Characterization of BcgI, a New Kind of Restriction-Modification System*

(Received for publication, June 7, 1993, and in revised form, August 30, 1993)

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The BcgI restriction enzyme from Bacillus coagulans is unusual in that it cleaves on both sides of its recognition site, CGAN6TGC, releasing a fragment that includes the site and several bases on each side. We report the organization and nucleotide sequences of the genes for the BcgI restriction-modification system and the properties of the proteins that they encode. The system comprises two adjacent, similarly oriented genes. The proximal gene, bcgIA, codes for a 637-amino acid protein (molecular mass = 71.6 kDa) that resembles certain mA-specific DNA-methyltransferases, particularly those that constitute the modification subunits of type I restriction-modification systems. The distal gene, bcgIB, codes for a 341-amino acid protein (molecular mass = 39.2 kDa) that comprises none of the sequences in the sequence data bases. The two genes overlap by several nucleotides. Alone, neither protein restricts or modifies DNA, but, together, they form a complex in the proportion A3B that does both. DNA binding assays showed that the DNA-protein complex can be formed only in the presence of both subunits, suggesting that the association of inactive subunits generates the active BcgI enzyme that can bind DNA and then either cleaves or methylates at target site.

Bacterial restriction and modification (R-M) systems are traditionally divided into three classes, designated types I, II, and III, on the basis of enzyme subunit composition, cofactor requirements, DNA specificity characteristics, and reaction products (1). Although convenient when introduced over a decade ago, this classification scheme has become somewhat out-of-date as the number of R-M systems and our understanding of their variety and relationships has grown. It became apparent some years ago, for example, that the major class actually comprises two classes (referred to today as types II and IIs (2)) and that the type III class, encompassing less than 0.2% of the known systems, barely merits a class of its own. Type II systems, in contrast, encompass almost 95% (3).

Why do R-M systems occur as distinct classes? One explanation is that each class represents a separate evolutionary tree, that R-M systems evolved only a few times, and that present day systems retained these ancestral organizations, while their target specificities diverged. The general lack of homology among restriction enzymes, in particular, argues against this explanation, however, and suggests instead that R-M systems evolved numerous times (4-6). Rather than reflecting separate evolutionary trees, then, the classes seem to represent nodes of evolutionary convergence, organizational formats of particular effectiveness toward which initially disparate systems gravitated. If this latter explanation is true, then we might expect to find rather more variety among R-M systems than the three- or four-class scheme would otherwise imply. And, indeed, as the number of well characterized systems increases, so too does our appreciation of their diversity (7-16).

Here we describe the properties of an R-M system with propertic distinct from those of other described systems, the BcgI system from Bacillus coagulans. BcgI enzyme recognizes the discontinuous, asymmetric sequence 5'-CGAN,TGTC-3', and, in the presence of Mg2+ and S-adenosylmethionine (AdoMet), it cleaves bilaterally and symmetrically outside the sequence to release a 34-base pair fragment (17). The unique properties of BcgI enzyme make it an attractive system for further study. We have cloned the genes for the BcgI R-M system into Escherichia coli. In this paper, we report the organization and nucleotide sequences of the two genes, bcgIA and bcgIB, and the properties of the related A and B subunits that they encode. The results of this study suggest that the BcgI R-M system is different from all characterized R-M systems not only in its unique enzymic properties but also in its unique gene composition and subunit functions.

EXPERIMENTAL PROCEDURES

Bacteria—B. coagulans, the natural host for the BcgI R-M system, was isolated from a soil sample and has been deposited with the American Type Culture Collection, with accession number ATCC 55055 (18). E. coli RR1 was from the New England Biolabs collection. RR1(DE3) lysS (19) was obtained from J. Forney (New England Biolabs); E. coli ER1821 was obtained from E. Raleigh (New England Biolabs).

Plasmids—pBR322 and pUC19 were from the New England Biolabs collection. pLM1, a pUC19-derivative containing a phage T7 promoter and a downstream transcription terminator, was the kind gift of G. Verdine (Harvard) (20). pSYX19, a pSC101-derivative containing the T7 promoter, was obtained from S.-Y. Xu (New England Biolabs).

Oligodeoxynucleotides—Complementary single-stranded oligodeoxynucleotides (New England Biolabs, Organic Synthesis division) were annealed by heating to 93 °C for 3 min and then slowly cooling to 25 °C over 1 h.

The specific duplex contains a single BcgI recognition sequence (shown in boldface type): 5' TTTGAGATAAGTGTATGCGGCGAG-GATCCCTGCTTGGCGCGCGCTAA3'; 3' ACTGTATTACA-CATACCGGCTCTTAGGACGAAACGGGCCGCAGTATGG-5'.
The nonspecific duplex differed from the specific one by one base pair, thus destroying the BcgI recognition sequence: 5′-TTTGGAC-
ATAGTGTATGCGGCGGATTCGTCTGATTCGCGCCGCTCA-
ATA 3′; 3′ ACTCTTATCATAACCACGCACCGACAGAGA-
ACGGGGCGGATTTAGG 5′.

Duplicate plates were filled in with [32P]dATP and DNA polymerase I Klone fragment.

Enzymes and Other Reagents—Restriction enzymes and DNA pol-
ymerase I Klone fragments were obtained from New England Biolabs. [32P]dATP (500 Ci/mmol), [32P]dATP (3000 Ci/mmol), and [32P]methionine (1200 Ci/mmol) were purchased from DuPont NEN.

BcgI Endonuclease and Methylation Transferase Assays—One unit of BcgI endonuclease is the minimum amount needed to completely digest 1 μg of plasmid DNA in 50 μl of BcgI cleavage buffer in 1 h at 37 °C. BcgI cleavage buffer contained 10 mM Tris-HCl (pH 8.4), 10 mM MgCl2, 100 mM NaCl, 1 mM dithiothreitol (DTT), and 20 μM AdoMet. One unit of BcgI methylase is the minimum amount needed to completely modify 1 μg of plasmid DNA in 50 μl of BcgI methylation buffer in 1 h at 37 °C. BcgI methylation buffer contained 10 mM Tris-HCl (pH 8.4), 10 mM NaBDTA, 100 mM NaCl, 1 mM DTT, 80 μM AdoMet.

Cloning and Sequencing the BcgI R-M Genes—The genes encoding the BcgI R-M system were cloned on an 8-kilobase Clal fragment of B. coagulans DNA by selecting for protectively modified, BcgI-resistant plasmid recombinants (21). A 3.5-kilobase HpaI-RsrII fragment containing the CIR plasmid was cut with Eco RI to generate a CIR plasmid cloning kit (New England Biolabs). The first strand was sequenced using the dideoxy chain termination method (22) or by primer extension using the Circumvent thermo-cycle sequencing system. The second strand was sequenced using custom synthesized primers.

Purification of BcgI Enzyme—BcgI endonuclease activity was purified from E. coli ER1821 containing pbcgIAB-10, a recombinant plasmid containing the BcgI R-M genes downstream of the lac pro-
moter in pUC18. All operations were performed at 4 °C unless other-
wise noted. 1) Frozen cells (327 g) from cultures grown at 37 °C for 40 min. 2) The superna-

tant was applied to a DEAE cellulose column (15 × 5 cm) equilibrated with buffer A containing 50 mM Tris-HCl (pH 7.5), 0.1 mM Na’EDTA, 1 mM DTT, 0.1 mM L-tryptophan (22) or 50 mM NaCl and applied to a Sephadex G-100 gel filtration column (23,24) equilibrated with buffer B containing 50 mM Tris-HCl (pH 7.4), 10 mM 2-mercaptoethanol, 1 mM Na’EDTA, 20 μM Sinefungin at 37 °C. The reaction mixtures were incubated at 30 °C for 1 h. Glycerol was added to 10%, and the DNA-protein complexes were loaded onto a POROS R column, 7040 kDa, was 3.653, 3.655). The fixed gels were dried and exposed to x-ray films. To

Electrophoretic Mobility Shift Assays—DNA binding and mobility-

shift assays were performed with either labeled proteins or labeled DNA in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM 2-mercap-

toethanol, 1 mM Na’EDTA, 20 μM Sinefungin at 37 °C for 30 min. The reaction mixtures were incubated at 30 °C for 1 h. Glycerol was added to 10%, and the DNA-protein complexes were loaded onto a POROS R column, 7040 kDa, was 3.653, 3.655). The fixed gels were dried and exposed to x-ray films. To

RESULTS

Cloning and Sequencing the BcgI Genes—The genes for the BcgI R-M system were cloned into E. coli by the methylation selection procedure as detailed in Ref. 21. Clal fragments of B. coagulans DNA were ligated to pBR322 and transformed into E. coli RR1. The transformants were plated and grown overnight to allow plasmids carrying the methyltransferase gene to become protectively modified, and then the plasmid population was purified, digested with BcgI, and transformed back into RR1 to recover BcgI-insensitive survivors (pBR322 contains three BcgI sites, and, therefore, cleavage by BcgI destroys unmodified plasmids). Plasmids thus recovered car-

ried an 8-kilobase Clal fragment in common, bore BcgI-specific modification, and expressed BcgI endonuclease activity.

The fragment from one of these clones was transferred to pUC19 to form pbcgIAB-10 (Fig. 1). The Clal fragment was subcloned to determine the bound-

aries of the BcgI genes and to remove extraneous DNA. The minimum subclone that expressed both endonuclease and methyltransferase activities, pbcgIAB-HR, comprised a 3.5-

kilobase HpaI-RsrII fragment in pUC19. The nucleotide se-

quence of this fragment was determined (Fig. 2). It was found to include two large, similarly oriented open reading frames (ORFs), bclLA (nt 173–2086) and bclLB (nt 2079–3104), cod-

ing for proteins of 637 amino acids (71,559 Da) and 341 amino acids (39,161 Da), respectively. Both ORFs are preceded by putative ribosome binding sites, AGGTTG (nt 160–164) 8 nt upstream of the A gene and AAGAGG (nt 2064–2069) 9 nt upstream of the B gene. The two ORFs overlap by several nt, and, therefore, the ribosome-binding site and the start codon of the B ORF lies within the 9' terminus of the A ORF. Such an intimate gene association is common among R-M systems.

Purification and Characterization of the BcgI Enzyme—The BcgI endonuclease activity was purified to near-homogeneity from E. coli ER1821 cells carrying pbcgIAB-10. It was found to comprise two proteins of 70- and 40-kDa in the molar ratio 2:1. The molecular masses of these proteins are close to those predicted for the BcgI A and B proteins on the basis of nt sequence (Fig. 3). Five-volume column chromatography steps were performed during the purification, and the fact that the two proteins stayed together throughout suggested that they were tightly associated. In an unsuccessful attempt to dissociate them, the endonuclease preparation was treated with 1 M urea and 1 M NaCl and applied to a Sephadex G-100 gel filtration column equilibrated in the same buffer. The two proteins again eluted in the same fractions, confirming their tight
The modification enzymes of the type I1 R-M systems AccI, belonging to the class (27). Members of this class include BanIII, HincII, PaeR71, and TaqI (28-32) and the modification subunits of the E. coli and Salmonella type I systems (33, 34), among others. These proteins are relatively large and heterogeneous, but all include the two-amin acid sequence motifs that are hallmarks of m6A-MTases, . . . LEP-G-G-F . . . and . . . NPPY . . . , or equivalents, 50-70 amino acids apart at their N termini. The match with the type M subunits was the closest, registering a score of 25% amino acid sequence identity in alignment with EcoA M (Fig. 4). This, albeit scant, similarity between BcgI A and characterized modification enzymes implies that it functions as a methylase domain in the BcgI system.

The B protein resembled none of the sequences in the NCBI data bases, nor did it resemble any of those in our personal data base of restriction and modification enzymes. Restriction enzymes are, as a rule, quite different from one another, and, therefore, the very absence of homology between BcgI B and other proteins might indicate, perversely as it were, that BcgI B functions as the endonucleolytic domain.

BcgI Methylation Produces N-6 Methyladenine—To identify the product of BcgI methylation, double-stranded duplexes were incubated with 3H-labeled AdoMet, purified BcgI enzyme, and analyzed for modified bases. Methylation was found to be sequence-specific in that 10 times more radioactivity was incorporated into the specific duplex that contained a BcgI site than the nonspecific duplex (data not shown). Following methylation, the specific duplex was digested to mononucleosides with phosphodiesterase I and alkaline phosphatase, unlabeled methyldeoxynucleosides were added to provide visual markers, and the mixture was separated by thin-layer chromatography (35, 36). The m6A, m4C, and mSC spots were counted for radioactivity, 95% of which was found to comigrate with m6A, indicating that N-6 methyladenine is the sole product of BcgI methylation (Table I). This result confirms the identification of BcgI as an m6A-methyltransferase, based on amino acid sequence comparisons.

The BcgI recognition sequence, CGAN\textsubscript{T}TGC, includes 1 adenine residue in each strand. We do not know whether the adenines are methylated in both strands or in only one. In type I systems, both strands are methylated (37, 38), but in type III systems, only one strand is methylated (39).

**Fig. 1.** Partial restriction map of the BcgI restriction and modification genes. Open boxes represent the two open reading frames, and their directions are indicated by arrows. The fragments present in various plasmid derivatives are shown below the map by hatched boxes, together with the BcgI endonuclease (R) and methyltransferase (M) phenotypes they express. +, expression of the activity; −, lack of expression.
The BeqIL R-M System

FIG. 2. Nucleotide sequence of the HpaI-RsrII fragment encompassing the begI A and B genes and the deduced amino acid sequences of the corresponding proteins. begI A spans nt 173–2086 (product = 341 amino acids; 39,161 kDa); begI B spans nt 2079–3104 (product = 39,161 kDa).

Both genes are preceded by putative ribosome binding sites (underlined) at nt 161–164 and 2061–2069. The genes over-lap by several nt. The two principal conserved amino acid sequence motifs present in the A protein and characteristic of m3A methyltransferases are shown in boldface type.
Activities of the Individual BcgI Proteins—Most characterized R-M systems comprise two genes, one for the endonuclease and one for methyltransferase. Usually, these genes occur side-by-side, and they code for enzymes that function independently. The BcgI system comprises two adjacent genes; to test whether they code for enzymes that also function independently, polymerase chain reaction fragments containing the \textit{bcgIA} and \textit{bcgIB} genes were separately cloned into the plasmid expression vector, pLM1, to form \textit{pbcgIA-2} and \textit{pbcgIB-7}, respectively (Fig. 1). Plasmid \textit{pbcgIA-2} (carrying just \textit{bcgIA}, the putative modification gene) expressed no detectable \textit{BcgI} endonuclease, nor did it express \textit{BcgI} methyltransferase, judging by its complete sensitivity to \textit{BcgI} digestion. This was true whether the adjacent, upstream, phage T7 promoter was induced or uninduced. Plasmid \textit{pbcgIB-7} (carrying just \textit{bcgIB}, the putative endonuclease gene) behaved similarly and expressed neither endonuclease nor methyltransferase, again regardless of induction.

We were not able to test the combination of \textit{pbcgIA-2} and \textit{pbcgIB-7} in the same cell, because their origins of replication were incompatible, but we were able to do so with two compatible plasmids, \textit{pbcgIA-31} and \textit{pbcgIB-8}. \textit{pbcgIA-31} is an exonuclease III deletion derivative of \textit{pbcgIAB-10} containing the \textit{bcgIA} gene; \textit{pbcgIB-8} is a pSC101 derivative expressing the \textit{bcgIB} gene in \textit{pSYX19} (Fig. 1). When \textit{pbcgIA-31} and \textit{pbcgIB-8} were transformed into the same cell, they complemented; the plasmid DNA became resistant to \textit{BcgI} digestion, signifying methyltransferase expression, and \textit{BcgI} endonuclease activity was readily detected in cell extracts. This suggests that the \textit{BcgI} proteins function in a mutually dependent fashion, such that both genes, in either cis or trans, are needed for the manifestation of either activity.

Complementation between the \textit{A} and \textit{B} proteins was also demonstrated in \textit{vitro}. \textit{BcgI} \textit{A} and \textit{B} subunits were separately purified by heparin-Sepharose chromatography, and the fractions were assayed by complementation using crude cell extracts of cells expressing the alternate subunit from \textit{E. coli} cultures carrying either plasmid \textit{pbcgIA-2} or plasmid \textit{pbcgIB-7}.

\textbf{Fig. 3.} Coomassie-stained 10–20% gradient SDS-polyacrylamide gel electrophoresis of purified \textit{BcgI} endonuclease (\textit{BcgI proteins lane}). The sizes of the molecular weight markers (MW) are given in kDa. Two proteins are present in the endonuclease preparation, \textit{BcgI A} (70 kDa) and \textit{BcgI B} (40 kDa). The ratio of the absorbances of the two peaks was found by scanning densitometry to be -3.3, indicating that the two proteins are present roughly in the proportion 3.3 + (70/40) = 1.9 A1 B. This is close to the value of 2:1 obtained by POROS R Chromatography.

\textbf{Fig. 4.} Amino acid sequence alignment between \textit{BcgI A} (upper sequence), the modification subunit of the type I \textit{EcoA} system (lower sequence), the sequence to which \textit{BcgI A} exhibits greatest similarity. The alignment was computed by the Genetics Computer Group software package (help@gcg.com; Madison, Wisconsin) program GAP, which returned a score of 25% identity. The two amino acid sequence motifs characteristic of m^{A}m^{G}-methyltransferases are shown in \textit{boldface} type. \textit{Solid vertical lines} emphasize identical amino acids; \textit{dotted vertical lines} emphasize equivalent (-) or similar (.) amino acids. \textit{BcgI A} is 148 amino acids longer than \textit{EcoA} A; most of the extra amino acids occur in the N-terminal half of the protein.
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Purified BcgI enzyme complex was incubated with [3H]-labeled AdoMet and the specific duplex containing BcgI site. Following methylation, the duplex was digested to mononucleosides, mixed with unlabeled methyldeoxynucleosides, and separated by thin-layer chromatography. Two different solvents were used to distinguish all possible methylated bases (35). The spots, visible under ultraviolet light, were scraped from the TLC plates and counted for radioactivity. Greater than 95% of the radioactivity comigrated with m6A, confirming that N-6 methyladenine is the sole product of BcgI modification.

| Solvent* G | Solvent D |
|------------|-----------|
| m6A<sup>a</sup> | m6A + m6C<sup>b</sup> |
| Relative mobility<sup>c</sup> | 1.3 | 1.0 |
| 3H incorporation<sup>d</sup> | 1067 | 24 |

<sup>a</sup>Solvent G is 66:33:1 isobutyric acid:water:ammonium hydroxide, v/v/v; solvent D is 80:20 ethanol:water, v/v (45).

<sup>b</sup>The methylated deoxynucleosides are as follows: m6C, N4-methyldeoxycytosine; m5C, 5-methyldeoxycytosine; m6A, N6-methyldeoxyadenosine.

<sup>c</sup>Mobility shown is relative to thymidine. m6C + m6C effectively comigrate in solvent G; m6A + m6C comigrate in solvent D.

<sup>d</sup>Data given in counts/min. Raw data is shown; background counts of 20 cpm have not been subtracted.

**DISCUSSION**

We show here that BcgI is a bifunctional restriction and modification enzyme comprising two subunits of 637 and 341 amino acids in the proportion 2:1. The subunits are encoded by adjacent, similarly oriented genes; the larger 1914-base pair bcgIA gene lies upstream of the smaller 1026-base pair bcgIB gene. The two genes overlap by several nucleotides. Separately, neither gene expresses endonuclease or modification activity in *vivo*, but, together, they complement and express both activities. The large subunit (calculated PI = 5.07) and the small subunit (calculated PI = 9.66) copurify as a tight complex. Both subunits are required for specific endonuclease and methyltransferase activity. Neither subunit alone binds to DNA, but, together, they bind in a nonspecific manner. This suggests, therefore, that the formation of the A<sub>H</sub>B complex somehow changes the conformation of the proteins such that they could now bind to and cleave or methylate DNA containing its recognition sequence.

BcgI does not belong to any of the traditional classes of R-M systems. With respect to gene organization, it resembles a
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Fig. 7. Electrophoretic mobility shift assays of [32P]-labeled double-stranded DNA with purified, unlabeled BcgI enzyme complex. Specific duplex or nonspecific duplex (see "Experimental Procedures") were end-labeled with [32P]dATP and Klenow fragment, and then aliquots containing 3 nM duplex were incubated with various dilutions (4–300 nM) of purified BcgI enzyme at 30 °C for 30 min. Aliquots, 20 μl, of the incubated DNA-BcgI complex were then removed from each reaction, and a 125-fold molar excess of the second unlabeled duplex was added. After an additional 10-min incubation at 30 °C, glycerol was added, and the DNA-enzyme complexes were electrophoresed in a 6% polyacrylamide gel cast in 45 mM Tris, 45 mM boric acid, 2 mM Na3EDTA and then visualized by autoradiograph. Binding to the duplexes is nonspecific, and it can be competed away equally well by excess specific or nonspecific oligos.

type II system; with respect to subunit composition, a type III system; with respect to recognition sequence, a type I system. However, it differs from each of these classes in important ways (42). Thus, type II enzymes act separately and recognize symmetric or continuous asymmetric (type IIs) DNA sequences; BcgI acts as a combined enzyme and recognizes an asymmetric discontinuous sequence. Type I systems recognize asymmetric, discontinuous sequences, but they cleave nonspecifically, and they comprise three subunits; BcgI cleaves specifically and comprises only two subunits. Type III enzymes comprise two subunits and recognize asymmetric, albeit continuous, sequences, but, like type I systems, they require ATP for cleavage, and they methylate in the absence of the R subunit; BcgI does not require ATP for cleavage, and neither of its subunits can methylate in the absence of the other. Finally, BcgI differs from another unique system, Eco67I (suggested as type IV in Refs. 10 and 11), which cleaves DNA on one side of its asymmetric recognition sequence with its bifunctional R subunit that also methylates one DNA strand, while its M subunit methylates both strands. Distinct from all type R-M systems, BcgI is the only characterized R-M system that cleaves bilaterally at a specific site with its unique A2B complex composed of two individually inactive subunits. Therefore, BcgI should be regarded as a new kind of R-M system.

Because the BcgI subunits are catalytically inert on their own, assigning a function to each is problematic. The A subunit includes amino acid sequence motifs characteristic of m7A-methyltransferases, and, therefore, it doubtless contributes much, if not all, of the modification capacity of the complex. In the simplest scenario, the A subunit would catalyze modification and determine the DNA sequence specificity (A = M + S), and the B subunit would catalyze cleavage (B = E). This scenario is unlikely, because it implies that the A subunit should bind to DNA and methylate on its own, abilities that the A subunit does not possess. For similar reasons, it is unlikely that the A subunit catalyzes modification (A = M), and that the B subunit catalyzes cleavage and determines specificity (B = R + S). Considering that the complex cleaves on both sides of the recognition sequence and that the subunit stoichiometry is 2 A:1 B, the A subunit might catalyze both methylation and cleavage (A = M + R), and the B subunit alone might determine specificity (B = S). That the B subunit does not bind to DNA separately could be explained by invoking a conformational change upon its association with the A subunit, but this explanation could equally well be applied to the other scenarios, too. Last, and, to our minds, most likely, in view of the discontinuous nature of the recognition sequence, it is possible that the A subunit catalyzes modification and contributes part of the specificity (A = M + S'), and the B subunit catalyzes restriction and contributes the rest of the specificity (B = R + S'). This model might explain why the B protein alone degrades DNA in a nonspecific manner (Fig. 5), and the A2B complex directs site-specific cleavage. It also accounts for the increased DNA binding affinity of BcgI when the A2B complex is formed (Fig. 6).

The unusual features of BcgI are (i) AdoMet is required for cleavage, (ii) the subunits are individually inactive, and (iii) cleavage is bilateral. What is the biological relevance of these features? The requirement for AdoMet might serve as a safety measure that couples restriction to modification. Thus, if the cell became undermodified for want of AdoMet, the capacity of the endonuclease to digest the cell’s DNA is reduced. The interdependency of the subunits might also have safety aspects (the endonuclease is active only when the system is capable of modification) and it also promotes AdoMet conservation, since the methyltransferase is active only when the system is able to restrict. In addition, if the B subunit does determine specificity, the system is modular and has the potential to switch specificities by the acquisition of new B subunits. Finally, bilateral cleavage could prove to be a particularly effective means for restricting infectious DNA molecules, because it diminishes the opportunities for cleaved fragments to rejoin. Two more restriction endonucleases have been discovered recently that, like BcgI, require AdoMet and cleave bilaterally. They are CjeI and CjeII from Campylobacter jejuni.2 Thus, BcgI-like systems may not be as rare as were once thought.

BcgI is a bifunctional enzyme that contains both cleavage and modification activities. The cleavage reaction occurs preferentially in vitro (17). What factor(s) and how it (they) is (are) involved in the regulation of BcgI cleavage and methylation activities in vivo is still a puzzle. The unique bilateral

2 J. M. B. Vitor, R. D. Morgan, and I. Schildkraut (New England Biolabs), personal communication.
cleavage and other enzymatic properties may be potentially useful in manipulating DNA, for example, in the construction of overlapping clone libraries in genomic research (43) and in analyzing polymerase chain reaction products to detect mutations with a different number of identical nucleotides (44).

Acknowledgments—We thank Drs. Ira Schildkraut and William Jack for helpful advice and discussions, David Landry for assistance in analyzing methylation products, Richard Roberts and Elizabeth Raleigh for critical reading of the manuscript, and Donald Comb for encouragement. We applaud the efforts of the personnel at the BLAST and RETRIEVE, and the CD-ROM ENTREZ data bases.

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