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Molecular Cloning and Characterization of the *dox*A Cytochrome P-450 Hydroxylase Gene in *Streptomyces peucetius* subsp. *caesius* ATCC 27952

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Abstract DNA sequence analysis of *dox*A from *Streptomyces peucetius* subsp. *caesius* ATCC 27952 revealed a 95% amino acid identity with that of *Streptomyces* strain C5. DoxA from *S. peucetius* subsp. *caesius* ATCC 27952 encodes a peptide with both conserved heme-binding and dioxygen-binding motifs. Expression of this gene in *S. lividans* 1326 resulted in bioconversion of daunorubicin to doxorubicin.

Key words: *dox*A, cytochrome P-450, hydroxylase, *Streptomyces peucetius* subsp. *caesius*

Daunorubicin and its C-14 hydroxylated derivative doxorubicin are important antitumor agents used for the treatment of many different cancers, in spite of undesirable acute and long-term toxic effects [1]. Doxorubicin was first isolated from *Streptomyces peucetius* subsp. *caesius* ATCC 27952, and is formed by C-14 hydroxylation of its immediate precursor, daunorubicin [2]. Although a number of organisms are known to produce daunorubicin, *S. peucetius* subsp. *caesius* has been the only organism reported to produce doxorubicin [4], which is currently obtained by the chemical conversion of the more abundant daunorubicin. Since doxorubicin is expensive, the development of efficient biotransformation process of daunorubicin to doxorubicin would be beneficial. One approach to accomplish this goal is the cloning and characterization of the gene responsible for the conversion of daunorubicin to doxorubicin. Recently, *dox*A encoding a cytochrome P-450-like enzyme has been cloned from *Streptomyces* sp. strain C5, which does not produce doxorubicin but produces daunorubicin [3]. Expression of this gene in *S. lividans* resulted in bioconversion of daunorubicin to doxorubicin. The *dox*A was also cloned from *S. peucetius* ATCC 29050, a daunorubicin-producing organism [8], however, it was not from the strain *S. peucetius* subsp. *caesius* ATCC 27952, the only organism to produce doxorubicin.

To clone the *dox*A gene from the *S. peucetius* subsp. *caesius* ATCC 27952, the *dox*A gene was first cloned from *Streptomyces* sp. strain C5 by PCR using the primer of 5'-GGTCGAACTGCGGGAGGCGT-3' for the upstream of *dox*A and that of 5'-TCACAGGGGGAGCGAGCGTC-3' for the downstream of the termination codon [3]. The PCR product of 1,423-bp was confirmed by a standard sequencing method [11], and inserted into the *Bam*HI site of pWHM3 [12], a high copy number of the *E. coli-Streptomyces* shuttle vector, to give pWHM-C5C14. To confirm the function of the cloned gene, *S. lividans* 1326 [5] was transformed with the plasmid pWHM-C5C14 and bioconversion activity of the transformant was tested by a feeding experiment. Briefly, recombinant strains were grown in 50 ml of R2YE medium [5] containing thiostrepton (25 μg/ml) and the flasks were incubated at 28°C and 250 rpm for 3 days. Daunorubicin was added to the final concentration of 50 μg/ml. After incubation at 28°C for 2 more days, the broth was extracted with a chloroform and methanol (9:1) mixture and then the dried residue was dissolved in methanol. The bioconversion activity was monitored by HPLC analysis according to the condition of Hwang *et al.* [6]. A control experiment was also carried out with a culture of *S. lividans* transformed with vector only. HPLC analysis showed that daunorubicin was converted to doxorubicin by 27% in the *S. lividans* expressing PCR fragment, indicating that the transformant with the plasmid pWHM-C5C14 confers an activity to hydroxylate daunorubicin at C-14 to give doxorubicin (Table 1).

In order to clone the *dox*A gene from *S. peucetius* subsp. *caesius* ATCC 27952, a clone carrying ca. 20 kb insert DNA (pKC-H1) was isolated with the PCR product as a

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Table 1. Comparison of bioconversion activities between DoxA of *Streptomyces* sp. strain C5 and *S. peucetius* subsp. *caesius* ATCC 27952.

Strains	Plasmids	Bioconversion rate (%)[a]	
		13-Dihydrodaunorubicin	Doxorubicin
C5	pWHM3	10±3.1	-
	pWHM-C5C14	16±3.8	27±5.3
	pWHM-27C14	11±3.0	21±4.5
Nu23	pWHM3	16±3.5	-
	pWHM-C5C14	18±4.5	51±8.2
	pWHM-27C14	21±4.8	43±6.5

[a]Biotransformation (%)

$$= \frac{\text{HPLC peak area of product}}{\text{Sum of HPLC peak area of daunorubicin and products}} \times 100$$

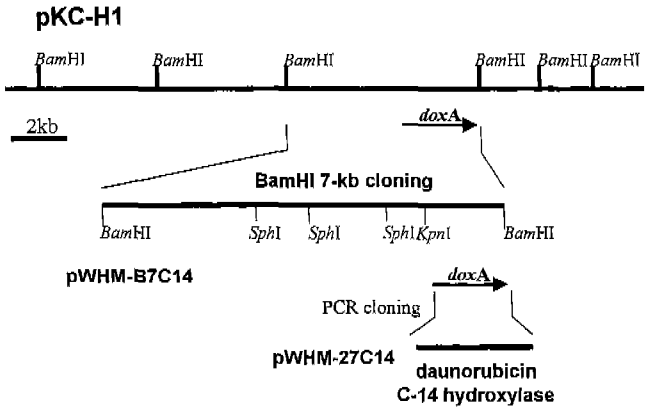

Fig. 1. Restriction maps and cloning strategy of the *dox*A gene locus from *Streptomyces peucetius* subsp. *caesius* ATCC27952.

probe from a cosmid library, which was constructed in pKC505 [10] with Gigapack II Gold Packaging Extract (Stratagene). The *dox*A gene could be located in the 7-kb *Bam*HI fragment (pWHM-B7C14) of the cosmid clone and its location was narrowed down to the 2-kb *Sph*I-*Bam*HI fragment by further analysis (Fig. 1). The nucleotide sequencing of the 2-kb *Sph*I-*Bam*HI fragment, followed by codon preference analysis using the FramePlot 2.3 program [7], revealed one complete ORF (*dox*A) and one partial ORF, which was originally identified as *orf*A in *Streptomyces* sp. strain C5 [3]. A probable start codon (GTG) and termination codon (TGA) for *dox*A are located at nt. 591–593 and nt. 1851–1853, respectively (Fig. 2). The *dox*A encodes a peptide of 420 amino acids (molecular weight 45,759 Da), which have a 95% amino acid identity with that of *Streptomyces* strain C5. It also belongs to the superfamily of cytochrome P-450 monooxygenase and contained a heme-binding motif (FGDGPHYCIG) centered at cysteine 369, and a dioxygen-binding motif (AGHERT) with the threonine 265 (Fig. 2). The latter motif appeared to have amino acid changes

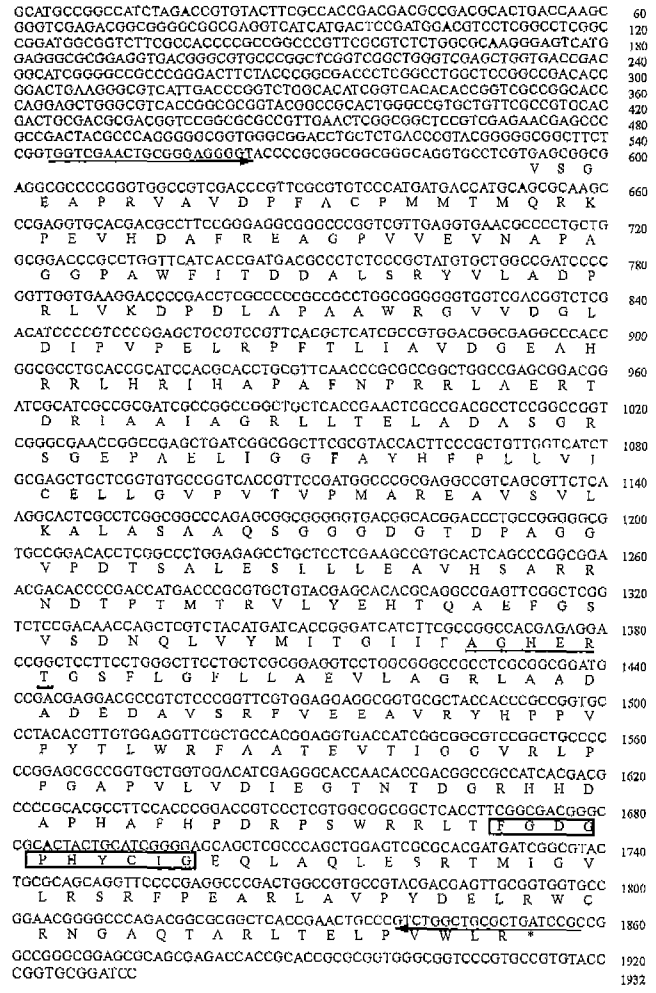

Fig. 2. Nucleotide sequence of the *doxA* gene from *Streptomyces peucetius* subsp. *caesius* ATCC 27952.

The deduced amino acid sequence of the *dox*A gene is given below the nucleotide sequence. The numbers on the right and left indicate nucleotide and amino acid positions, respectively. The solid line and box indicate the O_2 binding region and the heme binding pocket, respectively. The arrows indicate the PCR primer used for cloning of the *dox*A gene. The DNA sequence data have been deposited at GenBank under an accession number AF403708.

from that (AGHDTT) of DoxA of the *Streptomyces* sp. strain C5.

The open reading frame of *dox*A from *S. peucetius* subsp. *caesius* was cloned by PCR using primers of nt. 545–564 (5'-GGTCGAACTGCGGGAGGGGT-3') and nt. 1858–1849 (5'-GCGGATCAGCGCAGCCAGAC-3'). The PCR product of 1,314-bp was inserted in pWHM3 to give pWHM-27C14. *S. lividans* 1326 transformed with the plasmid pWHM-27C14 was tested for bioconversion activity in parallel with transformant of the plasmid pWHM-C5C14. *S. lividans* expressing DoxA from the *Streptomyces* sp. C5 converted about 27% of daunorubicin to doxorubicin, while recombinant strain expressing DoxA from the *S. peucetius* subsp. *caesius* converted approximately 21% of

substrate to doxorubicin (Table 1). The difference of the conversion rate between them did not deviate much from the error range, indicating that both DoxA expressed heterologously in *S. lividans* showed a similar degree of bioconversion activity, regardless of whether the donor strain was a doxorubicin-producer or not. Previous report suggested that, in the doxorubicin producing strain, the C-14 hydroxylation activity of DoxA would require to have unknown factor(s), which is necessary for achieving an efficient expression and/or catalytic activity of DoxA in the anthracycline producing organism [8]. To test this possibility, DoxA was expressed in an anthracycline-nonproducing mutant Nu23 strain that was derived from *S. peucetius* subsp. *caesius* ATCC 27952 [6]. The mutant strain Nu23 does not produce ε-rhodomycinone, daunorubicin, or doxorubicin; however, the Nu23 strain does convert intermediates such as ε-rhodomycinone and daunorubicin to doxorubicin, indicating that the mutant has nonfunctional polyketide synthase, but still maintains biosynthetic pathways intact between ε-rhodomycinone and doxorubicin [6]. Because the mutant Nu23 strain maintains intact biosynthetic pathways, which are necessary for the conversion of ε-rhodomycinone to doxorubicin, the Nu23 strain was transformed with the plasmid pWHM-C5C14 and then tested for bioconversion activity. The added daunorubicin was converted by more than 50% to doxorubicin in the Nu23 strain expressing the DoxA. The Nu23 strain transformed with pWHM-27C14 showed slightly lower conversion activity than that transformed with pWHM-C5C14, however, it was still at a much higher conversion rate than *S. lividans* transformants (Table 1). Meanwhile, the Nu23 strain with vector alone converted 16% of daunorubicin added to 13-dihydrodaunorubicin. These results reflect the effect of copy number of *dox*A. Moreover, our data also suggest that DoxA has a possibility of interacting with an unknown protein or factor, which is necessary for attaining efficient expression and/or catalytic activity of DoxA in the anthracycline-producing organism. Many bacterial cytochrome P-450 enzymes require a ferredoxin and NADPH:ferredoxin oxidoreductase for hydroxylase activity [9]. Furthermore, by analogy to other cytochrome P-450 enzymes, DoxA converts 13-deoxydaunorubicin to doxorubicin *in vitro* in the presence of only NADPH and suitable electron transport proteins [13]. Therefore, a sufficient NADPH source would be necessary for an efficient hydroxylase activity of DoxA. In addition, an active export system of doxorubicin is required since DoxA activity is inhibited by the end product [13]. Thus, there is a possibility that a high yield of doxorubicin could result from, not only the increasing expression of DoxA, but also an efficient expression of other proteins such as NADPH:ferredoxin oxidoreductase and active transport protein. In summary, *dox*A was isolated from the only doxoubicin-producing strain, *S. peucetius* subsp. *caesius* ATCC 27952, and a high conversion of daunorubicin to doxorubicin could be achieved by expression of *dox*A in the Nu23 strain, a mutant of *S. peucetius* subsp. *caesius* ATCC 27952.

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