Characterization of the chromosomes of *Bacillus subtilis* merodiploid strains by quantitative DNA–DNA hybridization

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The position of junctions and the extent of the duplicated chromosomal regions in *Bacillus subtilis* merodiploid strains were studied by quantitative DNA–DNA hybridization. We describe a method which allows (i) the identification of genes present in two copies per chromosome and (ii) the measurement of the amount of additional DNA in chromosomes with relatively large duplicated regions (about 10% or more). Analysis of previously described *B. subtilis* merodiploid strains GSY1127, GSY1800 and GSY1835 revealed that the duplicated segments represent $29 \pm 2\%$, $7 \pm 2\%$ and $13 \pm 2\%$ of the chromosome, respectively. Small discrepancies between these and previous genetic linkage data are discussed. Support for a role of prophage SP β in the formation of merodiploid GSY1835 is provided. In conclusion, the described method confirmed the genetic maps of the merodiploids previously obtained by transduction and transformation crosses and showed that a duplication of a segment is not accompanied by large deletions of other chromosomal regions, providing direct evidence that a cell can accommodate genomes of substantially increased size.

Keywords: *Bacillus subtilis*, merodiploids, chromosome size, prophage SP β

INTRODUCTION

In bacteria, the presence of additional DNA, either plasmidic or a duplication of a chromosomal region, is frequently accompanied by reduced growth rate of the cells. In the absence of any selective pressure, additional DNA is often lost, as if the cell tends to restore a normal DNA/volume ratio, which apparently ensures the highest growth rate. For instance, it was recently shown that *Bacillus subtilis* cells are unable to accommodate two complete chromosomes; multiple chromosomal copies, present in fused protoplasts, are segregated in separate cells at the time of cell wall regeneration (Hauser & Karamata, 1992). Therefore, assuming that a cell tends to restore an optimal DNA/volume ratio, it is conceivable that diploidy of a given region could be compensated by deletion(s) in other, possibly non-essential, regions of the chromosome. The existence of *B. subtilis* merodiploid strains, shown to contain diploid regions of up to 29% of the genome (Anagnostopoulos, 1990; see below), offers a possibility to test his hypothesis by comparing the

chromosomal size of merodiploid to haploid cells by physical methods.

We report a method, based on DNA–DNA hybridization, which allows accurate determination of the size of merodiploid chromosomes and the identification of markers present in two copies. The results presented show unambiguously that duplicated regions correspond to additional DNA. They largely confirm the size of the duplicated regions obtained by genetic linkage (Anagnostopoulos, 1990).

METHODS

Bacterial strains, plasmids and bacteriophages. These are listed in Table 1. All recombinant plasmids and bacteriophages contain chromosomal fragments of strains isogenic to *B. subtilis* 168.

Media, growth conditions and strain conservation. Rich SA medium and minimal medium S were as described by Karamata & Gross (1970). SR, the complex medium of Schaeffer *et al.* (1965), supplemented with 20 µg thymidine ml⁻¹ and solidified with Gibco agar (1.2%, w/v), was used for strain conservation after sporulation at 30 °C. For conservation of the merodiploid strain GSY1127, cells growing exponentially in minimal medium S were supplemented with glycerol (15%, v/v) and stored

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Table 1. Bacterial strains, plasmids and bacteriophages

Strain, plasmid or bacteriophage	Genotype or description	Source, reference or remarks
<i>Bacillus subtilis</i>		
L1440	Prototroph	Mauël & Karamata (1984)
GSY1269	<i>trpE26 ilvC1</i>	Trowsdale & Anagnostopoulos (1975)
GSY1127	<i>hisH2 ilvC1/ilvC⁺</i>	Anagnostopoulos & Trowsdale (1976)
GSY1800	<i>trpE26 hisH2 tyr.A⁺/trpE26⁺ hisH⁺ tyr.A1</i>	Schneider <i>et al.</i> (1982)
GSY1835	<i>trpE30</i>	Schneider & Anagnostopoulos (1981)
Plasmids		
pPRTR1	pJH101 with a 0.7 kb insert carrying gene <i>degR</i> (formerly <i>prrR</i>)	BGSC*; Yang <i>et al.</i> (1987)
pUF7-5	pJH101 with a 1.7 kb insert from the <i>flaA</i> locus	Gianni & Galizzi (1986); <i>outI</i> was shown to correspond to the <i>flaA</i> locus (Albertini <i>et al.</i> , 1991; Hauser <i>et al.</i> , 1991)
pMP33/25	pJH101 with a 4.5 kb insert carrying gene <i>dnaB</i>	A. Galizzi, Università di Pavia, Italy; Ogasawara <i>et al.</i> (1986)
pMP60/13	pJH101 with a 11 kb insert carrying gene <i>dnaN</i> (formerly <i>dnaG</i>)	Perego <i>et al.</i> (1987)
pO.P	pBR322 with a 3.6 kb carrying gene <i>outB</i>	Ferrari <i>et al.</i> (1985b)
pSU12	pBR322 with a 9.5 kb insert carrying gene <i>hsdM</i> (formerly <i>bsrM</i>)	Kiss <i>et al.</i> (1985)
pTRP-H3B2†	pBR322 with a 2.2 kb insert carrying genes <i>trpE</i> and <i>trpD</i>	BGSC*; Band <i>et al.</i> (1984); the 2.9 kb fragment from the <i>EcoRV</i> site of the insert the most distant from gene <i>trpD</i> to the <i>PvuII</i> site of the vector was used as probe for gene <i>trpE</i>
pTRPD	pMTL20EC with a 0.9 kb insert carrying gene <i>trpD</i>	This work; the 0.9 kb fragment of plasmid pTRP-H3B2 from the <i>PvuII</i> site of the insert to the <i>SphI</i> site of the vector was subcloned and used as probe for gene <i>trpD</i>
pBD54	pBR322 with a 5.2 kb insert carrying gene <i>cotA</i>	Donovan <i>et al.</i> (1987)
pphe ⁺ 1	pMTL20EC with a 1.4 kb insert carrying gene <i>pheB</i> and part of gene <i>pheA</i>	Hauser & Karamata (1992)
p8104	pMTL20EC with a 1.5 kb insert from the region of gene <i>tagB</i>	Young <i>et al.</i> (1989); fragment J
pC8	pMTL20EC with a 1.4 kb insert from prophage PBSX genome	Longchamp (1990)
pDALsub1	pBS42 with a 1.8 kb insert carrying gene <i>dal</i>	BGSC*; Ferrari <i>et al.</i> (1985a)
pFM1	pBR325 with a 6 kb insert carrying gene <i>purM</i>	Saxild & Nygaard (1988)
pLUM20	pHV32 with a 3.2 kb insert carrying genes <i>glpD</i> and <i>glpK</i>	Holmberg & Rutberg (1989)
pUC12sspE	pUC12 with a 1.2 kb insert carrying gene <i>sspE</i>	Hackett & Setlow (1987)
pPAT83	pJAB1 with a 1.8 kb insert carrying gene <i>spoIIE</i>	Collins <i>et al.</i> (1985)
Bacteriophages		
λC4BsF14	λCharon4A with a 13.3 kb insert carrying gene <i>dnaC</i>	Perego <i>et al.</i> (1987)
λ970	λCharon4A with a 17 kb insert from prophage SPβ genome	M. G. Sargent; isolated from the DNA bank of Ferrari <i>et al.</i> (1981)
λ378	λCharon4A with a 16 kb insert from prophage SPβ genome	As for λ970; the fragment of prophage SPβ present in this phage shows no overlap with that present in phage λ970

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† The *trpE* and *trpD* genes present on this plasmid were found to be those of strain 168. Band *et al.* (1984) raised the possibility that the *trp* genes of the sequenced W168 strain derive from W23. However, Southern experiments with strain W168 *trp* genes as probes and strain 168 chromosomal DNA (results not presented) revealed that in strain W168 only part of gene *trpC* derives from W23.

at -80°C after being frozen in liquid nitrogen. When required, media were supplemented with $20\text{ }\mu\text{g ml}^{-1}$ of amino acids.

Quantitative DNA-DNA hybridization. Chromosomal DNA was prepared from stationary-phase cultures (about 3×10^8 cells ml^{-1} , at least 3 h after the end of the exponential phase), grown in SA medium at 37°C . Following extraction (Marmur, 1961), DNA was purified on a CsCl density gradient (Maniatis *et al.*, 1982), measured by the diphenylamine method, and stored at -80°C . Plasmid and bacteriophage DNA were prepared as described by Del Sal *et al.* (1988) and Grossberger (1987), respectively. Radioactive probes were synthesized by the random priming method (Feinberg & Vogelstein, 1983), i.e. restriction fragments corresponding to *B. subtilis* DNA inserts, purified by a GeneClean kit (supplied by Lucerna Chem, Switzerland), were labelled with $5'[\alpha\text{-}^{32}\text{P}]\text{deoxyadenosine triphosphate}$ (3000 Ci mmol^{-1} , 111 TBq mmol^{-1} ; Amersham). Quantitative hybridization was performed as described by Young *et al.* (1989). For each strain to be probed, DNA samples of 0.05, 0.15 and 0.5 μg were adsorbed onto filters. Probes were generated from cloned fragments of different strain 168 derivatives and used at the highest concentration. After hybridization, filters were washed three times in $2 \times \text{SSC}/0.1\%$ SDS for 10 min at room temperature, once in $0.5 \times \text{SSC}/0.1\%$ SDS for 60–90 min at 65°C , and their radioactivity measured. The saturation of the samples by probe DNA was assessed by the proportionality of the hybridization counts to the quantity of adsorbed DNA. Since in some instances 0.5 μg samples were not saturated, counts obtained from the 0.15 μg samples, providing higher and thus more accurate counts than the 0.05 μg samples, were used throughout this study (see Table 2).

RESULTS AND DISCUSSION

Description of the system and theoretical correlation between the size of the duplicated region and quantitative hybridization counts

The experiments were performed on merodiploid strains GSY1127, GSY1800 and GSY1835 of *B. subtilis* (Fig. 1), shown by genetic linkage to contain duplicated regions corresponding to 25%, 4% and 7% of the genome (Anagnostopoulos, 1990). They were obtained by transformation crosses between strains with the chromosomal structure of strain 168 and those carrying chromosomal rearrangements associated with the *trpE26* mutation, i.e. translocation of segment C of the chromosome, involving disruption of the *trpE* gene, and inversion of the segment A–I flanking the replication origin (Audit & Anagnostopoulos, 1972; Anagnostopoulos & Trowsdale, 1976; Schneider & Anagnostopoulos, 1981; Anagnostopoulos, 1977; Schneider *et al.*, 1982). The genetic maps of merodiploid strains presented in Fig. 1 were deduced by the latter authors from transduction and transformation linkages.

Duplication of a chromosomal fragment corresponding to $x\%$ of the haploid genome (relative size = 100) leads to a merodiploid cell whose relative chromosome size is $100+x$. The number of such chromosomes per unit weight of DNA is reduced by $100/(100+x)$ compared to the number of haploid chromosomes. Therefore, when a given probe is hybridized to a unit weight of merodiploid DNA, the number of counts obtained will not only depend on the chromosomal localization of the probe (haploid or diploid region), but also on the size (x) of the

duplicated segment. The ratios of hybridization counts obtained with haploid to those obtained with merodiploid DNA will be $R_1 = (100+x)/100$ or $R_2 = 0.5 \cdot (100+x)/100$ for genes present in one or two copies in the merodiploid chromosome, respectively. Thus, the relative size (x) of the duplicated fragment can be calculated from hybridization counts ratios as follows:

$$x = 100(R_1 - 1) = 100(2R_2 - 1) \quad (1)$$

Determination of the size of duplicated regions in merodiploid strains and the identification of specific genetic markers present in two copies

DNA preparations were obtained from stationary-phase cultures of haploid strains L1440 and GSY1269, and merodiploid strains GSY1127, GSY1800 and GSY1835, and their concentration determined by the diphenylamine method. A 0.15 μg sample of each DNA preparation, adsorbed on filters, was hybridized with radioactive probes at a saturating concentration (see Methods). The probes were obtained from clones whose map positions, previously ascertained by physical mapping (not presented), are distributed relatively uniformly around the chromosome (Fig. 1). For all the probes shown in Table 2, the ratio between counts obtained with the relevant haploid strain and the merodiploid strains was calculated. In addition, these ratios were obtained (not presented) for a further 28 probes arbitrarily chosen from a DNA library of strain 168 (Mauël *et al.*, 1989). For each merodiploid strain, the mean of the ratios obtained with all probes present in one (R_1) or in two copies (R_2), with the exception of *trpD* and *trpE* (see below), were introduced into equation (1) derived here above. The calculation provides the size of the duplicated region, i.e. the additional DNA in merodiploid strains GSY1127, GSY1800 and GSY1835, which amounts, respectively, to $29 \pm 2\%$, $7 \pm 2\%$ and $13 \pm 2\%$ of the haploid 168 chromosome (Table 3). For strain GSY1127, this is in good agreement with data obtained by mapping, while for strains GSY1835 and GSY1800, the calculated figures are higher than those obtained by genetic methods (Table 3). The latter discrepancy can be readily accounted for by the fact that linkage was obtained from crosses between markers too distant from the junctions for adequate precision. In particular, due to absence of a suitable marker, the size of segment C was probably underestimated.

Inspection of Table 2 reveals that genes *pheA* and *dnaB* of strain GSY1127, gene *degR* of strain GSY1800, and genes *bsdM*, *purM*, *cotA* and *sspE* of strain GSY1835 are present in two copies.

The anomalies obtained with *trpE* and *trpD* probes, i.e. apparently non-integer numbers of corresponding genes (Table 2), have their explanation in the fact that the probes were derived from strains homologous to *B. subtilis* 168, while part of the *trp* region present in merodiploids originates from *B. subtilis* W23. Firstly, the construction of merodiploids GSY1127 and GSY1800 involves the correction of the *trpE26* mutation by a wild-type allele. The latter was derived from different 168/W23 hybrid

Fig. 1. Chromosomal structure of merodiploid strains GSY1127, GSY1800 and GSY1835 and their parent *trpE26* and 168 type strains as revealed by genetic linkage in appropriate transformation and transduction crosses. '*trpE26* type' refers to the mutant 166 *trpE26* isolated by X-ray irradiation of spores of the Marburg strain (Burkholder & Giles, 1947), as well as to strains of 168 genetic background into which the rearrangements associated with the *trpE26* mutation of strain 166 have been introduced by transformation. Letters A to D and roman numerals I and II indicate different segments of the chromosome. Segments B, C and Ib are duplicated in merodiploids GSY1127, GSY1800 and GSY1835, respectively. O and T are the origin and terminus of replication. Known genes probed in this work by quantitative DNA-DNA hybridization are indicated on the map of strain 168.

strains (C. Anagnostopoulos, personal communication), in which the original, strain 168, *trpC2* mutation was corrected by DNA from strain W23 (often designated 23, C. Anagnostopoulos, personal communication). This event was accompanied by replacement of part, or all, of the strain 168 *trpD* and *trpE* genes, closely linked to *trpC*, by the analogous but heterologous region of strain W23. Secondly, control hybridizations of 168 probes with homologous (168) and heterologous W23 DNA revealed, with the latter, a 50% reduction of hybridization counts. Thirdly, the mapping of the B-C junction between residues 1554 and 1555 in the 1751 bp sequence of gene *trpE* (Henner *et al.*, 1984; Jarvis *et al.*, 1990) and the maps of relevant merodiploids (Fig. 1) suggest that in GSY1127 the major part of *trpE* and the *trpD* gene are present in two and one copies, respectively, while in GSY1800, the respective genes are present in one and two copies.

Therefore, since the fragment of gene *trpE* used as probe is localized on segment B, the apparently anomalous hybridization counts for the *trp* markers (Table 2) reveal that, of the two *trpE* genes in GSY1127 and the two *trpD* genes in GSY1800, one derives from 168 and the other from W23. In addition, the high ratio of 2.30 (Table 2), i.e. the low hybridization of the probe to merodiploid DNA, shows that the single *trpD* copy in strain GSY1127 originates from W23. Preliminary evidence obtained by Southern hybridization is compatible with these conclusions. Further investigations should assess whether the heterologous nature of the *trp* genes, present at the junctions of the two duplicate regions, played any role in the stability of the merodiploids.

Inspection of Table 2 reveals that the target of probe λ 970, which includes part of prophage SP β , is present in two

Table 2. Quantitative DNA–DNA hybridization of specific cloned 168 markers and *B. subtilis* merodiploid strains GSY1127, 1800 and 1835

Probed gene	Hybridization counts (c.p.m.)*					Ratio of haploid/merodiploid hybridization counts‡		
	Haploid strains		Merodiploid strains			GSY1127	GSY1800	GSY1835
	L1440	GSY1269†	GSY1127	GSY1800	GSY1835			
<i>dnaN</i>	3575	—	3265	3498	3109	1.09	1.02	1.15
<i>spoIIIE</i>	1208	—	988	1268	1006	1.22	0.95	1.20
<i>outB</i>	2949	—	2438	2917	2552	1.21	1.01	1.16
<i>dal</i>	144	—	120	123	130	1.20	1.17	1.11
<i>bsdM</i>	109	—	97	137	214	1.12	0.80	0.51
<i>cotA</i>	4141	—	3515	4482	7039	1.18	0.92	0.59
<i>purM</i>	1627	—	1395	1637	3108	1.17	0.99	0.52
<i>sspE</i>	1852	—	1332	1434	3424	1.39	1.29	0.54
<i>glpD,K</i>	3117	—	2729	2990	2287	1.14	1.04	1.36
PBSX	2406	—	1978	1961	2012	1.22	1.23	1.20
<i>flaA</i>	3470	—	2662	3260	3060	1.30	1.06	1.13
SPβ-λ378	—	906	799	1020	1109	1.13	0.89	0.82
SPβ-λ970	—	1612	1203	1302	3114	1.34	1.24	0.52
<i>degR</i>	2005	—	1411	3963	1833	1.42	0.51	1.09
<i>trpD</i>	—	1791	780	2427	1373	<u>2.30</u>	<u>0.74</u>	1.30
<i>trpE</i> §	—	642	770	630	562	0.83	1.02	1.14
<i>pheA</i>	2490	—	3357	2485	2077	0.74	1.00	1.20
<i>dnaB</i>	2757	—	3675	2430	2527	0.75	1.13	1.09
<i>tagB</i>	2514	—	1850	1759	2133	1.36	1.43	1.18
<i>dnaC</i>	1514	—	1293	—	—	1.17	—	—

*Chromosomal DNA was extracted from stationary-phase cultures in SA medium. DNA samples of each merodiploid and reference haploid strain were adsorbed on filters. Probes were generated from cloned strain 168 chromosomal fragments. Counts obtained with the 0.15 µg DNA samples were used (see Methods). The background was between 14 and 45 c.p.m. Each figure refers to a single experiment.

† In the course of this study, it has appeared that strain L1440, the reference haploid 168 type strain, (i) is either cured of SPβ or bears a deletion at the prophage level (data not presented) and (ii) possesses *trpE* and *trpD* genes which originate from strain W23 (data not presented). Therefore, hybridization counts for SPβ and *trp* markers were determined with the DNA from the haploid *trpE26* type strain GSY1269, which is lysogenic for SPβ and whose *trp* region is isogenic to that of strain 168.

‡ Bold characters denote duplicated DNA homologous to that of the probes used. The underlined ratios correspond to *trp* probes which yielded an apparently non-integer number of copies of corresponding genes.

§ The fragment of gene *trpE* used as probe belongs to segment B (see text and Fig. 1).

Table 3. Excess DNA in merodiploids as percentage (x) of the haploid chromosome

The mean values of x were obtained from values for genes located in the haploid as well as in the diploid regions.

Method	Merodiploid strain:		
	GSY1127	GSY1800	GSY1835
Quantitative DNA–DNA hybridization*	29.0 ± 2.2	6.7 ± 1.9	12.9 ± 1.8
Genetic mapping (Anagnostopoulos, 1990)	25	4	7

* Figures obtained from data in Table 2, with the exception of *trpD* and *trpE* markers, as well as for the 28 randomly chosen probes (see text). The means are shown ± SD.

copies in the merodiploid GSY1835. Segment Ib of strain 168 includes at its extremity proximal to the origin of replication a secondary SPβ attachment site (Lipsky *et al.*, 1981), unoccupied by a prophage. This is also true for *trpE26* strains, GSY1127, GSY1800 and one of the two copies of fragment Ib of strain GSY1835 (data not presented). Therefore, the second copy of a fragment of SPβ found in the latter strain cannot but have arisen from the prophage located in segment D. The model for the generation of GSY1835 (Schneider & Anagnostopoulos, 1981) proposed a recombination near prophage SPβ which could induce precisely such a translocation. Preliminary evidence in favour of the role of prophage SPβ in the generation of GSY1835 has already been obtained (Schneider & Anagnostopoulos, 1983).

In conclusion, the quantitative, physico-chemical method devised has revealed that duplicated chromosomal regions of all *B. subtilis* merodiploid strains examined correspond

to additional DNA which, in strain GSY1127, is equivalent to about 30% of the chromosome. It follows that *B. subtilis* cells, with an apparently normal morphology (Hauser, 1991), can accommodate genomes of substantially increased size. Probably, the largest duplication, amounting, according to this contribution, to a total of 36% of the chromosome, is present in the unstable merodiploids designated Trp^+ and $\text{IlvA}^+ \text{Hom}^+$ (Anagnostopoulos, 1990). However, strain GSY1127 grows significantly more slowly than the wild-type (Hauser, 1991) and its DNA/cell volume ratio, presently under investigation, should throw new light on the importance of this ratio for cell growth.

ACKNOWLEDGEMENTS

This contribution is dedicated to C. Anagnostopoulos for his 75th birthday. We are grateful to him for strains, fruitful discussions and critical reading of the manuscript. We thank A. Galizzi, C. Holmberg, A. Kiss, P. Longchamp, P. Margot, P. Nygaard, R. J. Roberts, P. Setlow, H. Zalkin, L. Zheng and the *Bacillus* Genetic Stock Center (Ohio State University) for providing recombinant plasmids and bacteriophages.

This work was submitted by P. M. Hauser as part fulfilment for a PhD degree at the University of Lausanne.

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Received 23 August 1993; revised 10 January 1994; accepted 18 January 1994.