A new sequence-specific endonuclease from *Gluconobacter suboxydans*

A. Janulaitis, J. Bitinaitė and B. Jaskelevičienė

Institute of Applied Enzymology, 232028 Vilnius, Fermentu 8, Lithuanian SSR, USSR

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1. INTRODUCTION

The isolation of sequence-specific endonucleases from *Gluconobacter dioxyacetonicus* (IAM 1814 and IAM 1840) and *Gluconobacter oxydans* sub. *melanogenes* (IAM 1836) has been reported [1]. We have examined six *Gluconobacter suboxydans* strains for the presence of the enzymes of this type and discovered in two of them restriction endonucleases of identical specificity (named *Gsu*I and *Gsb*I).

Here, we describe the isolation procedure of the new site-specific endonuclease *Gsu*I, which recognizes a hexanucleotide sequence 5′...CTCCAG.

2. MATERIALS AND METHODS

The *G. suboxydans* strains (755M, 755T, H-15M, H-15T, AsM and Ig22) used were obtained from Dr M.S. Loytsianskaya (Leningrad State University). Restriction endonucleases *Pst*I, *Bcn*I, *Bsp*I and *Msp*I were isolated in this laboratory using standard procedures [2–5], *Taq*XI [6] (isoshizomer of *Bst*NI) was a gift from Dr V.G. Korobko (Institute of Bioorganical Chemistry, Moscow). Heparin–agarose was prepared as in [7] and kindly donated by O. Sudžiuvienė. Phage fd DNA was a gift from K. Sasnauskas.

The various materials and methods (including computer programme) used were as in [8].

2.1. *Bacteria growth*

All *Gluconobacter* strains used for specific endonuclease screening were grown at 30°C in aqueous medium containing (g/l): yeast extract, 10; peptone, 5; glucose, 10; agitated in New Brunswick G25 Gyrotory shaker at 250 rev./min overnight.

G. suboxydans H-15T strain used as a source for specific endonuclease preparative isolation was grown at 30°C in LKB 1601-013 10 l laboratory fermentor with aeration 14 l/min, agitation of 600 rev./min to an $A^{0.5\ cm}_{540\ nm}$ of 3.3. The cells were harvested by centrifugation and stored at −20°C.

2.2. *Enzyme purification*

G. suboxydans H-15T cells (20 g) were thawed in 40 ml buffer A (10 mM K-phosphate (pH 7.7), 1 mM EDTA, 1 mM dithiothreitol). They were disrupted by sonication (100 W MSE desintegrator at maximal output, 6 min) and centrifuged at 48000 × *g* for 1 h. The supernatant, brought to 100 ml with buffer A, was applied onto a 2.5 × 20 cm column of DEAE-cellulose equilibrated with the same buffer. The column was washed with 100 ml of buffer A and developed with 750 ml KCl linear gradient (0–0.25 M) in buffer A. The active fractions which eluted at 0.12–0.14 M KCl were pooled and dialysed overnight against buffer A. The enzyme solution was chromatographed on a 1 × 10 cm column of heparin–agarose, equilibrated with buffer A, using a linear 100 ml 0–0.04 M KCl gradient in buffer A. The active fraction, eluted at 0.17 M KCl, was dialysed against buffer A, containing 50% glycerol (v/v) and stored at −20°C.

2.3. *Assay of* Gsu*I activity*

Endonuclease activity was assayed by adding

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5–10 μl enzyme solution to 40 μl reaction mixture (10 mM Tris–HCl (pH 7.8), 6 mM $MgCl_2$, 20 mM NaCl, 1 mM dithiothreitol, 0.02% Triton X-100, 200 μg/ml of bovine serum albumin) containing 2 μg phage λ DNA. Incubations were routinely performed at 30°C for 1 h and terminated by adding 20 μl of a solution of 60% sucrose, 60 mM EDTA and 0.025% bromphenol blue. Restriction fragments were separated by electrophoresis in 1% agarose as in [8]. One unit of endonuclease activity is defined as the amount of enzyme which would completely digest 1 μg λ DNA in 1 h. Double digests were performed by simultaneous addition of *Gsu*I and another enzyme to the above mentioned reaction mixture.

3. RESULTS AND DISCUSSION

Among 6 strains of *G. suboxydans* examined, 2 strains (H-15T and H-15M) were found to produce site-specific endonucleases (names *Gsu*I and *Gsb*I). They appeared to yield identical λ DNA fragmentation pattern. *G. suboxydans* H-15T producing more enzyme than H-15M, was used as a source for enzyme isolation.

Purified endodeoxyribonuclease *Gsu*I, prepared as in section 2, appears to be essentially free from contaminating non-specific nucleases, since DNA samples digested with 10-fold excess of the enzyme for 20 h give sharp bands without smear in agarose gels. The yield of enzyme was 80 units/g wet wt cells. Activity did not decrease after 6 months storage at −20°C.

3.1. *Identification of the recognition site of* Gsu*I*

Digestion of various DNAas of known nucleotide sequence with *Gsu*I yielded: pBR322-4, φX174-3 fragments (fig.1). Phage fd DNA was cleaved once. Comparison of this data with the tables given [9], showed that the 5′...TCATGA and 5′...YCGCGR, where Y is pyrimidine, and R-purine, were candidates for the recognition sequence of the enzyme. However, comparison of the observed sizes of φX174 and pBR322 fragments with the distances between palindromes concerned in φX174 [9] and in pBR322 (tables for pBR322, analogous to those for φX174 in [9], were prepared by A. Mironov) excluded both these sequences as possible recognition sites for *Gsu*I. These data indicated that *Gsu*I recognizes an unusual nucleotide sequence not listed in the palindrome tables used.

The following step in determining the recognition sequence was to map *Gsu*I sites in φX174 DNA. The position of *Gsu*I-1 and *Gsu*I-2 sites was

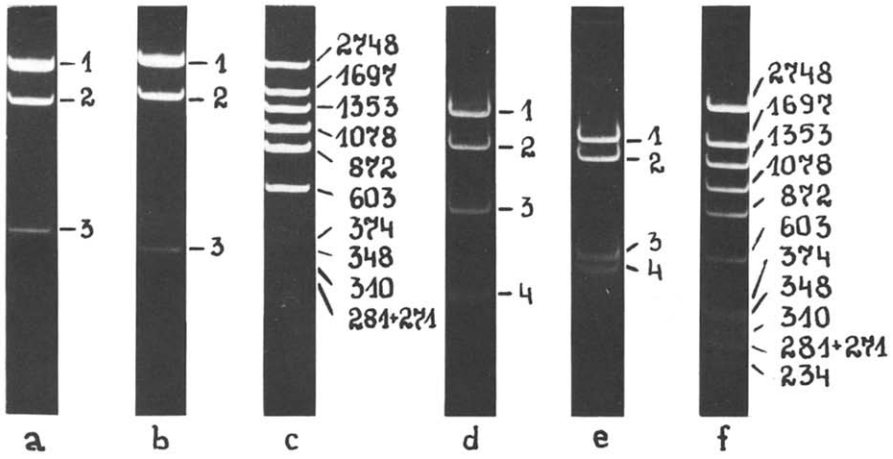

Fig.1. Gel electrophoresis of φX174 and pBR322 digests. Samples were analysed in a slab of 3% (a–c) and 2% (d–f) agarose gels: (a) φX174 + *Gsu*I; (b) φX174 + *Gsu*I + *Bcn*I; (c) φX174 + *Msp*I and φX174 + *Bsp*I; (d) φX174 + *Gsu*I + *Pst*I; (e) pBR322 + *Gsu*I; (f) φX174 + *Msp*I and φX174 + *Bsp*I. Sizes of the markers in (c) and (f) are indicated on the right in base pairs (bp). The sizes of *Gsu*I fragments in (a),(b),(d) and (e) are given in table 1.

estimated on the basis of ϕX174 DNA fragmentation data (fig.1,a,b,d) and it was localized as indicated in fig.2. There was insufficient information to map the third *Gsu*I cleavage site unambiguously, which was 880 bp from *Pst*I site. Two possible alternative localizations are shown in fig.2, as *Gsu*I-3 or *Gsu*I-4. The nucleotide sequence in the regions of these sites was inspected manually and as a result the common nucleotide sequence 5′...CTCCAG was found at *Gsu*I-1, *Gsu*I-2 and *Gsu*I-3 sites.

A further confirmation of this sequence was obtained by comparing the number and sizes of the *Gsu*I restriction fragments generated by ϕX174 and pBR322, and fragments generated by ϕX174 double digests with *Gsu*I + *Bcn*I and *Gsu*I + *Pst*I, respectively with computer predicted ones for 5′...CTCCAG sequence (fig.1, table 1). It is obvious that the fragment sizes and number given in table 1 are in close agreement with experimental data, but for one exception, which applies to two shortest *Gsu*I generated pBR322 fragments. As predicted, there should have existed two essentially equally sized (582 base pairs (bp) and 590 bp) *Gsu*I fragments, however app. M_r of these two fastest moving *Gsu*I fragments as determined by their relative electrophoretic mobility markedly differed from those predicted. This uncoincidence was reproducible: the sizes of fragments under consideration, as determined in 7 independent experiments was found to be 551 ± 18 and 633 ± 28 ($n = 7$, $q < 0.05$). Double digest experiments with *Gsu*I + *Taq*XI were performed in order to check whether 551 and 633 bp experimentally determined *Gsu*I fragments correspond to those predicted 582 bp and 590 bp. As it is shown in fig.3 one of the *Taq*XI sites is localized about in the middle of the predicted 590 bp *Gsu*I fragment and the other in the vicinity of 582 bp *Gsu*I fragment end. Thus, it could be predicted that after *Taq*XI cleavage, one of the *Gsu*I shortest fragments under consideration would yield two easily distinguishable subfragments and the other would become slightly shortened. The data shown in fig.3 and table 1 demonstrate that 551 bp *Gsu*I fragment was cleaved by *Taq*XI yielding two subfragments (330 bp and 235 bp). 633 bp *Gsu*I fragment was practically unaffected by *Taq*XI: the experimentally determined subfragment size was found to be 625 bp. These data allowed us to assign the experimentally determined 633 bp and 551 bp *Gsu*I pBR322 fragments to 582 bp and 590 bp predicted ones, respectively. All other *Gsu*I + *Taq*XI fragment sizes approach quite closely those predicted. The 582 bp *Gsu*I fragment definitely runs anomalously in the gels. It could be suggested that there is apparently something about the composition of this fragment that lowers the mobility in the gels used. Marked uncoincidences between the predicted and the observed sizes of some DNA fragments are well documented [10].

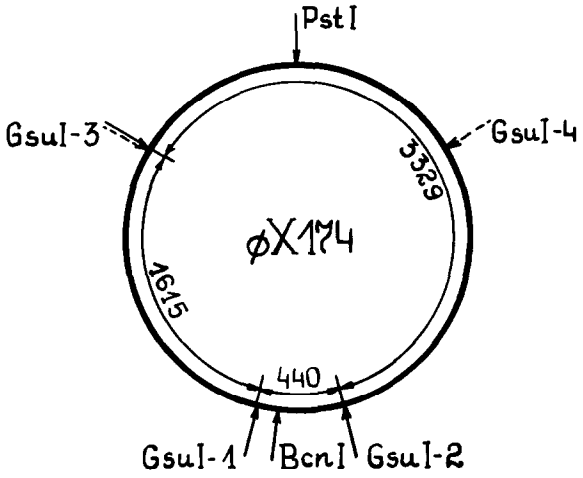

Fig.2. Coordinates of *Gsu*I cleavage sites on ϕX174. Distances between cleavage sites are expressed in base pairs.

Double digest data (see fig.3 and table 1) are especially convincing in confirming that 5′...CTCCAG is recognized by *Gsu*I, as it relates the postulated location at the predicted 3 *Gsu*I recognition sites within ϕX174 and 4 within pBR322, to 1 *Pst*I and 1 *Bcn*I in ϕX174 and to 6 *Taq*XI sites in pBR322, which location is known.

To check whether the existence of some other sequence different from 5′...CTCCAG but yielding the same fragmentation pattern of pBR322, ϕX174 and fd is possible, a computer search was performed on them. The analysis generated only the above mentioned sequence.

Obvious possibilities as that the proposed recognition sequence is in some way related to a true recognition sequence, were eliminated by manual inspection of nucleotide sequences at proposed sites in pBR322, ϕX174 and fd. In particular any

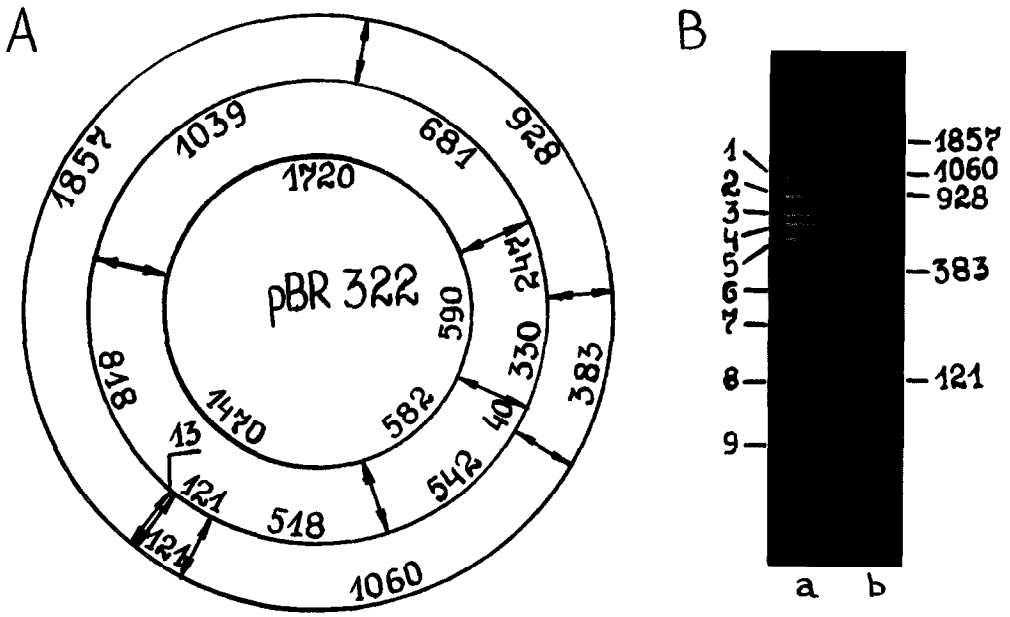

Fig.3. (A) Location of *Gsu*I and *Taq*XI sites on pBR322. Distances between recognition sequences are expressed in base pairs: outer ring, *Taq*XI cleavage map; inner ring, *Gsu*I; middle ring, *Gsu*I + *Taq*XI. (B) Gel electrophoresis of pBR322 + *Gsu*I + *Taq*XI. Samples were analysed in a slab of 2–16% polyacrylamide linear gradient gel: (a) pBR322 + *Gsu*I + *Taq*XI; (b) pBR322 + *Taq*XI. Sizes of the markers in (b) are indicated on the right in base pairs (bp).

Table 1

Comparison of computer-generated (C) and experimentally determined (E) *Gsu*I and double digest patterns of ϕX174 and pBR322 DNAs (fragment sizes in bp)

No.	ϕX174						pBR322			
	*Gsu*I		*Gsu*I + *Bcn*I		*Gsu*I + *Pst*I		*Gsu*I		*Gsu*I + TaqXI	
	C	E	C	E	C	E	C	E[b]	C	E
1.	3369	3329[a]	3369	3329	2471	2350	1720	1738 ± 42	1039	1050
2.	1577	1615	1577	1615	1577	1630	1470	1479 ± 61	818	850
3.	440	440	330	360	898	880	590	551 ± 16	681	700
4.			110	–[c]	440	420	582	633 ± 28	542	625
5.									518	540
6.									343	330
7.									247	235
8.									121	128
9.									40	40
10.									13	–

[a] Fragment size obtained by subtracting from the total molecule length the sum of the lengths of all the other fragments, deduced from electrophoresis experiments

[b] Fragment sizes represent mean values determined in 5 independent expts for 1738 bp and 1479 bp fragments ($n = 5$, $q < 0.05$) and in 7 expts for 633 bp and 551 bp fragments ($n = 7$, $q < 0.05$)

[c] – = Fragments missed on the gel

one nucleotide (N) is found at the right and left end of the sequence 5'...NCTCCAGN. We detected no similarities in the regions other than the hexanucleotide sequence postulated.

No other restriction enzyme has been described with the specificity of *Gsu*I and so this constitutes a new addition to the collection of specific endonucleases.

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