4444–4446 *Nucleic Acids Research, 1997, Vol. 25, No. 22*

*Bpl*I, a new *Bcg*I-like restriction endonuclease, which recognizes a symmetric sequence

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Received September 5, 1997; Accepted September 29, 1997

ABSTRACT

***Bcg*I and *Bcg*I-like restriction endonucleases cleave double stranded DNA specifically on both sides of their asymmetric recognition sequences which are interrupted by several ambiguous base pairs. Their heterosubunit structure, bifunctionality and stimulation by AdoMet make them different from other classified restriction enzymes. Here we report on a new *Bcg*I-like restriction endonuclease, *Bpl*I from *Bacillus pumilus*, which in contrast to all other *Bcg*I-like enzymes, recognizes a symmetric interrupted sequence, and which, like *Bcg*I, cleaves double stranded DNA upstream and downstream of its recognition sequence (8/13)GAGN$_5$CTC(13/8). Like *Bcg*I, *Bpl*I is a bifunctional enzyme revealing both DNA cleavage and methyltransferase activities. There are two polypeptides in the homogeneous preparation of *Bpl*I with molecular masses of ~74 and 37 kDa. The sizes of the *Bpl*I subunits are close to those of *Bcg*I, but the proportion 1:1 in the final preparation is different from that of 2:1 in *Bcg*I. Low activity observed with Mg^{2+} increases >100-fold in the presence of AdoMet. Even with AdoMet though, specific cleavage is incomplete. *S*-adenosyl-homocysteine (AdoHcy) or sinefungin can replace AdoMet in the cleavage reaction. AdoHcy activated *Bpl*I yields complete cleavage of DNA.**

Type II restriction endonucleases, by definition, recognize nucleotide sequences 4–8 bp in length, cleave within them, require only Mg^{2+} as a cofactor, and interact with target duplexes as monomers (1,2). As the number of characterized type II restriction endonucleases increases, evidence accumulates that they are a non-homogenous group of enzymes. Some of them have been demonstrated to manifest such unusual properties that the suggestion has been made that they should be classified as new kinds of restriction endonucleases: type IIS, type IV, *Bcg*I-like (2–4). Of note is the fact that all enzymes belonging to the suggested designations are distinguished by their recognition of asymmetric nucleotide sequences. Specifically, *Bcg*I-like enzymes (*Bcg*I, *Bsp*24I, *Bae*I, *Cje*I and *CjeP*I) recognize asymmetric sequences interrupted by 4–7 ambiguous bp, require Mg^{2+} for cleavage activity, are stimulated by AdoMet, and cleave DNA on both sides of their recognition sequence, thereby excising a short DNA fragment (5–9). The genes coding for *Bcg*I have been cloned and sequenced (6). The *Bcg*IA gene codes for a 71.6 kDa protein that resembles certain m6A-specific DNA-MTases. The *Bcg*IB gene encodes a 39.2 kDa protein. Neither protein can cleave or modify DNA by itself, but together they form a complex of composition A_2B that can perform both functions. Two subunits of *Bae*I have also been identified (~80 and 55 kDa), subunits a little larger than those of *Bcg*I. *Bae*I activity also includes both endonucleolytic and DNA methylation activities (8). Only specificity and cofactor requirements for the remaining *Bcg*I-like enzymes (7,9) have been determined so far.

We now describe a new *Bcg*I-like restriction endonuclease *Bpl*I, isolated from *Bacillus pumilus* P126-132, which in contrast to all other known *Bcg*I-like enzymes recognizes a symmetric interrupted nucleotide sequence and cleaves upstream and downstream of the recognition sequence (8/13)GAGN$_5$CTC(13/8) leaving 5 nt 3′ extensions. The *Bpl*I protein has been purified to apparent homogeneity by heparin-Sepharose, blue-Sepharose, phosphocellulose, hydroxyapatite and AH-Sepharose column chromatography. SDS-PAGE of the purified preparation reveals two polypeptides of *Bpl*I with molecular masses of 74 (subunit α) and 37 kDa (subunit β) (Fig. 1A). Thus, *Bpl*I like *Bcg*I and *Bae*I contains two protein subunits (6,8). In addition, the sizes of the *Bpl*I subunits are close to those of *Bcg*I. Using scanning densitometry to characterize the *Bpl*I subunit mass ratio, a ratio of absorbencies of the two peaks of ~2:1 was observed, a figure which corresponds to a subunit mass ratio of 74:37, indicating that the two proteins are present in the final preparation in the proportion 1:1. This finding indicates that the *Bpl*I is a dimer of composition αβ, while *Bcg*I is a trimer of composition A_2B (6).

Purified *Bpl*I maintains an absolute requirement for Mg^{2+}, for cleavage activity, which is stimulated by AdoMet (data not shown), and which, in parallel manifests methylation activity, as determined by the DNA protection assay (Fig. 1B). Only a minor number of the *Bpl*I sites were modified. Resistance to *Bpl*I cleavage of modified DNA should not, however, be interpreted as an experimental artefact, since plasmid DNA when added to the *Bpl*I premodified λ DNA yields the same restriction pattern as that resulting from separate cleavage of the same plasmid by *Bpl*I (data not shown). The recognition sequence of all five *Bcg*I-like enzymes contains a pair of adenines, which is located symmetrically relative to the centre of the double-stranded duplex that is being cleaved (8). This pair of adenines in the *Bcg*I recognition sequence is the target site for methylation (6). Thus, it is reasonable to speculate that the symmetrically located adenines in the *Bpl*I recognition sequence possibly also constitute the methylation site of the *Bpl*I enzyme.

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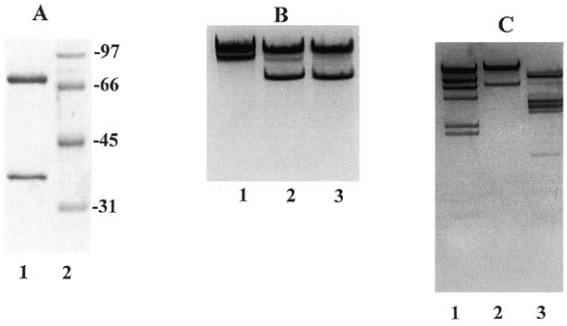

Figure 1. (**A**) Coomassie-stained 10% SDS-PAGE of purified *Bpl*I endonuclease (lane 1) with size standard (lane 2). (**B**) Methylation of λ DNA with *Bpl*I. Lane 1, λ DNA + *Xho*I; lane 2, λ DNA + *Xho*I incubated with 50 ENase units of *Bpl*I in reaction mixture with 0.1 mM AdoMet, without Mg^{2+} and afterwards cleaved with *Bpl*I; lane 3, λ DNA + *Xho*I + *Bpl*I. In reaction mixtures used in the cleavage step AdoMet was replaced by AdoHcy to obtain complete cleavage of unmodified DNA (see text). (**C**) Cleavage of various DNAs with *Bpl*I. Lane 1, λ DNA–*Hin*dIII digest as size markers; lane 2, λ DNA + *Bpl*I; lane 3, Ad-2 DNA + *Bpl*I.

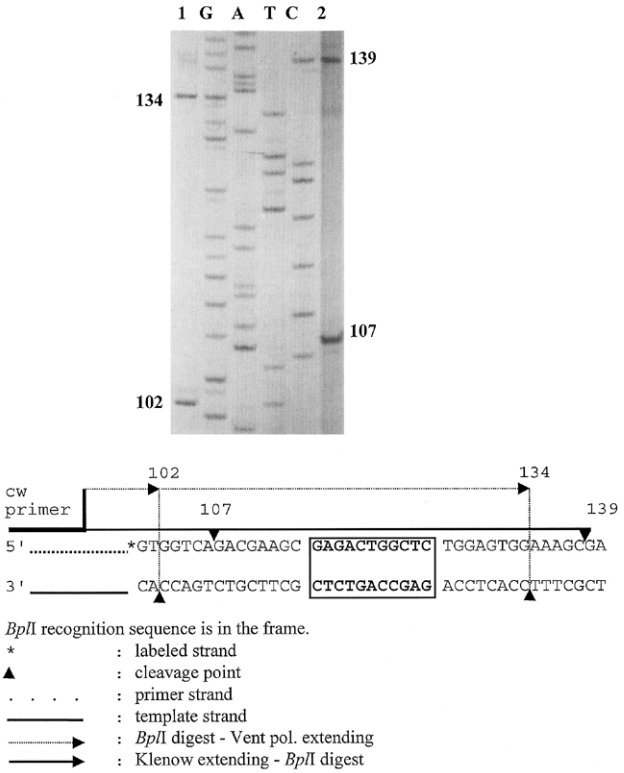

Figure 2. Determination of the cleavage site of *Bpl*I. λ DNA and a synthetic primer (40 920–40 940, cw strand) was used in the sequencing reaction based on the dideoxynucleotide chain termination reaction, through the *Bpl*I site. An additional extension reaction was carried out in the presence of four deoxynucleotides and [α-^{33}P]dATP containing the same λ DNA and primer. Lane 1: λ DNA – *Bpl*I digest was extended with Vent polymerase in the presence of [α-^{33}P]dATP. It should terminate the reaction leaving blunt ends at the points where *Bpl*I cleaved DNA. This result shows cleavage points on the complementary strand. Lane 2: the labeled DNA was digested with *Bpl*I. Both probes were mixed with sequencing dye and loaded on an 8% denatured polyacrylamide gel along with standard GATC ladders.

Optimal enzyme activity is observed in 33 mM Tris–HCl buffer (pH 7.9), containing 10 mM magnesium acetate, 66 mM potassium acetate at 37°C, with 0.05 mM AdoMet. Even at optimal AdoMet concentration though, which increases purified *Bpl*I activity >100-fold, complete cleavage of DNA is not achieved. *Bsp*24I also cleaves DNA incompletely (7), though incomplete cleavage is not a characteristic shared with other *Bcg*I-like restriction endonucleases. Like all characterized *Bcg*I-like restriction endonucleases activity does not require ATP.

AdoHcy and sinefungin can replace AdoMet as a cleavage cofactor in *Bpl*I digestion (optimal concentrations: 0.2 mM AdoHcy and 0.2 mM sinefungin). Of note is the fact that AdoHcy-activated *Bpl*I yields complete cleavage of DNA, indicating that binding of the cofactor rather than methyl group transfer is essential for cleavage. Incomplete cleavage in the presence of AdoMet therefore may be attributed to the concomitant methylation of some target nucleotides. AdoHcy can replace AdoMet in the activation of *Bae*I (8) but has no effect at low concentrations or inhibits *Bcg*I activity at high concentrations (5). The effect of sinefungin has been characterized only in the case of *Bcg*I, where it was shown to reliably replace AdoMet. All the data described above therefore indicate some diversity in the effects of cofactors on the activities of *Bcg*I-like enzymes.

Of the DNA substrates used (λ, pBR322, pUC18, M13mp18 and ϕX174) only λ phage DNA was cleaved by *Bpl*I at only one site, yielding two fragments of ~7500 and 41 000 bp (Fig. 1C). Double digestion of λ DNA with *Bpl*I and *Ehe*I, *Xho*I, *Nhe*I, *Eco*47III and *Eco*31I localized the *Bpl*I recognition site to a position around 41 000. Using data on frequencies of occurrence of palindrome sequences in the DNA substrates used (10) we identified only one sequence that was consistent with our observations, 5′-GAGN$_5$CTC-3′. The numbers of cleavage sites and lengths of DNA fragments resulting from treatment of Ad-2 DNA with *Bpl*I are consistent with the predicted recognition sequence (Fig. 1C).

λ phage DNA was used as a template to characterise *Bpl*I's point of cleavage. The 20mer oligodeoxyribonucleotide complementary to λ DNA at the position 40 920–40 940, 5′-gctttgtggtaataggccag (cw strand) was used for forward sequencing (11), which was performed through the *Bpl*I site, located at the position 41 035. The cleavage site of *Bpl*I was determined by comparison of dideoxy sequencing ladders with polymerized extension products, cleaved with *Bpl*I. Two DNA bands were observed following digestion with *Bpl*I (Fig. 2, lane 2): a 107 base fragment containing the primer and terminating eight bases before the recognition sequence; and a second 139 base fragment which included a 32 base fragment together with the recognition sequence and corresponded to the partial digestion product. The results obtained indicate that the second *Bpl*I DNA cut occurred 13 bases away from the recognition sequence. To determine cut points on the complementary strand, λ DNA digested with *Bpl*I was used as the template in the extending reaction using Vent polymerase, and the same primer. The reaction terminates such as to leave blunt ends at the point where *Bpl*I cleaves DNA. Two DNA bands, a 102 base fragment and a 134 base fragment, resulted, revealing that *Bpl*I cleaves DNA 13 bases upstream and eight bases downstream of the recognition sequence (Fig. 2). Similar experiments to those described above were carried out using a ccw-strand primer 5′-gcttttgctccattagccag complementary at the position 41 150–41 130, which yielded the same results

(data not shown). We therefore conclude that *Bpl*I cleaves double stranded DNA on both strands 8/13 bases before and 13/8 bases beyond the recognition sequence: (8/13)GAGN$_5$CTC(13/8).

Twenty two prototypes of type II enzymes are listed in REBASE which recognize palindromic interrupted nucleotide sequences, in which the ambiguous sequence contains >1 bp (12, REBASE ver.708, 1997). For all prototypes (except *Hgi*EII) and for many of their isoschizomers, cleavage sites are known and they are located within the variable part of the recognition sequence or at the junction between the variable and unique components of the recognition sequence (*Alw*NI, *Apa*BI and *Dra*III). To date, only a few such restriction endonucleases have been characterized beyond the characterization of their specificity. Information currently available gives no indication as to their heterosubunit composition (13–17). In asymmetric nucleotide sequences 5′-to-3′ recognition sequences are different in each strand. This precludes the possibility that symmetrically organized homodimers are involved in their recognition and rationalizes the heterosubunit composition of *Bcg*I. Recognition of a symmetric nucleotide sequence and symmetric cleavage by *Bpl*I implies that this enzyme probably interacts with it's target duplex as a symmetrically organized oligomer composed of two α and two β subunits. The crucial issue in the subunit composition selection of *Bpl*I and the other enzymes recognizing interrupted palindromes would therefore seem to be the location of the cleavage site relative to the recognition sequence. An interdependence of enzyme structure and location of cleavage site in enzymes recognizing asymmetric sequences supports this notion. The monofunctional type II restriction endonuclease *Bpu*10I which recognizes an asymmetric sequence and cleaves within that sequence is composed of two heterosubunits (18), while type IIS ENase *Fok*I, which cleaves outside its asymmetric recognition sequence interacts with its target duplex as a monomer (19).

ACKNOWLEDGEMENTS

The authors would like to thank Barbara Richmond-Smith for help in preparation of the manuscript. Special thanks to Jurate Markauskiene for help in protein purification.

REFERENCES

1 Yuan,R. (1981) *Annu. Rev. Biochem.* **50**, 285–315.
2 Pingoud,A. and Jeltsch,A. (1997) *Eur. J. Biochem.* **246**, 1–22.
3 Szybalski,W., Kim,S.C., Hasan,N. and Podhajska,A.J. (1991) *Gene* **100**, 13–26.
4 Janulaitis,A., Petrusyte,M., Maneliene,Z., Klimasauskas,S. and Butkus,V. (1992) *Nucleic Acids Res.* **20**, 6043–6049.
5 Kong,H., Morgan,R.D., Maunus,R.E. and Schildkraut,I. (1993) *Nucleic Acids Res.* **21**, 987–991.
6 Kong,H., Roemer,S.E.,Waite-Rees,P.A., Benner,J.S., Wilson,G.G. and Nwankwo,D.O. (1994) *J. Biol. Chem.* **269**, 683–690.
7 Degtyarev,S.Kh., Rechkunova,N.I., Zernov,Y.P., Dedkov,V.S., Chizikov,V.E., Van Calligan,M., Williams,R. and Murray,E.(1993) *Gene* **131**, 93–95.
8 Sears,L.E., Zhou,B., Alliota,J.M., Morgan,R.D. and Kong,H. (1996) *Nucleic Acids Res.* **24**, 3590–3592.
9 Vitor,J.M.B. and Morgan,R.D. (1995) *Gene* **157**, 109–110.
10 Schildkraut,I. (1984) *Genet. Eng. Plenum, Setlow,J.K., Hollaender,A. New York* **6**, 117–140.
11 Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
12 Roberts,R.J. and Macelis,D.(1997) *Nucleic Acids Res.* **25**, 248–262.
13 Nwankwo,D.O., Lynch,J.J., Moran,L.S., Fomenkov,A. and Slatko,B.E. (1996) *Gene* **173**, 121–127.
14 Lau,P.C.K., Forghani,F., Labbe,D., Bergeron,H., Brousseau,R. and Holtke,H.J. (1994) *Mol. Gen. Genet.* **243**, 24–31.
15 Striebel,H.M., Seeber,S., Jarsch,M. and Kessler,C. (1996) *Gene* **172**, 41–46.
16 George,J., Hamada,Y.Y.T. and Chrikjian,J.G. (1981) *Fed. Proc.* **40**, 1848.
17 Bae,M. and Song,E.S. (1994) *Korean J. Microbiol.* **32**, 297–300.
18 Stankevicius,K., Lubys,A., Timinskas,A. and Janulaitis,A. (1996) *Biologija* **2,** 51–53.
19 Wah,D.A., Hirsh,J.A., Dorner,L.F., Schildkraut,I. and Aggarwal,A.K. (1997) *Nature* **388**, 97–100.