpse into the uncultured microbial population but also enabling the high-throughput discovery of new enzymes for industrial bioconversions (4). A genomics-in-

spired strategy has been successful in unveiling a new (+)- γ -lactamase that was overlooked using standard screening methods. We believe that as more genome sequences become available, more novel (+)- γ -lactamases will be found in underexplored or neglected organisms.

In this article, we describe the identification of a novel (+)- γ -lactamase from *B. japonicum* USDA 6 based on sequence-structure guided genome mining for the synthesis of optically pure lactam. This is the third (+)- γ -lactamase gene to be reported, and it is the first (+)- γ -lactamase found by a genome-mining method.

In silico screening of DNA sequence databases for the putative (+)- γ -lactamase genes in microorganisms was performed. A BLAST search using (+)- γ -lactamase from *S. solfataricus* as a template was performed in NCBI, and an unnamed protein product (GenBank accession no. BJ6T_02120) was found from *B. japonicum* USDA 6 that showed 49% identity to the template (Fig. 2). *B. japonicum* USDA 6 is a Gram-negative, rod-shaped, nitrogen-fixing bacterium that forms a symbiotic relationship with *Glycine max*. It is a slow-growing bacterium and therefore not suitable for application in biotechnology. However, neglected organisms might contain effective (+)- γ -lactamase. In order to confirm our prediction, the whole cell of *B. japonicum* USDA 6 (purchased

FIG 3 Coomassie-stained gel after SDS-PAGE analysis of expressed proteins from transformed bacteria using gentle lysis. M, protein marker; lane 1, *E. coli* BL21(DE3) cells transformed with empty pET-30a vector; lane 2, *E. coli* BL21(DE3) cells transformed with pET-30a-(+)- γ -lactamase vector.

from China General Microbiological Culture Collection Center) was cultured to catalyze the racemic γ -lactam. High (+)- γ -lactamase activity was detected. Based on the gene of the unnamed protein product, the gene was amplified from *B. japonicum* USDA 6 genomic DNA by the use of forward primer 5'-GGAATTCCATATGGTGACAGTTGTCCTTCC-3' and reverse primer 5'-CCCAAGCTTTCACATCTTCTTCCAGTCGCC-3' (the NdeI restriction site is underlined in the former, and the HindIII restriction site is underlined in the latter). Then, the gene was cloned into plasmid pET-30a(+) and transformed into *E. coli* BL21(DE3) for expression, which was induced by 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) at 37°C for 4 h. The cells were harvested by centrifugation (4,000 $\times$ g for 10 min) and then analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The recombinant (+)- γ -lactamase ran as a single band on an SDS-PAGE gel (Fig. 3), corresponding to the predicted molecular mass of 50 kDa. The (+)- γ -lactamase activity was assayed by high-performance liquid chromatography (HPLC). Assay conditions for enzyme activity were as follows: wet recombinant cells (7 mg) were added to 500 μ l of the 100 mM substrate solution (pH 7.0, 50 mM phosphate buffer) with an *E. coli* wild-type strain as a negative control. The reaction solution was incubated at 37°C for 24 h. The reaction solution was extracted with 200 μ l ethyl acetate. The ethyl acetate extract (10 μ l) was applied to a Daicel Chiralpak AS-H column (Daicel Corp., Tokyo, Japan) and eluted with a mobile phase consisting of 90% acetonitrile and 10% isopropanol (volume ratio) at a flow rate of 0.6 ml/min. The UV absorbance of eluted γ -lactam was measured at 230 nm. The (+)- γ -lactam and (-)- γ -lactam had retention times of 11 and 12.7 min, respectively. As shown in Fig. 4, the (+)- γ -lactamase from *B. japonicum* USDA 6 appears to have potential for the production of chirally pure γ -lactam by hydrolyzing the (+)- γ -lactam specifically with no action against the (-) isomer. This high specificity and activity resulted in a yield of 49% and an enantiomeric excess of >99%.

As predicted by rational genome mining, a novel (+)- γ -lactamase was found and cloned from *B. japonicum* USDA 6. While only two (+)- γ -lactamases without similarity have been reported in previous literature, this third novel (+)- γ -lactamase will give us

FIG 4 Chiral HPLC analysis of the resolution.

more information about this kind of enzyme. Also, while thousands of other industrial biocatalysts have been studied (e.g., esterase), research on (+)- γ -lactamase is relatively scarce. Genome-mining methods provide an opportunity to examine these useful industrial biocatalysts in nature. The (+)- γ -lactamase from *B. japonicum* USDA 6 was expressed in *E. coli*, and its industrial applications could include the production of chirally pure (-)- γ -lactam for the synthesis of carbocyclic nucleosides such as the anti-HIV agent abacavir.

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