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Chromosomal Loci of Genes Controlling Site-Specific Restriction Endonucleases of *Bacillus subtilis*

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Summary. We constructed transformants of *B. subtilis* 168 which acquired genes for site-specific restriction endonucleases. These endonucleases originated from various strains of *B. subtilis* and were classified into five groups based on the specificity of the sequences recognized by the enzymes. We examined the loci of genes for site-specific restriction endonucleases belonging to different groups: *hsrE* determined Endo.R.*Bsu*1231(I), *hsrB* Endo.R.*Bsu*1247(I), *hsrR* Endo.R.*Bsu*R and *hsrC* Endo.R.*Bsu*1247(II). One gene, *hsrE*, was located between *sacA* and *purA* by transduction crosses with phage PBS1, and another gene, *hsrB*, between *hsrE* and *purA*.

Genes *hsrR* and *hsrC* had been suggested to be allelic or closely linked by previous studies with transformation. We located *hsrR* and *hsrC* between *purB* and *tre*.

Our previous observation and this study show that *B. subtilis* 168 has at least three independent loci on the chromosome for four genes for site-specific restriction endonucleases in addition to the locus for the original restriction activity (*Bsu*168-specific restriction) of strain 168.

Introduction

Since the first site-specific endo-deoxyribonuclease (type II-restriction endonuclease) Endo.R.*Hin*dII was discovered by Smith, Wilcox and Kelly (Smith and Wilcox 1970; Kelly and Smith 1970), hundreds of site-specific endonucleases had been found in various microorganisms (see Roberts 1980 for review) and widely used by many investigators in various studies to cleave DNA at specific sites.

Although site-specific endonucleases are commonly called restriction endonucleases, only a few of them are proven to be involved in the biological phenomenon of "restriction" against a foreign genetic entity. Some investigators suppose that site-specific endonucleases may have roles in cellular functions other than restriction (Shibata et al. 1976; Chang and Cohen 1977; Miller and Cohen 1978).

Strains of *Bacillus subtilis* and their relative species have been found frequently to have site-specific endonucleases (Bron and Murray 1975; Bron et al. 1975; Wilson and Young 1975; Shibata and Ando 1975, 1976; Shibata et al. 1976; Ikawa et al. 1976). In order to investigate the genes and the biological functions of site-specific endonucleases, we introduced their genes by transformation into *B. subtilis* 168 using restriction activity on phage ϕ105C for selection (Shibata et al. 1979; Ikawa et al. 1980; see Table 1). The genetic structure of *B. subtilis* 168 has been extensively analyzed (see Kejzlarová-Lepesant et al. 1975; Young and Wilson 1975; Henner and Hoch 1980 for review). All the transformants we isolated possessed the modification activity corresponding to newly introduced restriction endonuclease activity.

The site-specific restriction endonucleases introduced into *B. subtilis* 168 have been classified into five groups based on the specificity of nucleotide sequence recognized by the enzymes. All of the newly introduced restriction endonucleases recognized different sequences from that recognized by the original restriction enzyme of *B. subtilis* 168 (Table 1, Ikawa et al. 1980).

We could construct a transformant which had three restriction and modification systems, namely *Bsu*R-, *Bsu*1247(I)-, and *Bsu*1231(I)-systems in addition to the original system of *B. subtilis* 168 (*Bsu*168-system). On the other hand, *Bsu*R-, *Bsu*1247(II)- and *Bsu*1231(II)-systems could not coexist within a cell, suggesting that the genes controlling these systems are allelic, closely linked or incompatible. These results indicate that *B. subtilis* 168 has at least three independent genetic loci for the site-specific restriction endonucleases (Ikawa et al. 1980).

In this study, we describe the mapping of the genes for site-specific restriction endonucleases which had been introduced into *B. subtilis* 168.

Materials and Methods

Bacterial Strains and Phages. Bacterial strains used in this study are listed in Table 2. Phage ϕ105C is a clear plaque mutant of a temperate *Bacillus* phage ϕ105 (Shibata and Ando 1974).

Media. Spizizen's medium with minor modifications (MM) was used to test auxotrophic markers of strains (Shibata and Saito 1973). Amino acids (50 μg/ml), adenine (200 μg/ml) or thiamine (20 μg/ml) was added to MM, if required. *Tre* or *sac* marker was tested as described by Lepesant et al. (1974) and Lepesant-Kejzlarová et al. (1975).

Transduction Mediated by Phage PBS1. We carried out PBS1 transduction experiments using the method described by Dubnau (1971).

Test of Host-Specific Restriction Activities. Restriction activities of strains were examined by cross-streaking the culture of the tested strain with appropriately modified ϕ105C, as described by Wood (1966).

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Table 1. Summary of specificities and genes of site-specific endonucleases or restriction activities of *Bacillus subtilis*

Gene	Related site-specific endonuclease or restriction activity (r)	Original[a] *B. subtilis* strain	Nucleotide[b] sequence recognized	Group[c]	*B. subtilis* strains from which isoschizomer was isolated	Note and references
hsrB	Endo.R.*Bsu*1247(I) $r_{1247(I)}$	IAM1247	5′ CTGCAG 3′ 3′ GACGTC 5′	3		Isoschizomer of Endo. R. *Pst*I Shibata et al. 1976, 1979;
hsrC	Endo.R.*Bsu*1247(II) $r_{1247(II)}$	IAM1247	N.D.	2C		Shibata et al. 1979;
hsrE	Endo.R.*Bsu*1231(I) $r_{1231(I)}$	IAM1231	N.D.	4	ATCC6633 IAM1192 IAM1193	Ikawa et al. 1980; Shibata et al. 1976;
hsrF	Endo.R.*Bsu*1231(II) $r_{1231(II)}$	IAM1231	N.D.	2B	IAM1192	Shibata et al. 1976; Ikawa et al. 1980;
hsrM	Endo. unknown r_{168}	168	N.D.	1		original restriction activity of *B. subtilis* 168
hsrR	Endo.R.*Bsu*R r_R	R	5′ GGCC 3′ 3′ CCGG 5′	2A	IAM1076 IAM1114	Isoschizomer of Endo. R.*Hae*III. Bron et al. 1975, Shibata et al. 1976;

[a] The endonuclease activity had been found from the indicated strain. The ability to produce the enzyme had been introduced into *B. subtilis* 168 by transformation using DNA from the indicated strain

[b] Arrows indicate the phosphodiester bonds cleaved; N.D., not determined

[c] See Ikawa et al. 1980

Table 2. Strains of *Bacillus subtilis* 168[a]

Strains	Phenotype relating to restriction	Genotype relating to restriction	Other genotype	Reference and origin
101	r^+_{168}	wild type (*hsrM*$^+$)	*purB6 leuA8 metB5*	See ref. Saito et al. 1979
1012	r^-	*hsrM*1	*leuA*8 *metB*5	ref. Saito et al. 1979
ISB 8	$r^+_{1247(I)}$	*hsrB*$^+$*hsrM*1	*leuA*8 *metB*5	ref. Shibata et al. 1979
ISC 45	$r^+_{1247(II)}$	*hsrC*$^+$*hsrM*1	*leuA*8 *metB*5	$r^+_{1247(II)}$-transformants of 1012
ISE 15	$r^+_{1231(I)}$	*hsrE*$^+$*hsrM*1	*leuA*8 *metB*5	ref. Ikawa et al. 1980
ISR 11	r^+_R	*hsrR*$^+$*hsrM*1	*leuA*8 *metB*5	ref. Ikawa et al. 1979
ISMRB 9	r^+_{168} r^+_R $r^+_{1247(I)}$	*hsrR*$^+$ *hsrB*$^+$	*purB*6 *leuA*8 *metB*5	ref. Shibata et al. 1979
ISMRBE 17	r^+_{168} $r^+_{1247(I)}$ $r^+_{1231(I)}$ r^+_R	*hsrB*$^+$ *hsrE*$^+$*hsrR*$^+$	*purB*6 *leuA*8 *metB*5	ref. Ikawa et al. 1980
LMAH		wild type	*purA*16 *leuA*8 *metB*5 *hisA*3	from Dr. H. Yoshikawa (Kanazawa univ.)
61448		wild type	*cysA lin*	from Dr. E. Freese (NIH, Bethesda)
61516		wild type	*purB tre*	from Dr.E. Freese
61517		wild type	*sacA mtlA narB*	from Dr. E. Freese
61553		wild type	*purA*16 *leuA*8 *metB*5 *lys*	from Dr. E. Freese
61560		wild type	*purA*16 *nov*1 *leuA*8 *metB*5	from Dr. E. Freese
QB885		wild type	*purA*16 *thiC*5 *sacA*321	from Dr. R. Dedonder (IRBM, Paris)

[a] Abbreviations; r^+ and r^- indicate restriction proficiency and deficiency, respectively; r_{168}, $r_{1247(I)}$, $r_{1247(II)}$, $r_{1231(I)}$, $r_{1231(II)}$ and r_R indicate *Bsu*168-, *Bsu*1247(I)-, *Bsu*1247(II)-, *Bsu*1231(I)-, *Bsu*1231(II)- and *Bsu*R-specific restriction activities; *pur, leu, met, arg, cys, lys, thi* and *his* indicate requirement of adenine, leucine, methionine, arginine, cysteine, lysine, thiamine and histidine for growth, respectively; *lin, mtl* and *nov* indicate resistance against Lincomycin, 5-methyl-tryptophan and novobiocin, respectively; *sacA* is the structural gene for sucrase; *tre* indicates ability to assimilate trehalose

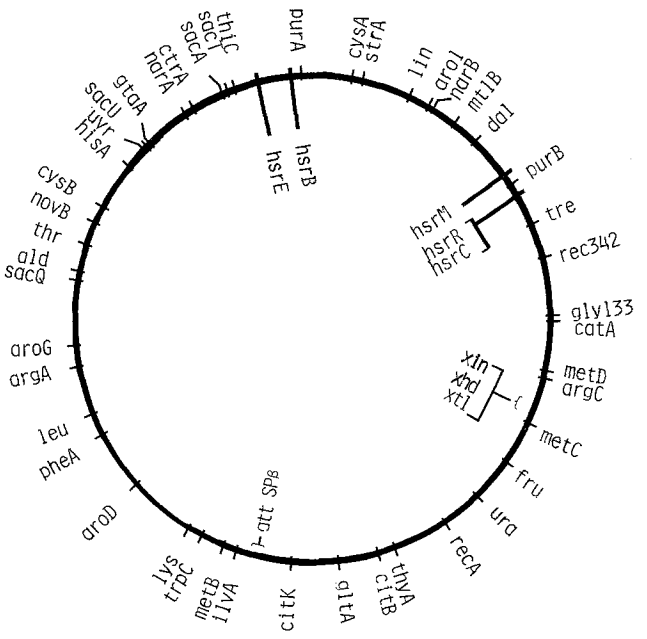

Fig. 1. Linkage map of the chromosome of *B. subtilis* 168 based on Kejzlarova-Lepesant et al. (1975). *xin*, *xhd* and *xtl* are the genes of defective prophage PBSX. att SPβ indicate the integration site of prophage SPβ

Table 3. Two-factor transduction crosses among *hsrE*, *purA*, *thiC*, *sacA* and *cysA*

Donor (11)[a]	Recipient (00)[a]	Selection (No. of samples)	Recombinant classes		No.	Percentage linkage
			*purA*16	*hsrE*		
ISE 15	LMAH	Ade$^+$	1	0	201	14
		(233)	1	1	32	
ISE 15	QB885	Ade$^+$	1	0	127	14
		(147)	1	1	20	
			*purA*16	*thiC*5		
			1	0	147	0
			1	1	0	
			*thiC*5	*hsrE*		
ISE 15	QB885	Thi$^+$	1	0	48	11
		(54)	1	1	6	
			*thiC*5	*purA*16		
			1	0	54	0
			1	1	0	
			sacA	*hsrE*		
ISE 15	61517	Sac$^+$	1	0	258	4
		(269)	1	1	11	
			cysA	*hsrE*		
ISE 15	61448	Cys$^+$	1	0	131	0
		(131)	1	1	0	

[a] "0" and "1" refer to recipient and donor markers, respectively

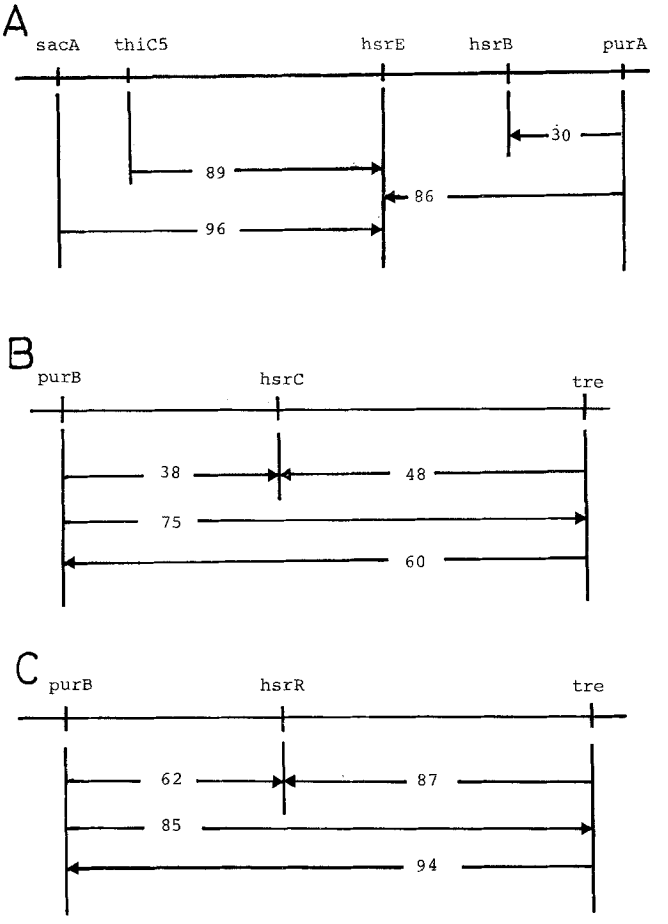

Fig. 2A–C. PBS1 transduction map of genes for site-specific endonucleases or restriction activities on the chromosome of *B. subtilis* 168. **A** *hsrB* and *hsrE*. **B** *hsrC*. **C** *hsrR*. Values are given as percentage recombination. Percentage recombination = 1-(percentage linkage)

Results

Genetic Locus of hsrE

We found that various strains of *B. subtilis* have site-specific endo-deoxyribonucleases and constructed a series of transformants of *B. subtilis* 168 to obtain one or more of the genetic determinants for these site-specific endonucleases (Table 1, Shibata et al. 1976; Ikawa et al. 1980).

A gene controlling *Bsu*1231(I)-specific restriction endonuclease was named *hsrE*. We mapped *hsrE* by two-factor transduction crosses, with *purA*16, *thiC*5, *sacA* or *cysA* marker of strain LMAH, QB885, 61517 or 61448. The gene order of this region is *sacA-sacT-thiC-sacS---purA*, and *thiC* is closely linked with *sacA* (R. Dendonder, personal communication). Transducing phage PBS1 was grown on ISE15 (*hsrE*$^+$). As shown in Table 3, *hsrE* was co-transferred with both *thiC* and *purA* (percentage linkages were 11 and 14, respectively). Since *thiC* and *purA* were not co-transferred at all by phage PBS1 (Table 3), the *hsrE* locus seems to be between *thiC* and *purA* (Fig. 2A).

We then used *sacA* as a reference marker for the region outside of the *thiC-purA* region. Linkage between *hsrE* and *sacA* was 4% and lower than that between *hsrE* and *thiC*, supporting the conclusion that *hsrE* was between *thiC* and *purA* (Table 3 and Fig. 2A). Another reference marker, *cysA* was not co-transferred at all with *hsrE* (Table 3).

Table 4. Two factor transduction crosses among *hsrB* and *purA*

Donor (11)[a]	Recipient (00)[a]	Selection (No. of samples)	Recombinant classes		No.	Percentage linkage[b]
			*purA*16	*hsrB*		
ISB 8	61553	Ade$^+$ (95)	1	0	22	77
			1	1	73	
ISMRB 9	61553	Ade$^+$ (34)	1	0	11	68
			1	1	23	
ISB 8	LMAH	Ade$^+$ (103)	1	0	22	79
			1	1	81	
ISMRB 9	LMAH	Ade$^+$ (54)	1	0	16	70
			1	1	38	
ISB 8	61560	Ade$^+$ (235)	1	0	46	80
			1	1	189	

[a] "0" and "1" refer to recipient and donor markers, respectively.
[b] Percentage linkage is the frequency (%) of donor type transductants among all transductants having selected markers (Dubnau et al. 1967)

Genetic Locus of hsrB

A gene termed *hsrB* which controls *Bsu*1247(I)-specific restriction endonuclease was mapped by two-factor transduction crosses with *purA*16 of strains 61553, 61560 and LMAH. Transducing phage PBS1 was grown on ISB8 and ISMRB9, both of which were *hsrB*$^+$. The linkage between *hsrB* and *purA* was around 72% (Table 4), irrespective of the DNA donor strains. ISB8 and ISMRB9 had been independently isolated by transformation with *B. subtilis* IAM1247 DNA.

We then located *hsrB* by three-factor transduction crossing using *hsrE* and *purA* as reference markers. Phage PBS1 was grown on ISMRBE17 (*hsrE*$^+$, *hsrB*$^+$). Strain 61560 (*purA*) was the recipient. As shown in Table 5 and Fig. 2A, *hsrB* was located between *purA* and *hsrE*.

Genetic Loci of hsrR and hsrC

The gene for *BsuR*-specific restriction endonuclease was termed *hsrR*, and that for *Bsu*1247(II)-specific restriction endonuclease *hsrC*. We found that either *hsrR* or *hsrC* was cotransferred with *purB* and *tre* by transducing phage PBS1. Then we determined

Table 5. Three factor transduction cross among *hsrB*, *hsrE* and *purA*

Donor (111)[a]	Recipient (000)[a]	Selection (No. of samples)	Recombinant classes			No.	Suggested gene order
			hsrE	*hsrB*	*purA*		
ISMRBE 17	61560	Ade$^+$ (124)	1	1	1	17	*hsrE – hsrB – purA*
			0	1	1	58	
			1	0	1	0	
			0	0	1	49	

[a] "0" and "1" refer to recipient and donor markers, respectively

Table 6. Three factor transduction crosses to determine the order of *hsrR* and *hsrC* in relation to *purB* and *tre*

Donor (111)[a]	Recipent (000)[a]	Selection (No. of samples)	Recombinant classes			No.	Suggested gene order
			*purB*6	*hsrR*	*tre*		
ISR 11	61516	Ade$^+$ (117)	1	0	0	72	*purB – hsrR – tre*
			1	1	0	26	
			1	0	1	1	
			1	1	1	18	
		Tre$^+$ (70)	0	0	1	61	*purB – hsrR – tre*
			0	1	1	5	
			1	0	1	0	
			1	1	1	4	
			*purB*6	*hsrC*	*tre*		
ISC 45	61516	Ade$^+$ (95)	1	0	0	35	*purB – hsrC – tre*
			1	1	0	36	
			1	0	1	1	
			1	1	1	23	
		Tre$^+$ (25)	0	0	1	12	*purB – hsrC – tre*
			0	1	1	3	
			1	0	1	0	
			1	1	1	10	

[a] "0" and "1" refer to recipient and donor markers, respectively

the order of the genes, *purB*, *hsrR*, *hsrC* and *tre* by three factor crosses mediated by phage PBS1, which had been grown on ISR11 (*hsrR*$^+$) or ISC45 (*hsrC*$^+$). Strain 61516 (*purB6*, *tre*) was used as recipient. As shown in Table 6 and Figs. 2B and C, the gene orders were determined to be *purB-hsrR-tre* and *purB-hsrC-tre* respectively.

Discussion

In the present study, we mapped four genes controlling restriction endonucleases of *B. subtilis* belonging to four different groups (Table 1, Ikawa et al. 1980); Those were Endo.R.*Bsu*1247(I), Endo-R.*Bsu*1231(I), Endo.R.*Bsu*R, and Endo.R.*Bsu*1247(II). We located these four genes at three loci near *purA* locus or *purB* locus on the chromosome of *B. subtilis* 168 (Figs. 1 and 2). All are different from the locus of *hsrM* which controls *Bsu*168-specific restriction enzyme, the original enzyme of *B. subtilis* 168 (Shibata and Ando 1974; Saito et al. 1979; Ikawa et al. 1979).

We had supposed that some, if not all, genes of the site-specific restriction endonucleases of *B. subtilis* might be encoded by a prophage or plasmid, since two well-studied genes of site-specific restriction endonucleases of *Escherichia coli* (Endo.R.-*Eco*RI and Endo.R.*Eco*RII) are not coded for by the chromosome but by a plasmid (Betlach et al. 1976; Bigger et al. 1973) and some of the restriction enzymes of *E. coli* and *B. stearothermophilus* have been shown to be coded for by temperate phages (Lederberg 1957; Lees and Welker 1973). Meanwhile, *B. subtilis* 168 is known to have defective prophage PBSX and prophage SPβ both of which might have as yet undetermined functions (Seaman et al. 1964; Warner et al. 1977). Our present and previous studies, however, show that all of the restriction endonuclease genes (*hsrB*, *hsrC*, *hsrE*, *hsrR* and *hsrM*) are located separately from the genes of the defective prophage PBSX or from the attachment site of prophage SPβ. It is known that the genes of PBSX are clustered between *argC* and *metC* (Garro et al. 1970; Thurm and Garro 1975) and that the attachment site of SPβ is located between *ilvA* and *kauA* (Zahler et al. 1977). Therefore, the restriction enzyme genes are obviously independent of these prophages.

To map *hsrB*, the gene for site-specific endonuclease *Bsu*1247(I), we used two transformants, ISB8 and ISMRB9, as DNA donor strains of transduction. These transformants were independently isolated using DNA from *B. subtilis* IAM1247. We found that the percent linkage between *hsrB* and *purA* is the same within experimental error, irrespective of DNA donor strain. Therefore, it seems that *hsrB* is integrated at only one locus on the chromosome of *B. subtilis* 168.

From similar reasoning, *hsrE* of Endo.R.*Bsu*1231(I) seems to have a unique locus. *HsrR* for Endo.R.*Bsu*R was located between *purB* locus and *tre* locus (Fig. 2B and Table 6). Although Trautner et al. (1974) had mapped *hsrR* (by their notation, *hsdR*) between *purA* and the replication origin of *B. subtilis* 168 chromosome, they recently obtained the same results as ours and corrected their former conclusion (C. Anagnostopoulos, personal communication).

We do not know whether inactive alleles of the exogenous restriction genes such as *hsrB*, *hsrC*, *hsrE* and *hsrR* are present on the original chromosome of *B. subtilis* 168 or whether the exogenous genes are inserted at the specified sites by partial sequence homology of neighboring region. We are now examining these possibilities and the possible allelism of *hsrR*, *hsrC* and *hsrF* (Ikawa et al. 1980; see below.) by means of gene cloning techniques.

As shown in Figs. 2B and C, the percentage recombination between *purB* and *tre* varied according to the donor strain, ISR11 or ISC45. These variations may be due to partial nonhomology of the DNA of the donor strain for transduction to that of the recipient strain around *hsrR* locus or *hsrC* locus. In ISR11 and ISC45, DNA in this region had originated from *B. subilis* R and *B. subtilis* IAM 1247 respectively (Ikawa et al. 1979; Shibata et al. 1979). Such nonhomology between donor DNA and recipient DNA is known to affect extensively the efficiency and linkage of markers in transduction by phage PBS1 (Dubnau et al. 1969). Therefore, we should not conclude that *hsrR* and *hsrC* are separated on the basis only of the difference in percentage recombination between *hsrR* or *hsrC* and *purB* or *tre* (Figs. 2B and C). We rather re-emphasize our previous suggestion that *hsrR* and *hsrC* may be allelic or closely linked. We had examined 100 *Bsu*R-specific restriction proficient transformants derived from *Bsu*1247(II)-specific restriction proficient strain (ISC30) and found that all the transformants had lost *Bsu*1247(II)-specific restriction activity without exception (Ikawa et al. 1980).

We failed to locate *hsrF* for *Bsu*1231(II)-specific restriction endonuclease, since PBS1 did not multiply on *Bsu*1231(II)-specific restriction proficient strains.

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