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Nucleic Acids Research, 1993, Vol. 21, No. 4 987–991

A unique restriction endonuclease, *Bcg*I, from *Bacillus coagulans*

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Received October 9, 1992; Revised and Accepted January 16, 1993

ABSTRACT

We have purified and characterized a new restriction endonuclease, *Bcg*I, which has properties unlike those of the three recognized classes of restriction enzymes. *Bcg*I was isolated from *Bacillus coagulans*, and it recognizes the sequence CGAN$_6$TGC. *Bcg*I cleaves double stranded DNA on both strands upstream and downstream of the recognition sequence, so that the recognition sequence is released as a 34-base pair fragment with 2-base 3'-extensions. Mg^{++} and S-adenosylmethionine are required for cleavage. Sinefungin, a structural analogue of AdoMet which generally inhibits methylase activity, can replace AdoMet in the cleavage reaction. The apparent binding constant (K_β^{app}) for AdoMet is about 100 nM, while the K_β^{app} for sinefungin is about 500 nM.

INTRODUCTION

More than 2000 restriction endonucleases have been identified, mostly from bacterial sources (9). Three distinct types of restriction enzymes have been recognized on the basis of the subunit composition, cofactor requirements, and the type of DNA cleavage (Table 1; refs. 8, 10). Type I restriction–modification systems are composed of three protein subunits. Mg^{++}, ATP and AdoMet (S-adenosylmethionine) are required for DNA cleavage which occurs at non-specific sites often kilobases away from the recognition sequence. The type II restriction enzymes usually consist of homodimers of a single polypeptide subunit and require only Mg^{++} for DNA cleavage. This cleavage occurs within or close to the enzyme's recognition site. Type III restriction enzymes contains two different subunits and require Mg^{++} and ATP for DNA cleavage. Although not absolutely required, AdoMet stimulates cleavage activity of Type III enzymes. We report here a novel restriction endonuclease *Bcg*I whose DNA cleavage pattern and cofactor requirements differ from those of previously described restriction-modification classes.

MATERIALS AND METHODS

Biological materials

All restriction endonucleases, modifying enzymes, and oligonucleotides were from New England Biolabs. [^{35}S]-dATP (500 Ci/mmole) and [^{3}H]-AdoMet (12.5 Ci/mmole) was purchased from New England Nuclear.

Assays for *Bcg*I cleavage and methylation activity

*Bcg*I endonuclease activity was assayed by digestion of lambda DNA in *Bcg*I digestion buffer (100 mM NaCl, 10 mM Tris–HCl (pH 8. 4 at 25°C), 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM AdoMet). One unit of restriction endonuclease activity is defined as the amount of enzyme required to completely digest 1 μg lambda DNA in 50 μl in one hour at 37°C.

The assay for *Bcg*I methylation activity was carried out by incubation DNA with purified *Bcg*I in *Bcg*I methylation buffer (100 mM NaCl, 10 mM Tris–HCl (pH 8.4), 10 mM EDTA, 1 mM dithiothreitol and 80 μM AdoMet) at 37°C. The incubated DNA was subsequently extracted with phenol and chloroform to inactive enzyme. Because modified DNA was resistant to *Bcg*I cleavage, the level of *Bcg*I methylation was determined by digestion of treated DNA with *Bcg*I in *Bcg*I digestion buffer.

Purification of *Bcg*I

*Bcg*I was purified from *Bacillus coagulans* NEB # 545. All purifications were performed at 4°C. Frozen cells (450 g, wet weight), were thawed at room temperature and suspended in 900 ml buffer A (50 mM NaCl, 10 mM potassium phosphate (pH 6.5), 10 mM 2-mercaptoethanol, 0.1 mM Na_2EDTA). After sonication, insoluble material was removed from the crude extract by centrifugation at 30000×g for 30 minutes.

Crude extract was applied to a phosphocellulose column (50×160 mm) pre-equilibrated with buffer A. After sample loading, the column was washed with 300 ml buffer A. The enzyme was eluted with 3000 ml of a linear gradient from 0.05 to 1 M NaCl in buffer A. Endonuclease activity eluted in a peak centered around 0.35 M NaCl.

Active fractions were applied to a hydroxylapatite column (25×150 mm) equilibrated with buffer B (100 mM NaCl, 10 mM potassium phosphate (pH 7. 0), 10 mM 2-mercaptoethanol, 0. 1 mM Na_2EDTA). After washing with 70 ml buffer B, the column was eluted with 700 ml of a linear gradient of potassium phosphate from 0. 01 to 0. 7 M in buffer B. Active fractions, eluting around 0. 2 M potassium phosphate, were pooled and dialyzed against buffer C (50 mM NaCl, 10 mM Tris–HCl (pH 7. 4), 10 mM 2-mercaptoethanol, 0. 1 mM Na_2EDTA).

The enzyme pool from hydroxylapatite column was applied to a heparin sepharose column (15×100 mm), previously equilibrated with buffer C. The column was washed with 20 ml buffer C and eluted with 200 ml of a linear gradient from 0. 05 to 1 M NaCl. Active fractions were pooled and dialyzed against

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50% glycerol, 100 mM NaCl, 10 mM Tris−HCl (pH 7. 4), 1 mM dithiothreitol, and 0. 1 mM Na_2EDTA. The final preparation was stored at −20°C.

RESULTS

The recognition sequence and cleavage site of *Bcg*I

Analysis of the DNA banding patterns resulting from *Bcg*I-cleavage of φX174 RF (2 sites), pBR322 (3 sites) and pUC19 (1 site) DNAs suggested that the enzyme recognizes the asymmetric non-contiguous sequence: 5′ $CGA(N)_6TGC$ 3′. Additional digests of lambda (29 sites) and T7 (19 sites) DNAs produced fragments consistent with this recognition site assignment (Figure 1).

The cleavage site of *Bcg*I was determined by comparing dideoxy sequencing ladders with polymerized extension products cleaved with *Bcg*I (1, 7). The template DNA was pUC19 and a synthetic primer (2138→2155) was positioned upstream of the *Bcg*I site (2215). Two prominent DNA bands were observed following digestion with *Bcg*I (lane marked−): a 67-base fragment containing the primer, and terminating 11 bases before the recognition sequence, and a 34-base fragment which included the recognition sequence (12 bases), the 10 bases preceding it, and 12 bases following it. A third, fainter, 101-base fragment corresponded to the partial digest and was comprised of both fragments. The 34-base fragment was thus generated by complete cleavage on both sides of the recognition sequence (Figure 2, lane−). Figure 3 lane + shows the result of treating lane (−) products with *E.coli* DNA polymerase-Klenow fragment. All three fragments were reduced by two nucleotides, suggesting that a double strand cleavage occurred with *Bcg*I which left 2-base 3′-extensions. To determine directly the site of cleavage on the complementary strand DNA strand, a pUC19 primer (2274→2258) was used to generate a DNA strand initiating from the other side of the recognition sequence. Two cleavage sites were again observed (not shown), one 10 bp upstream of the recognition site and the other 12 bp downstream from the recognition sequence. A similar DNA sequencing analysis on pBR322 produced the same pattern of cleavage with respect to the *Bcg*I recognition sequence. To verify that cleavage occurs on both sides of the recognition sequence, lambda DNA, T7 DNA and pBR322 DNA were digested with *Bcg*I and loaded on a 20% polyacrylamide gel. The expected 34-base pair (bp) fragments were observed (Figure 3), the intensity of which suggested that one copy of the fragment arose from each cleavage. We conclude that *Bcg*I cleaves double stranded DNA on both strands upstream and downstream of its recognition sequence, thus:

5′...NNNNNNNNNNCGANNNNNNTGCNNNNNNNNNNNN...3′
3′...NNNNNNNNNNNNGCTNNNNNNACGNNNNNNNNNN...5′

The cofactors and their roles

Initial experiments showed that *Bcg*I cleavage activity was rapidly lost during purification. In an attempt to restore cleavage activity, the column fractions representing the flow-through from the phosphocellulose column were mixed with the most active fractions from the gradient elution. Nearly full *Bcg*I activity was recovered. We found that AdoMet could substitute for the phosphocellulose flow-through in restoring the activity. Cleavage by *Bcg*I was found to require both Mg^{++} and AdoMet (Figure 4).

*Bcg*I may methylate DNA during incubation for cleavage since AdoMet is a methyl donor in methylation reaction. The

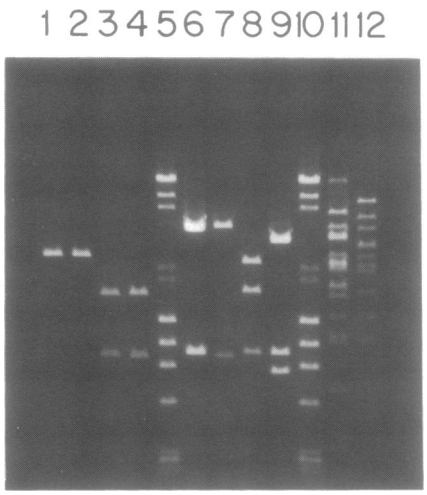

Figure 1. Identification of *Bcg*I recognition sequence. Lane 1: pUC19 + *Bcg*I. Lane 2: pUC19 + *Bcg*I and *Sca*I. Lane 3: pUC19 + *Bcg*I and *Alw*NI. Lane 4: pUC19 + *Bcg*I and *Pst*I. Lane 6: φX174 + *Bcg*I. Lane 7: φX174 + *Bcg*I and *Pst*I. Lane 8: φX174 + *Bcg*I and *Nci*I. Lane 9: φX174 + *Bcg*I and *Stu*I. Lane 11: lambda DNA digested with *Bcg*I. Lane 12: T7 DNA digested with *Bcg*I. Lane 5 and Lane 10: Lambda DNA digested with *Hin*dIII and φX174 digested with *Hae*III, size standard.

Table 1. Comparison between *Bcg*I and other types of restriction endonucleases

Restriction endonuclease	Cleavage factors	Recognition sequence	Cleavage characteristics
*Bcg*I	Mg^{++}, AdoMet	Asymmetric; interrupted $CGAN_6TGC$	Specific; ds; symmetric, on both sides of recognition sequence
Type I e.g. *Eco*K	Mg^{++}, ATP, AdoMet	Asymmetric; interrupted; bipartite e.g.$AACN_6GTGC$	Non-specific; ds; on both sides of recognition sequence
Type II e.g. *Bam*HI	Mg^{++}	Symmetric; continuous or interrupted e.g. GGATCC	Specific; ds; symmetric, within recognition sequence
Type IIs e.g. *Fok*I	Mg^{++}	Asymmetric e.g. GGATG	Specific; ds; asymmetric, on one side of recognition sequence
Type III e.g. *Eco*pI	Mg^{++}; ATP; (stimulated by AdoMet)	Asymmetric; continuous e.g. CTGAAG	Semi-specific; ds; ds; asymmetric, one side of recognition sequence
*Eco*57I, *Gsu*I, *Bsg*I	Mg^{++}; (stimulated by AdoMet)	Asymmetric; continuous e.g. CTGAAG	Specific; ds; asymmetric, on one side of recognition sequence

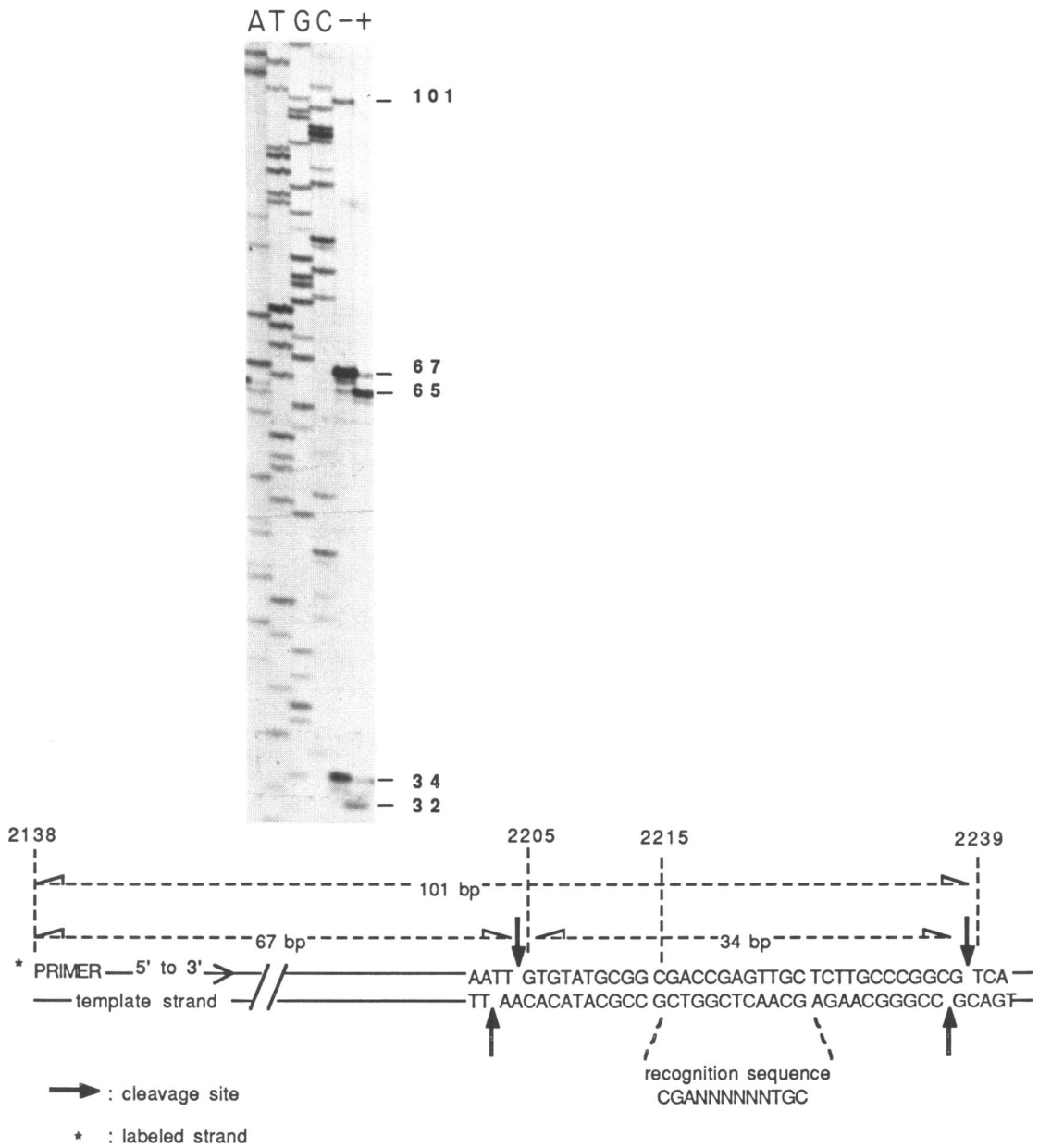

Figure 2. The cleavage site of *Bcg*I. pUC19 plasmid DNA and a synthetic primer (2138→2155) were used in sequencing reaction based upon the dideoxynucleotide chain termination method. Additional extension reaction was carried out in the presence of four deoxynucleotides and [^{35}S] dATP containing the same plasmid and primer. The labeled substrate was then digested with *Bcg*I. After inactivating *Bcg*I at 65°C for 15 minutes, the reaction mixture was divided into two aliquots: one was mixed with stop solution immediately (lane −); the other was treated with Klenow Fragment at room temperature for 10 minutes and then mixed with stop solution (lane +). Two *Bcg*I cleavage products were loaded to 8% denatured polyacrylamide gel along with standard A, T, G, C ladders.

methylation activity was tested using Lambda DNA incubated with 100 units of purified *Bcg*I restriction enzyme in *Bcg*I methylation buffer for one hour at 37°C. Only about 50% of the *Bcg*I sites were modified based on the resistance of incubated DNA to *Bcg*I cleavage (materials and methods). ATP did not stimulate methylation. Although purified *Bcg*I had both cleavage and methylation activities in the presence of both Mg^{++} and AdoMet in vitro, the DNA was completely digested by purified *Bcg*I, (Figure 4, lane 2), indicating that cleavage occurs preferentially under these conditions.

To determine whether the methylation occurs during the cleavage, the incorporation of [^{3}H]-AdoMet into DNA was monitored during the cleavage. pBR322 DNA was digested with *Bcg*I in the presence of both Mg^{++} and [^{3}H]-AdoMet for 15, 45 and 120 minutes. The cleavage products were separated by agarose gel electrophoresis. The cleavage was complete within 15 minutes as judged by the appearance of the expected 34-bp fragment along with other cleavage products (1153, 1321 and 1785 bp) (data not shown). Although cleavage was complete, [^{3}H]-methyl incorporation in 34-bp fragment continued to increase for 2 hours (Table 2). In contrast, no incorporation was noted in the large fragments lacking *Bcg*I recognition sequence (Table 2). The fact that *Bcg*I cleavage reaction was complete within 15 minutes but the methylation continued for 2 hours

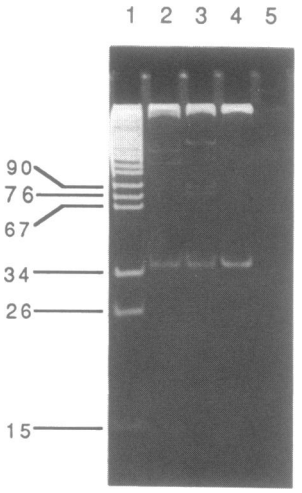

Figure 3. *Bcg*I cleaves double strand DNA on both sides of its recognition sequence, generating a 34 base-fragment. Lambda DNA (lane 2), T7 DNA (lane 3) and pBR322 DNA (lane 4) were digested with *Bcg*I in *Bcg*I digestion buffer at 37°C for 15 min. The digests were then loaded into a 20% polyacrylamide gel cast in TBE buffer (90 mM This, 90 mM boric acid, 4 mM EDTA). The gel was stained in TBE buffer containing 0. 5 μg/ml ethidium bromide after electrophoresis. Lane 1: pBR322 DNA digested with *Msp*I, size standard. Lane 5: *Bcg*I enzyme in digestion buffer without DNA.

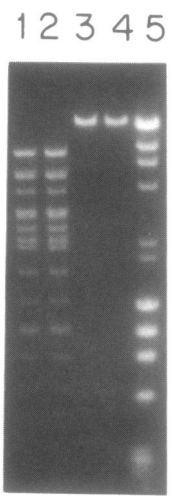

Figure 4. Assay of the cofactors which are required in *Bcg*I cleavage reaction. Lane 1: T7 DNA digested with *Bcg*I in the presence of 100 mM NaCl, 10 mM Tris−HCl (pH 8. 4 at 25°C), 1 mM dithiothreitol, 1 mM ATP, 20 μM AdoMet, and 10 mM Mg^{++}. Lane 2: Same components as in lane 2 without ATP. Lane 3: Same components as in lane 2 without AdoMet. Lane 4: Same components as in lane 2 without Mg^{++}. Lane 5: Lambda DNA digested with *Hin*dIII and φX174 RF DNA digested with *Hae*III, size standard.

indicates that the purified *Bcg*I enzyme binds to the 34-bp fragment after cleavage and methylates its recognition site. Assuming that each strands of the recognition sequence can be singly modified by *Bcg*I, the incorporation after 2 hours represents methylation of 15% of the available sites.

Sinefungin, a structural analog of AdoMet which generally inhibits methylase activity but can stimulate methylase DNA binding activity (2), was found to substitute for the AdoMet cofactor in the cleavage reaction. Thus binding of the cofactor

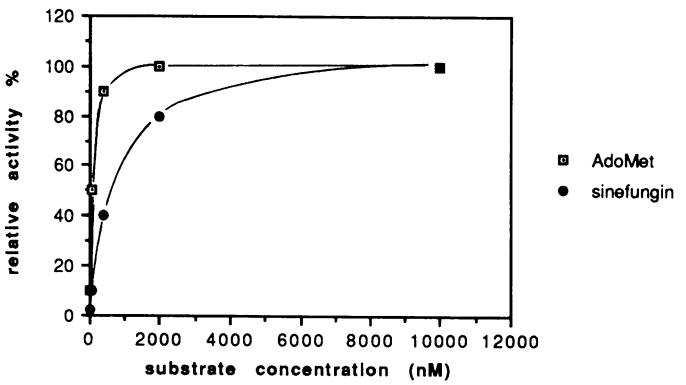

Figure 5. Comparison of kinetic parameters for AdoMet and sinefungin. Assays for K_b^{app} were carried out in *Bcg*I digestion buffer described in Materials and Methods containing 1 u *Bcg*I, 1 μg lambda DNA, and variable amounts of AdoMet, or sinefungin in 50 μl reaction volume. After incubation at 37°C for 15 minutes, 25 μl aliquots were mixed with loading buffer and then loaded on 1% agarose gel. Relative activities were measured based on the cleavage patterns on agarose gel.

Table 2. The 34-base pair fragment is methylated during and following cleavage

Time (min)	[^{3}H]-incorporation (cpm)	
	34-bp frag.	other frags.
15	923	110
45	2102	59
120	4969	100

pBR322 DNA was digested with *Bcg*I in *Bcg*I digestion buffer and [^{3}H]-AdoMet for 15, 45 and 120 minutes, respectively. The digestion mixtures were subsequently loaded to a 1. 8% low melting point agarose gel. The 34-base pair fragment and the other high molecular weight DNA fragments (1153-bp, 1321-bp and 1785-bp) were separated and purified using β-agarase I (New England Biolabs, protocol of manufacturer). The DNA was then spotted onto a DE81 filter (Whatman) and unincorporated [^{3}H]-AdoMet was removed by repeated washing with 0. 2 M NaCl and 20 mM Tris−HCl (pH 8. 0). The filters were then washed with alcohol, dried and counted.

rather than methyl transfer appears to be the key element in cleavage. Presumably AdoMet binding induces conformational changes necessary for cleavage. The apparent binding constant (K_b^{app}) for AdoMet and sinefungin was determined from the relative extent of cleavage in the presence of variable amount of activator. The K_b^{app} is defined as the amount of activator required to reach 50% of saturated extent of cleavage. The K_b^{app} for AdoMet is about 100 nM and the K_b^{app} for sinefungin is about 500 nM (figure 5), indicating a higher affinity of *Bcg*I for AdoMet.

S-adenosyl-homocysteine (AdoHcy), the product of methyl transfer reaction, was also tested to see if it would satisfy cofactor requirement. No stimulation to *Bcg*I cleavage by AdoHcy was observed. In fact, we found that most of the *Bcg*I cleavage activity was inhibited when AdoHcy concentration reaches 1 mM. Therefore *Bcg*I cleavage activity can be affected by AdoMet and AdoHcy.

DNA cleavage by *Bcg*I was not linear with respect to time. The extent of cleavage seen at 5 minutes was identical to that seen after 1 hour (not shown). Enzyme stability does not seem to account for this observation since the enzyme is not inactivated when incubated under the same conditions in buffer lacking DNA. The number of cleavage events appears to be approximately equal

to the number of enzyme molecules suggesting that turnover may not occur [enzyme molecules were determined based on specific activity and molecular weight, (Kong, H. and Nwankwo, D., unpublished observations)]. Similar observations have been made for type IIs endonucleases which do not destroy their recognition site during cleavage (Jack, W. E. and Noren, C. unpublished). The observation that *Bcg*I cleavage also does not destroy its recognition sequence and methylation by *Bcg*I occurs after cleavage suggests that *Bcg*I still binds the 34-bp fragment after cleavage so that turnover could not be observed.

DISCUSSION

Restriction endonucleases *Eco*57I and *Gsu*I recognize 6−base asymmetric nucleotide sequences and cleave double-stranded DNA on one side of their recognition site 14−16 bases away: CTGAAG16/14, CTGGAG16/14 (4, 5). The recognition sequence and cleavage site of restriction endonuclease *Bsg*I is similar to *Eco*57I and *Gsu*I: GTGACG16/14 (Kong H., McMahon, M. J. and Schildkraut, I. unpublished). All three enzymes' cleavage activities are stimulated by AdoMet. Another restriction enzyme, *Sgr*II, which appears to cleave on both sides of its recognition site to produce a 20−40 bp fragment, has been partially characterized by Orekhov, et. al (3). *Bcg*I is the first reported example of an enzyme which combines both of these features (Table 1). Although most of the restriction enzymes from bacteria can be classified into three types, further restriction enzyme variants undoubtedly exist. Since typical screening regimens do not include AdoMet, it is possible that other examples of this type of cleavage have been overlooked.

The unique cleavage properties of *Bcg*I could prove extremely useful in many contexts. For instance, the 34-bp fragments resulting from cleavage could serve as valuable landmarks for physical mapping of large DNAs (6). Recently the *Bcg*I restriction and modification genes have been cloned into *E.coli* (Kong, H. and Nwankwo, D., unpublished observations). It will be of considerable interest to see how these genes compare with known restriction-modification systems.

ACKNOWLEDGEMENTS

The authors would like to thank Drs. Geoffrey Wilson, William Jack and Richard Roberts for useful comments and critical reading on this manuscript. Special thanks to Dr. Donald Comb for his support.

REFERENCES

1. Brown, N. L. , Hutchinson, C. A. and Smith, M. (1980) *J. Mol. Biol.* **140**, 143−148.
2. Friedman, S. (1986) *Nucleic Acids Res.* **14**, 4543−4556
3. Orekhov, A. V. , Rebentish, B. V. and Debabov, V. G. (1982) *Dokl. Akad. Nauk.* SSSR, **263** 217−220.
4. Petrusyte, M. , Bitinaite, J. , Kersulyte, D. , Meukavice, C. , Butkus, V. and Janulaitis, A. (1987) *Dolk. Akad. Nauk.* SSSR, **295** 1250−1253.
5. Petrusyte, M. , Bitinaite, J. , Menkevicius, S. , Klimasauskas, S. , Butkus, V. , and Janulaitis, A. (1988) *Gene* **74**:89−91.
6. Roizes, G. , Charlieu, J. P. , Marcais, B. and Bellis, M. (1991) *J. DNA Sequencing and Mapping* **2**:65−67.
7. Sanger, F. , Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci.* USA **74**:5463−5467.
8. Roberts, R. J. (1976) *CRC Crit. Rev. Biochem.* **4**:123−164.
9. Roberts, R. J. and Macelis, D. (1992) *Nucleic Acids Res.* **20**:2167−2180.
10. Wilson, G. G. and Murray N. E. (1991). *Annu. Rev. Genet.* **25**:585−621.