

Antibacterial Activity of 4,5-Dihydroxy-2-cyclopentan-1-one (DHCP) and Cloning of a Gene Conferring DHCP Resistance in *Escherichia coli*

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Abstract

In the present study we report that 4,5-dihydroxy-2-cyclopentan-1-one (DHCP), which is derived from heat-treatment of uronic acid or its derivatives, has antibacterial activity against *Escherichia coli*. The compound causes complete growth inhibition at 350 μ M concentration. We have cloned a gene from *E. coli*, which confers DHCP resistance when present in multicopy. The putative protein encoded by this gene (*dep* - DHCP efflux protein) is a transmembrane efflux protein with a high homology to other antibiotic-efflux proteins including those for chloramphenicol, bicyclomycin and tetracycline. However, the *Dep* protein does not confer cross-resistance to any of the antibiotics tested.

Introduction

In spite of a number of antibiotics available against a variety of bacteria, due to emergence of multiple drug resistant strains, the search for newer and more effective antibacterial compounds has continued. 4,5-dihydroxy-2-cyclopentan-1-one (DHCP) (see Figure 1) is a compound having antibacterial activity against a variety of gram-positive and -negative bacteria, such as *Escherichia coli*, *Bacillus*, *Salmonella*, *Staphylococcus* etc. The process for manufacture and the properties of DHCP have been patented (Koyama *et al.*, 1999). It is prepared by the heat-treatment of uronic acid or its derivatives, wherein uronic acid is galacturonic acid, glucuronic acid, mannuronic acid or iduronic acid. It is also produced from roasted or parched vegetables, fruits, cereals, mushrooms, sea algae, cortex or cartilage. It has been demonstrated that this compound induces cancer cell differentiation and apoptosis. It has potential application as therapeutic or preventive agent against cancer and also as an antibacterial agent in antiseptics, dentrifices, cosmetics and bathing agents

(Koyama *et al.*, 1999).

We have isolated a multicopy suppressor from an *E. coli* genomic library for the DHCP toxicity. The putative protein encoded by this gene showed high homology to known efflux proteins conferring resistance to a number of antibiotics including chloramphenicol, bicyclomycin and tetracycline. The gene was mapped at 37.5 min on the *E. coli* chromosome. It is designated as *dep* for DHCP efflux protein. However, the *Dep* protein does not confer cross-resistance to any of the antibiotics tested.

Structure of DHCP

DHCP was obtained by the heat-treatment of uronic acid and the methods for production, purification and chemical characterization of DHCP are patented (Koyama *et al.*, 1999) and will be published elsewhere. DHCP as shown in Figure 1 is a cyclopentanone with two hydroxyl groups at positions 4 and 5.

Effect of DHCP on the Growth of *E. coli*

The *E. coli* wild-type strain JM83 [*F*⁻*ara* (*lac-proAB*) *rpsL*(*str*^r)] (Yanisch-Perron *et al.*, 1985) was grown in Luria broth (LB). Media were supplemented with ampicillin (final concentration of 50 μ g/ml) whenever required. To check the effect of DHCP on the growth of *E. coli*, cells grown overnight in LB medium were diluted into fresh LB medium. After the growth reached to the Klett unit of 50, DHCP was added at various concentrations (0-400 μ M) and growth was further monitored. After it reached to the Klett unit of 90-100, it was diluted 10-fold into media containing respective concentrations of DHCP. Figure 2A shows the effect of different concentrations of DHCP on *E. coli*. The growth was slowed after 3 h of incubation in the presence of 50 μ M DHCP, but it reached the maximum density after 8 h, similar to that without DHCP. The cells grew more slowly after 3 h incubation with 100 μ M of DHCP and the maximum cell density was lower than that without DHCP.

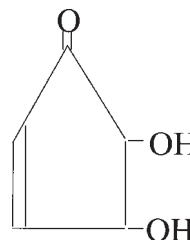


Figure 1. The structure of DHCP.

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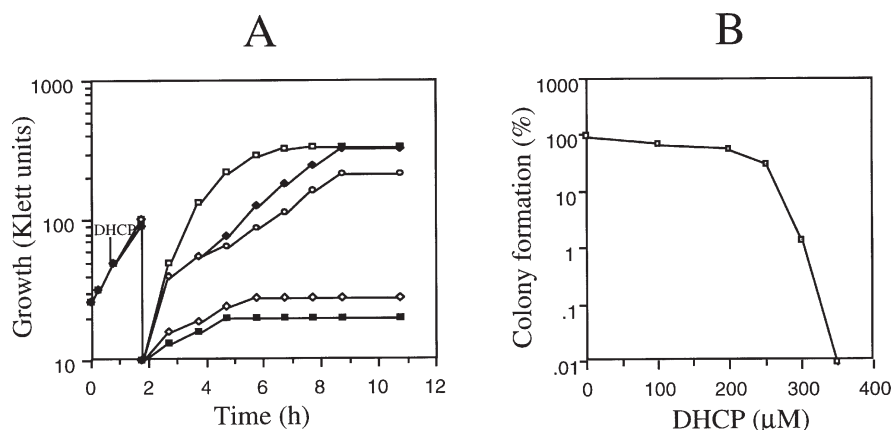


Figure 2. Effect of DHCP concentrations on the growth of *E. coli*.

A. The JM83 cells were grown in LB medium up to Klett unit of 50 and DHCP was added at various concentrations (0-400 μM). After the growth reached to Klett unit of 90-100, cells were diluted with medium containing respective concentrations of DHCP and growth was further monitored. DHCP concentration: 0 μM , open squares; 50 μM , closed diamonds; 100 μM , open circles; 250 μM , open diamonds; 400 μM closed squares.

B. Overnight grown cells of *E. coli* JM83 were diluted appropriately and plated on LB plates containing different concentrations of DHCP (0-350 μM). The number of colonies on the plate without DHCP was taken as 100% and the other numbers were expressed as relative percentages.

In the presence of 250 μM DHCP, growth was severely impaired after 3 h of incubation and cells stopped growing after 5 h. In the presence of 400 μM DHCP, cell growth stopped after 4 h of incubation. Microscopic examination of the cells grown with 250 μM DHCP for 8 h showed that the cells were elongated forming filaments, which are approximately 15-fold longer than the control cells. DAPI (diamidino phenylindole) (Hiraga *et al.*, 1989) staining of these cells showed that the chromosomal condensation of the cells might be impaired by DHCP (data not shown).

To check the colony formation ability of *E. coli* at various concentrations of DHCP, cells grown overnight in LB medium were diluted appropriately and plated on LB plates containing DHCP (0-350 μM). After incubation at 37°C, the number of colonies on each plate were counted. The number of colonies on the control plate without DHCP was taken as 100% and the other numbers were expressed as relative percentages (Figure 2B). In the presence of 300 μM DHCP, 100-fold decrease in the colony numbers was observed. When 1×10^4 cells were plated on LB medium containing 350 μM DHCP, no colonies were obtained.

Screening of an *E. coli* Genomic Library for Genes Conferring Resistance to DHCP

In order to examine if *E. coli* contains a gene(s) that confers resistance to DHCP, the *E. coli* genomic library was screened. The construction of *E. coli* genomic library was described previously (Lu and Inouye, 1998). The partially digested *Sau3A*I chromosomal DNA fragments from *E. coli* JM83 were cloned into the *Bam*HI site of pUC19. The JM83 cells were transformed with the genomic library. Transformants were isolated for their ability to grow on DHCP (400 μM) containing LB plates at 37°C. Plasmid DNA was isolated from the resistant colonies, purified and retransformed into JM83 cells to confirm its ability to confer resistance to DHCP. The plasmid was designated as pSP001 and was found to contain a 5.2-kb DNA fragment. This fragment was sequenced from both ends using

Sequenase and BLAST search was carried out for the analysis of homology of this fragment with the entire *E. coli* genome. It was found that this DNA fragment is located at 37.5 min on the *E. coli* chromosome and contains four ORFs (Figure 3): ORF389, *purR* encoding purine synthesis repressor, *ydhB* encoding a homologue of the *cyn* operon transcriptional activator and *ydhC* encoding a homologue of bicyclomycin resistance protein (Berlyn *et al.*, 1996).

To determine which gene is responsible for conferring resistance to DHCP, several deletion constructs were

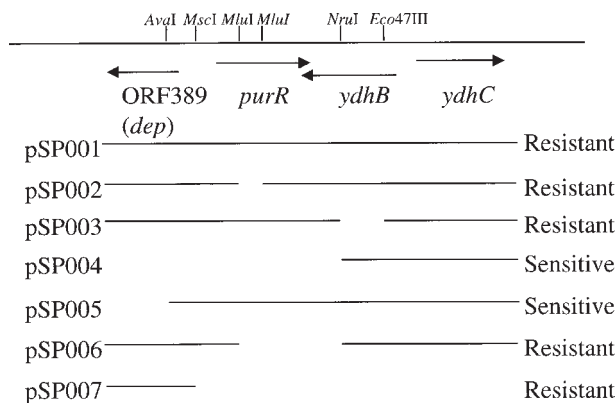


Figure 3. Restriction mapping of the plasmid pSP001 conferring resistance to DHCP. Four ORFs comprising the DNA fragment (5.2 kb) conferring resistance to DHCP and the flanking ORFs are shown. The orientation of each ORF is marked with an arrow. The restriction enzyme sites are also shown. The ORFs are not drawn to scale. The plasmid pSP001 containing the DNA fragment conferring resistance to DHCP was digested with restriction enzymes to disrupt each of four ORFs, religated and transformed into JM83 cells. The transformants were then examined for their sensitivity to DHCP (400 μM). The enzymes used for digestion were: for *purR*: *Mlu*I, for *ydhB*: *Nru*I-*Eco*47III, for ORF389, *purR*, and *ydhB*: *Nru*I and *Sma*I, for ORF389: *Ava*I, and for *purR* and *ydhB*: *Mlu*I and *Nru*I. For construction of plasmid with ORF389 (*dep*), the plasmid pSP001 was digested with *Sma*I and *Msc*I, the fragment was purified and cloned into pUC19 to yield plasmid pSP007.

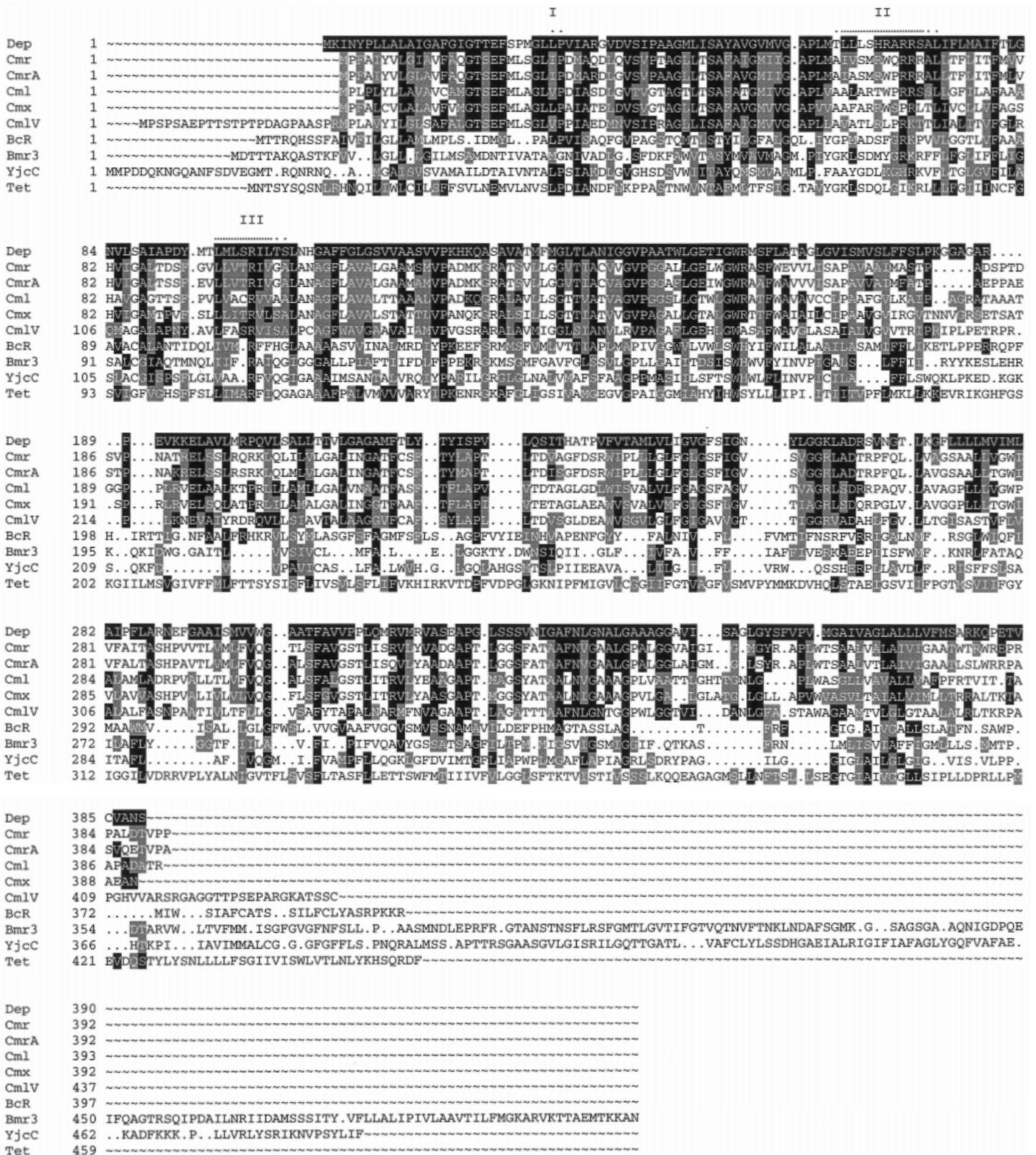


Figure 4. The sequence homology between Dep, Cmr, CmrA, Cmx, CmlV, BcR, Bmr3, YjcC and Tet. Identical and similar sequences are marked with black and gray boxes, respectively. The consensus sequences for transmembrane proteins are marked with dotted lines and are represented as I, II, and III stretches.

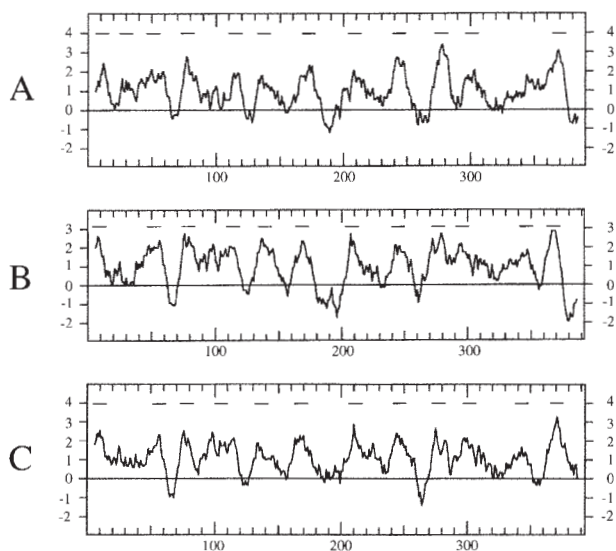


Figure 5. Hydropathic profiles of Dep (A), Cmr from *Rhodococcus fasciens* (B) (Desomer *et al.*, 1992) and Cml from *Streptomyces lividans* (C) (Dittrich *et al.*, 1991). Horizontal bars indicate predicted transmembrane regions.

prepared as shown in Figure 3. Disruption of *purR*, *ydhB* and both *purR* and *ydhB* had no effect on the resistance to DHCP (constructs pSP002, pSP003 and pSP006, respectively). However, disruption of ORF389 with *purR* and *ydhB* (pSP004) as well as disruption of ORF389 alone (pSP005) resulted in loss of DHCP resistance. We thus cloned ORF389 separately in pUC19 (pSP007), transformed the resultant plasmid in JM83 and checked sensitivity to DHCP. This plasmid conferred resistance to DHCP. These results clearly demonstrate that ORF389 is responsible for resistance to DHCP when cloned in a multicopy plasmid and further work was carried out using the plasmid pSP007. The ORF389 was named as *dep* – DHCP efflux protein (see below).

Homology Analysis of ORF389 with Other Genes Conferring Drug Resistance

Using BLAST-homology search computer program, we carried out a homology search for the putative protein encoded by *dep*. Figure 4 shows nine proteins showing significantly high homology with *dep*. Half of these proteins confer resistance to chloramphenicol. The proteins showing the highest degree of homology include: Cmr from

Rhodococcus fasciens (Desomer *et al.*, 1992), CmrA from *R. erythropolis* (Nagy *et al.*, 1997), Cml from *Streptomyces lividans* 1326 (Dittrich *et al.*, 1991), Cmx from *Corynebacterium striatum* (Accession no. U72639), and CmlV from *S. venezuelae* ISP5230 (Mosher *et al.*, 1995). As seen from Figure 4, *Dep* has the highest degree of homology with Cmr, product of chloramphenicol resistant gene (*cmr*) as compared to other proteins. Cmr protein was shown to contain three consensus sequences defined by Rouch *et al.* (1990) for transmembrane proteins. These sequences are at similar positions with respect to the predicted transmembrane domains. These are marked in Figure 4 with dotted lines and are designated as I, II, III. In case of *Dep*, the first stretch (I) comprising of LP is completely homologous with the stretch defined by these authors. The second stretch (II) shows 50% similarity with that of Cmr protein and the third stretch (III) is homologous between these two proteins except for one residue. According to the model proposed by Rouch *et al.* (1990), the stretches I and III are located on the outside of the cytoplasmic membrane and the stretch II is located on the inside of the membrane. The positions of the membrane loops for the putative protein encoded by *qacA* were ascertained by inspecting the antigenic index profile and turn prediction. Such regions have a high antigenic index and turn probability (Rouch *et al.*, 1990).

In addition to homology in the primary sequences, the hydrophobic profile of *Dep* (Figure 5A) is significantly similar to those of Cmr of *R. fasciens* (Desomer *et al.*, 1992) (Figure 5B) and Cml of *S. lividans* (Dittrich *et al.*, 1991) (Figure 5C). *Dep* is predominantly hydrophobic and probably contains 12 predicted transmembrane α -helices (Figure 5A).

The other proteins homologous to *Dep* include BcR (bicyclomycin- resistance protein) from *E. coli* (Bentley *et al.*, 1993), Bmr3 from *B. subtilis* involved in the multiple drug efflux pump conferring resistance to puromycin, tosulfoxacin, norfloxacin (Ohki and Murata, 1997), Tet from *Staphylococcus hyicus* conferring tetracycline resistance (Schwarz *et al.*, 1992) and YjcC conferring tetracenomycin-resistance (Accession no. D90826) (Figure 4). All of these are efflux proteins, which is one of the most common mechanisms for drug resistance. We speculate that *dep* encodes a putative efflux protein that forms a cytoplasmic channel specific for DHCP. The homologies are more prominent towards the N-terminal end of the proteins, which also is a common feature for efflux proteins (Desomer *et al.*, 1992).

Table 1. Minimum inhibitory concentrations (MICs) of various antibiotics for *E. coli* JM83 cells harboring pUC19 and pSP007.

	MICs ($\mu\text{g/ml}$)				
	kanamycin	spectinomycin	chloramphenicol	tetracycline	DHCP
cells with pUC19	25	12.5	6.25	3.125	25
cells with pSP007	50	12.5	6.25	3.125	200

MICs for both dilutions of the cells (3.5×10^5 and 3.5×10^3 cells) were the same.

Measurement of Minimum Inhibitory Concentrations for Cells Harboring pUC19 and pSP007

Since Dep shows homology to efflux proteins for multiple drug resistance, we checked if it confers resistance to other antibiotics as well. The *E. coli* wild-type cells harboring pUC19 or pSP007 plasmid were grown overnight in LB medium containing ampicillin. The cells were diluted 10- and 1000- times, and 5 μ l of each dilution (corresponding to 3.5×10^5 cells and 3.5×10^3 cells, respectively) was spotted on LB plates containing serial dilutions of kanamycin, chloramphenicol, spectinomycin, tetracycline and DHCP. Plates were incubated at 37°C for 20 h. As seen from Table 1, pSP007 did not confer significant cross-resistance to any of the antibiotics tested. The MIC values for cells harboring pUC19 and pSP007 were same for spectinomycin, chloramphenicol and tetracycline. The MIC value was two times higher for kanamycin for the cells harboring pSP007 than the cells with pUC19. The MIC value for DHCP on the other hand was 8 times higher for the cells harboring pSP007 than that for the cells with pUC19. It is interesting that Dep did not confer resistance to chloramphenicol, in spite of the high homology to *cmr*.

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