

## A new type II restriction endonuclease, *OfoI* from nonheterocystous cyanobacterium *Oscillatoria foreaui*

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**Although restriction enzymes are widely distributed in nature, many bacterial genera are yet to be explored for the presence of this important class of enzymes. We have purified and characterized a new type II restriction endonuclease, *OfoI* from a nonheterocyst cyanobacterium *Oscillatoria foreaui*. The recognition sequence has been determined by primer extension analysis. The purified enzyme *OfoI* recognizes and cleaves the palindromic hexanucleotide 5'-C↓YCGRG-3', generating 5'-protruding ends.**

THE primary role of restriction–modification systems (R–M) is to protect the host from invading foreign DNA molecules<sup>1</sup>. Three major types of R–M system are found in bacteria, viz. types I, II and III. Type II R–M systems comprise a separate restriction endonuclease and a methyltransferase that act independent of each other. Type II restriction endonucleases generally recognize palindromic sequences in DNA, cleave within or near their recognition sequences and produce DNA fragments of defined sizes. These useful properties have led to the screening of diverse bacterial species for new restriction enzymes. As a result, a large number of type II enzymes have been characterized<sup>2</sup>. Some of these enzymes have been studied extensively with respect to their structure, DNA binding and reaction characteristics. In spite of their importance to the host bacteria, restriction enzymes have not been detected in all bacterial species investigated; however, some strains have more than one enzyme<sup>3</sup>. A large number of bacterial species are yet to be subjected to careful screening methods to isolate new restriction enzymes.

Cyanobacteria, earlier known as blue-green algae, are widespread in freshwater, marine environment, soil, paddy fields and hot springs. The genus *Oscillatoria* comprises many species, which are nonheterocystous, filamentous organisms belonging to the order Oscillatoriales and family Oscillatoriaceae. So far, no restriction enzyme has been identified from this bacterial genus. Here, we report the identification and characterization of

a new restriction enzyme from *Oscillatoria foreaui*. The strain used in the study was isolated from rice fields of Kerala and maintained in the culture collection of the Centre for Advanced Study in Botany, University of Madras, Chennai and registered as *O. foreaui* A-1340. *O. foreaui* A-1340 appears as trichomes which are sparse, elongate, bent, distinctly constricted at the crosswalls, and apex not attenuated (Figure 1). *O. foreaui* cells were grown in BG-11 nitrogen-amended medium at  $27 \pm 1^\circ\text{C}$  (ref. 4) under a light intensity of  $30\text{--}40 \mu\text{Em}^{-2} \text{s}^{-1}$  provided by cool white fluorescent lamp with 12 h alternate light/dark regime. The growth was measured in terms of chlorophyll-*a* content<sup>5</sup>. The cell-free extracts of *O. foreaui* showed a consistent pattern of substrate DNA cleavage into discrete fragments, which is a characteristic feature of type II restriction enzymes.

The enzyme was purified using two column chromatography steps. Harvested cells were resuspended in 40 ml of buffer A (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM *b*-mercaptoethanol, 0.1 mM EDTA). Cells were lysed by sonication and insoluble material from crude homogenate was removed by centrifugation at  $92,000 g$  for 2 h. The supernatant was passed through phosphocellulose column, which was pre-equilibrated with buffer B (10 mM potassium phosphate, pH 7.4, 50 mM NaCl, 5 mM *b*-mercaptoethanol, 0.1 mM EDTA). After sample-loading, the column was washed with 400 ml of buffer B and then eluted with linear gradient of 0.05–1.6 M NaCl in buffer B. Fractions which showed distinct pattern of cleavage were pooled, dialysed against buffer A and then loaded onto heparin sepharose column. The column was washed with 400 ml of buffer A and eluted with linear gradient of 0.05–1.6 M NaCl in buffer A. The active fractions were pooled and dialysed against buffer A containing 50% glycerol and stored at  $-20^\circ\text{C}$ .

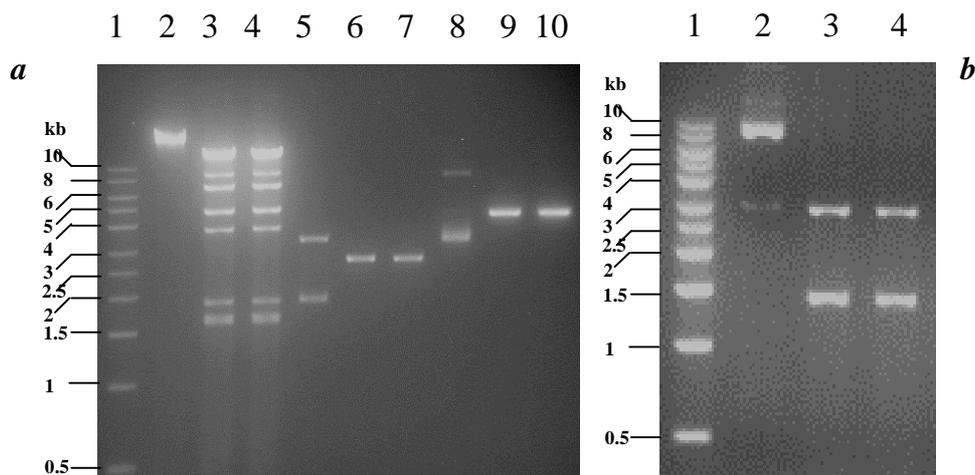
The restriction endonuclease assays were performed by incubating the enzyme with pUC18 DNA substrate in a 40  $\mu\text{l}$  reaction buffer (10 mM Tris-HCl, pH 7.4, 10 mM  $\text{MgCl}_2$ , 50 mM NaCl and 5 mM *b*-mercaptoethanol) for 1 h at  $37^\circ\text{C}$ . The unit activity of the enzyme was esti-



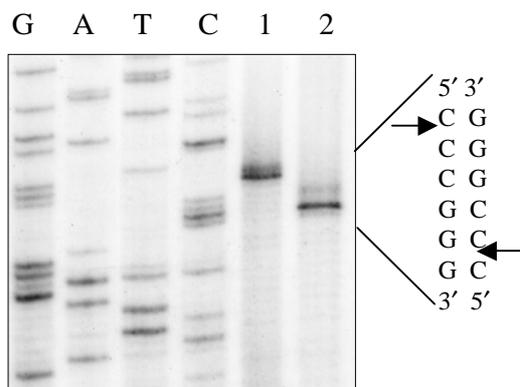
**Figure 1.** Photograph of *Oscillatoria foreaui*.

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**Figure 2.** Digestion profile of different DNA substrates with *OfoI* and *AvaI*. **a**, Comparative cleavage patterns of *OfoI* and *AvaI* using different DNA substrates. Lanes 2, 5, 8, Undigested *IDNA*, pUC18 and pBR322 DNA, respectively; lanes 3, 6, 9, *IDNA*, pUC18 and pBR322 DNA digested with *AvaI*; lanes 4, 7, 10, Corresponding *OfoI* digests; lane 1, DNA marker (0.5–10 kb), Novagen, Madison, USA. **b**, Double digestion of pBR322. Lanes 3, 4, Double digestion of pBR322 using *AvaI*–*EcoRI* and *OfoI*–*EcoRI*, respectively; lane 2, Undigested pBR322; lane 1, DNA marker as indicated. In all the cases, assays were terminated by adding 5  $\mu$ l of stop buffer (20% ficoll, 50 mM EDTA, bromophenol blue 0.1% w/v and xylene cyanol 0.1%). The reaction mixture was resolved by electrophoresis for 2 h at 100 V on 1% agarose gel in 40 mM Tris-acetate, pH 8.2, 1 mM EDTA. The gel was stained by 0.5  $\mu$ g/ml ethidium bromide.

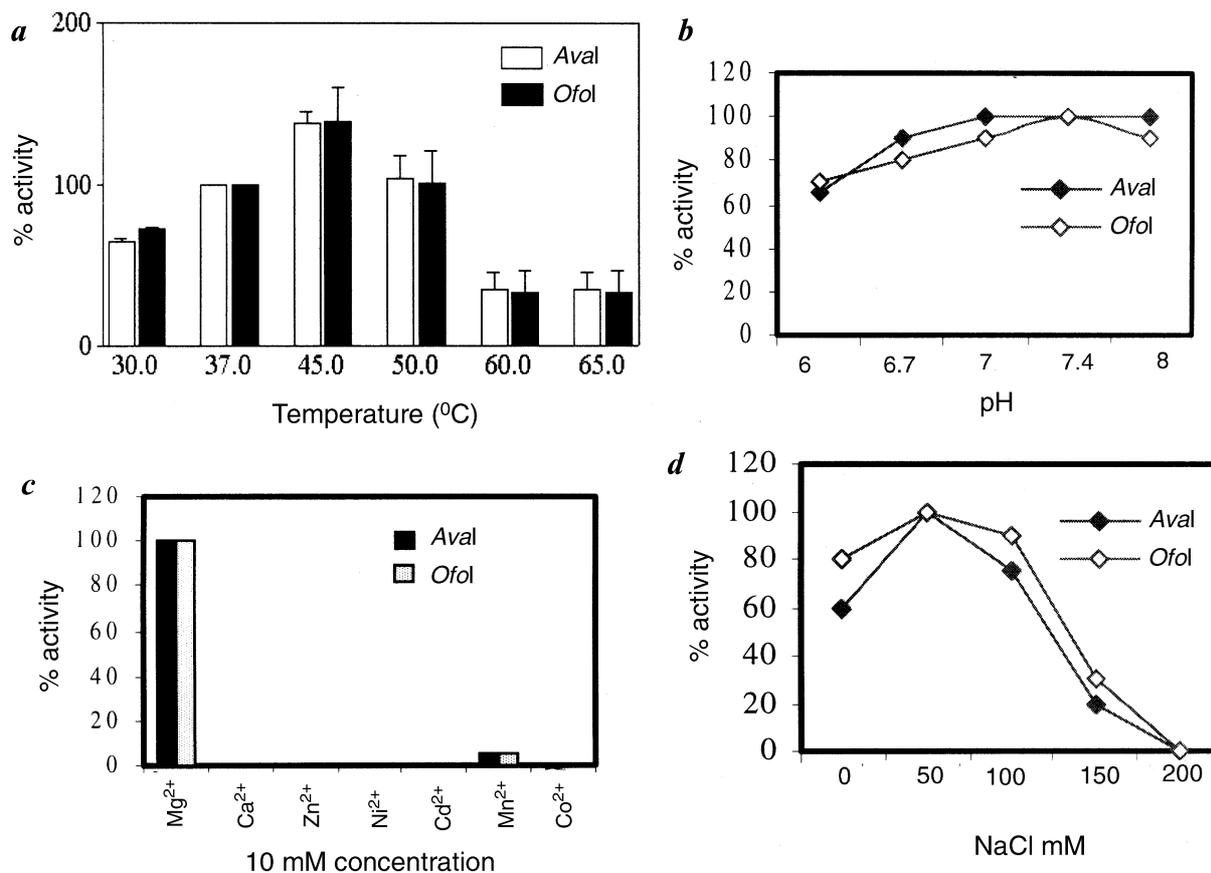


**Figure 3.** Determination of cleavage site of *OfoI*. The nucleotide position of cleavage of *OfoI* was determined by primer extension analysis. The plasmid pUC18, which has a single site for *OfoI*, was used for this analysis. The pUC18 forward primer (5'-GTT TTC CCA GTC ACG AC-3') was end-labelled using  $\gamma$ -<sup>32</sup>P-ATP and T4 polynucleotide kinase. The exact cleavage position of the enzyme *OfoI* was mapped by standard dideoxy sequencing reaction using Taq DNA polymerase. G, A, T, C represent the sequence ladder. To map the top strand cleavage site, pUC18 was digested with *OfoI*, the resulting cleavage products were used for primer extension (lane 1). To map the cleavage site in the bottom strand, first the primer was extended and then the resulting products were cleaved by *OfoI* (lane 2).

mated by incubating various amounts of the enzyme with 1  $\mu$ g of pUC18 DNA under the standard assay conditions described above. One unit of enzyme activity was defined as the amount of enzyme required to completely digest 1  $\mu$ g of *IDNA* in 1 h at 37°C under the standard assay conditions. A total of 18,000 units of enzyme was recov-

ered after purification, starting from 7.3 g of *O. foreaui*. The cleavage pattern of the new enzyme was determined using different DNA substrates such as pUC18, pBR322 and *IDNA* in the reaction. Plasmids pUC18 and pBR322 DNA have a single site, while *IDNA* contains eight sites. The cleavage pattern generated by the new enzyme was compared with the pattern of other type II restriction enzymes; it was identical to the pattern generated by *AvaI*. *AvaI* digestion of *IDNA* generated nine fragments of sizes 14677, 8614, 6888, 4720, 4716, 3730, 1881, 1674 and 1602 bp. Fragments of the same size were generated from *IDNA* with the new enzyme (Figure 2a). Double-digestion pattern of pBR322 obtained using the new enzyme and *EcoRI* was identical to that obtained from *AvaI* and *EcoRI* (Figure 2b). The exact cleavage site of the new enzyme was determined by primer extension analysis as described in Balke *et al.*<sup>6</sup>. The pUC18 DNA was digested by the enzyme and a primer (5'-GTT TTC CCA GTC ACG AC-3') was annealed to 76 bp upstream of the cleavage site and then extended with Klenow fragment of DNA polymerase I. To localize the bottom strand cleavage site, the primer was first extended and then cleaved with the new enzyme. Dideoxy sequencing reactions were carried out using the same primer, and the sequencing products were electrophoresed in parallel with the extended products (Figure 3). These data showed that the new enzyme recognizes and cleaves the following sequence





**Figure 4.** Comparison of *OfoI* and *AvaI* activities. *a*, Optimum temperature of the enzymes was determined by estimating the per cent activity at different temperatures (30 to 65°C) under standard assay conditions. *b*, Effect of pH on enzyme activity was evaluated by estimating per cent activity at different pH values such as 6.0, 6.7, 7.0, 7.4 and 8.0. *c*, Effect of divalent metal ions on *AvaI* and *OfoI* activity was assessed by estimating the total activity in the presence of different divalent metal ions such as Mg<sup>2+</sup>, Mn<sup>2+</sup>, Ca<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, Cd<sup>2+</sup> and Ni<sup>2+</sup>. *d*, Optimum ionic strength for activity of the enzymes was determined by adding 0 to 200 mM NaCl to the reaction mixture.

generating four nucleotide-protruding 5'-ends. The recognition sequence and cleavage pattern are identical to those of *AvaI* (ref. 7). Since this is the first restriction enzyme to be characterized from the *O. foreaui*, it is named as *OfoI*.

The properties of *OfoI* were compared with *AvaI*, since both were isolated from different cyanobacteria (Figure 4 *a-d*). The enzyme *AvaI* is isolated from *Anabaena variabilis* and has been well characterized<sup>8</sup>. A comparison of the activities of isoschizomer *OfoI* with *AvaI* is important to evaluate its origin and explore its potential applications. The temperature optimum for the enzyme *OfoI* was determined by carrying out assays with constant amounts of DNA and enzyme over a broad range of temperatures ranging from 30 to 65°C. The enzyme exhibits complete activity at 37°C; when temperature was increased to 45°C, it showed stimulated activity (Figure 4 *a*). *OfoI* is not inactivated by heating at 65°C for 20 min. Similar observations have been made with *AvaI*. Next, optimum pH for the enzyme was determined by estimating the per cent activity at different pH values ranging from 6.0, 6.7, 7.0, 7.4, 8.0 and 8.5. *OfoI* exhibits broad

pH optimum and was found to be most active at pH 7.4 (Figure 4 *b*). Type II restriction enzymes require divalent metal ions for their cleavage property. Hence, the enzyme *OfoI* was assayed for its activity in the presence of different divalent cations such as Mg<sup>2+</sup>, Mn<sup>2+</sup>, Ca<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, Cd<sup>2+</sup> and Ni<sup>2+</sup>. *OfoI* showed maximum activity in the presence of Mg<sup>2+</sup> and around 5% activity in presence of Mn<sup>2+</sup>, while no detectable activity was obtained with any other divalent cations tested (Figure 4 *c*). Another notable property of many Enases is that they exhibit star activity in presence of Mn<sup>2+</sup> ions<sup>9</sup>. We could not detect any star activity in the presence of Mn<sup>2+</sup>. The effect of ionic strength on enzyme activity was determined by estimating per cent activity at different salt concentrations. The enzyme is functional between 0 and 100 mM NaCl concentrations, exhibiting optimum activity at 50 mM NaCl concentration (Figure 4 *d*). Both *OfoI* and *AvaI* showed nearly identical biochemical properties (Figure 4 *a-d*).

The enzyme *OfoI* could be a convenient alternative to *AvaI* because of the following reasons. *A. variabilis* contains two restriction enzymes *AvaI* and *AvaII*, and hence

purification protocols have to be rigorous to avoid contaminating endonucleases. In contrast, *OfoI* purification is relatively simple compared to *AvaI* purification<sup>10</sup>, and the purified restriction enzyme *OfoI* is free from non-specific nucleases and any other restriction enzyme.

More than 200 cyanobacterial restriction endonucleases have been reported<sup>11</sup>. Amongst these, *AvaI* is the well-studied restriction enzyme from *A. variabilis*. Till date, thirteen isoschizomers and one neoschizomer (*Nli3877I*) of *AvaI* have been found in various cyanobacterial genera like *Agmenellum*<sup>12</sup>, *Anabaena*, *Anabaenopsis*, *Nostoc*<sup>13</sup>, and *Phormidium*. Widespread occurrence of these isoschizomers in various cyanobacterial species<sup>14</sup> belonging to different genera and families, strongly suggests that the genes for the enzyme are likely to be of common origin, and also indicates efficient horizontal gene transfer within cyanobacteria. Near identical behaviour in reaction characteristics of *AvaI* and *OfoI* provides further credence to this hypothesis.

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## Comparison of Etest with MIC method on Lowenstein–Jensen medium for susceptibility testing of *Mycobacterium tuberculosis*

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**The emergence of multiple drug resistance in tuberculosis (TB) has led to increased interest in determining the rifampicin and isoniazid susceptibility rapidly for management of individual cases as well as for epidemiological purposes. In TB, a major problem appears to be different cut-off levels recommended in different media/systems. To evaluate the comparative performance of Etest, it was compared to standard Lowenstein–Jensen (LJ) MIC method using 55 *Mycobacterium tuberculosis* clinical isolates, among which 25 (45.45%) were resistant to rifampicin. The concordance of Etest (1 µg/ml) with standard LJ MIC method (64 µg/ml) was 100%. In the case of INH there was 85% concordance between the two methods when 1 µg/ml on the Middlebrook agar by Etest was used as cut-off point, whereas 1 µg/ml was the cut-off MIC on LJ medium. However there was 100% concordance when the cut-off point was changed to 0.75 µg/ml. The study supports the utility of Etest for timely detection of drug resistance in *M. tuberculosis*.**

TUBERCULOSIS (TB) is a growing health problem both in terms of disease burden and increasing resistance to conventional chemotherapy<sup>1</sup>. The regions where TB is more prevalent lack the resources to implement appropriate measures to control the disease<sup>2</sup>. The standard treatment of TB as recommended by the World Health Organization (WHO) is a multi-drug regimen that includes two important drugs, rifampicin and isoniazid<sup>3,4</sup>, and the resistance to these two drugs together<sup>5</sup> has been defined as multi-drug resistance (MDR). The early recognition and appropriate treatment have been proven to be one of the most effective strategies to control MDR TB<sup>6</sup>, even in human immunodeficiency virus (HIV)-infected population<sup>7</sup>.

Currently recommended methods for susceptibility testing include the proportion method, resistance ratio method and MIC method on Lowenstein–Jensen medium<sup>8</sup> and on defined media by using Middlebrook agar method, including the BACTEC<sup>9</sup>. Although relatively expensive, the Etest method will be valuable for developing countries, as it is relatively easy to perform, provides

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