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Chromosomal Organization of rRNA Operons in *Bacillus subtilis*

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ABSTRACT

Integrative mapping with vectors containing ribosomal DNA sequences were used to complete the mapping of the 10 rRNA gene sets in the endospore forming bacterium *Bacillus subtilis*. Southern hybridizations allowed the assignment of nine operons to distinct *Bcl*I restriction fragments and their genetic locus identified by transductional crosses. Nine of the ten rRNA gene sets are located between 0 and 70° on the genomic map. In the region surrounding *cysA14*, two sets of closely spaced tandem clusters are present. The first (*rrnJ* and *rrnW*) is located between *purA16* and *cysA14* closely linked to the latter; the second (*rrnI*, *rrnH* and *rrnG*) previously mapped within this area is located between *att*SPO2 and *glpT6*. The operons at or near the origin of replication (*rrnO,rrnA* and *rrnJ,rrnW*) represent "hot spots" of plasmid insertion.

THE *Bacillus subtilis* genome contains 10 rRNA operons, each with genes coding for 16S, 23S and 5S rRNA subunits, as well as $tRNA^{ala}$ and $tRNA^{ile}$ found in operons *rrnO* and *rrnA* in the spacer region between 16S and 23S rDNA (LOUGHNEY, LUND and DAHLBERG 1982; STEWART, WILSON and BOTT 1982). While *Escherichia coli* contains seven scattered rRNA operons (NOMURA, GOURSE and BAUGHMAN 1984), the endospore-forming bacterium *B. subtilis* has 10 rRNA gene sets which are highly clustered near the origin of replication (BOTT, STEWART and ANDERSON 1984; LAFAUCI *et al.* 1986). *B. subtilis* and perhaps Gram-positive organisms in general, may have a unique type of rRNA and tRNA gene organization because of the sporulation and germination processes that they undergo. One unusual aspect of the translational apparatus of *B. subtilis* not seen in *E. coli*, is that the rRNA and tRNA genes are highly clustered (VOLD 1985). We have been studying the genetic and structural organization of the 10 rRNA gene sets and the functional role for this clustering near the origin of replication of *B. subtilis*.

Previous to this study, the following information was available: the operons *rrnO* and *rrnA* are located near the replication origin (HENKES *et al.* 1982; OGASAWARA, SEIKI and YOSHIKAWA 1983; WILSON, HOCH and BOTT 1981), the tandemly situated repeats *rrnI-rrnH-rrnG* are found near the attachment site of phage SPO2 (BOTT, STEWART and ANDERSON 1984; CHOW and DAVIDSON 1973) and two operons *rrnE* and *rrnD* are at 44° and 70° on the genetic map, respectively (LAFAUCI *et al.* 1986). Additional locations, *rrnB* and possibly *rrnC* map between *aroG* and *thrA* (BOTT, STEWART and ANDERSON 1984). One operon, *rrnR*, was believed to be located at the *ilvBC-leu* region (GOTTLIEB, LAFAUCI and RUDNER 1985; LAFAUCI *et al.* 1986; PIGGOT and HOCH 1985; SMITH *et al.* 1968; VOLD 1985).

To establish the identity of each *rrn* operon in *B. subtilis*, genomic DNA was digested with *Bcl*I, which restricts once at the 3′ end of each 23S gene (GREEN *et al.* 1985; LAFAUCI *et al.* 1986). Ten unique *Bcl*I DNA fragments are detected by Southern blot hybridization with radioactive cloned ribosomal DNA sequences (LAFAUCI *et al.* 1986). By integrative mapping and transductional crosses, we report the assignment of 9 of the 10 *Bcl*I fragments to specific genetic loci. The two proposed operons *rrnC* and *rrnR* are absent. Instead a cluster of two operons *rrnJ-rrnW* located upstream of *cysA14* represent new assignments. The second cluster *rrnI-rrnH-rrnG* which was previously known (BOTT, STEWART and ANDERSON 1984; CHOW and DAVIDSON 1973; PIGGOT and HOCH 1985; ZEIGLER and DEAN 1985) has been given a revised map position between *att*SPO2 and *glpT6*. These findings show that 9 of the 10 rRNA gene sets are located between 0 and 70° on the genomic map.

MATERIALS AND METHODS

Bacterial strains and plasmids: The *B. subtilis* strains and plasmids used in this study are described in Table 1. The parental strains are used either as representative sources of donor DNA or as recipients in transformational or transductional crosses. The table indicates the strains with a normal number of *rrn* operons, those with a natural deletion and those with an interrupted *rrn* gene set as determined from *Bcl*I Southern hybridizations (see below).

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TABLE 1

Strains and plasmids used in this study

Strains or plasmids	Genotype	Deleted or interrupted *rrn*[a]	Source
I. A. Parental strains			
NCTC3610	Prototroph		A. SONENSHEIN
168T	*trpC2*		K. BOTT
Kit1	*trpC2 purA16 cysA14*		D. DUBNAU
SB25	*trpC2 hisH2*		D. DUBNAU
E88	*trpE46 hisH2*		J. KANE
BD170	*trpC2 thr-5*	*rrnG**	D. DUBNAU
BD79	*leuB1 pheA1*	*rrnG**	D. DUBNAU
CU420	*trpC2 leuB6 ilvC4*	*rrnW**	A. GARRO
CU542	*trpC2 leuB12 ilvC4*	*rrnW**	A. GARRO
B. Mapping strains			
Kit 1 to kit 9	Mapping recipients		D. DUBNAU
1A474	*trpC2 amyE aroI906*		BGSC[b]
1A429	*trpC2 aroI906 glpT6*		BGSC
1A241	*cysA14 rpsE2*		BGSC
1A221	*lin-2*		BGSC
RJ4	*trpC2 amyE lin-2 aroI906*		This study[c]
RJ13	*trpC2 aroI906 glpT6 lin-2*		This study
RJ25	*trpC2 aroI906 glpT6 rpsE2*		This study
IS25	*cysA14 lin-2* (SP02)		I. SMITH
II. Integrant strains[d]			
No.	Plasmid		
168T-18	pGR102 *trpC2* Cm^r	*rrnD*	This study
168T-37	pWR103 *trpC2* Cm^r	*rrnG*	This study
168T-73	pWR103 *trpC2* Cm^r	*rrnE*	This study
168T-79	pWR103 *trpC2* Cm^r	*rrnH*	This study
168T-80	pWR103 *trpC2* Cm^r	*rrnW*	This study
168T-83	pGR102 *trpC2* Cm^r	*rrnB*	This study
168T-87	pGR102 *trpC2* Cm^r	*rrnA*	This study
168T-88	pGR102 *trpC2* Cm^r	*rrnJ*	This study
SB25-35	pGR110 *trpC2 hisH2* Cm^r	*rrnO*	This study
III. *E. coli* strains	*hsdS20 recA13 aro-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*		K. BOTT
HB101	*recB21 recC22 sbcB15*		J. CLARK
JC7623	*recB21 recC22 sbcA23 recA56*		J. CLARK
JC9604			

[a] Operons, *i.e.*, *rrnG** or *rrnW** denote genes where naturally occurring or induced deletions due to plasmid insertions occur (WIDOM *et al.* 1988); those without denote operons interrupted by plasmid insertion. Assignments are based on the absence of a unique *BclI* fragment (see Table 2 and Figure 2).

[b] Bacillus Genetic Stock Center, Columbus, Ohio.

[c] Mutations introduced by transformation with donor DNA from strain 1A221 or 1A241.

[d] Isolated as Cm^r transformants after plasmid transformation with the recombinant plasmids shown in Figure 1a.

The integrant strains were obtained by transformation with one of the six integrable plasmids described below, all derivatives of the parent vector pJH101 (FERRARI *et al.* 1983) (Figure 1a). These plasmids were transformed into *Escherichia coli* HB101 or into strains JC7623 and JC9604 containing mutations in the *recBC* and *sbc* enzymes (COHEN and CLARK 1986) and clones selected according to drug resistance markers (Figure 1a). We designate the various *B. subtilis* transformants as the parent strain dash (—) clone number and the specific plasmid integrated in the next column (Table 1).

Culture methods, transformation and transduction: All genetic methods in *B. subtilis* and *E. coli* were done as previously described (LAFAUCI *et al.* 1986). Selection and scoring of drug resistance markers was done on either tryptose blood agar base (TBAB, Difco Laboratories, Detroit, Michigan) or LB plates containing 10 μg of chloramphenicol, or 125 μg of spectinomycin (Sigma Chemical Corp., St. Louis, Missouri) or 50 μg of lincomycin per ml (gift from Upjohn). The *rpsE2* mutation confers resistance to spectinomycin. Resistance to lincomycin was also scored on minimal plates with 0.5% glycerol, 0.025% vitamin-free casamino acids, the required amino acids and 50 μg of lincomycin per ml as described by LINDGREN (1978). $GlpT^+$ recombinants are sensitive and $GlpT^-$ are resistant to 40 μg of fosfomycin per ml (Sigma) added to a low-phosphate medium (MIKI, MINAMI and IKEDA 1965) containing peptone (10 g/liter; Difco) as the main carbon source (LINDGREN 1978). The *amyE* gene was scored on TBAB plates containing 1.0% potato starch (Sigma) and after overnight incubation, the plates were flooded with approximately 2 ml of a solution of 0.5% (wt/vol) I_2 and 5.0% (wt/vol) KI (NICHOLSON and CHAMBLISS 1985). Colonies exhibiting an unstained halo of starch hydrolysis were scored as $AmyE^+$. Lysogeny for phage SPO2 was scored by replicating recombinant colonies on MB plates containing tryptone (10 g/liter, Difco)

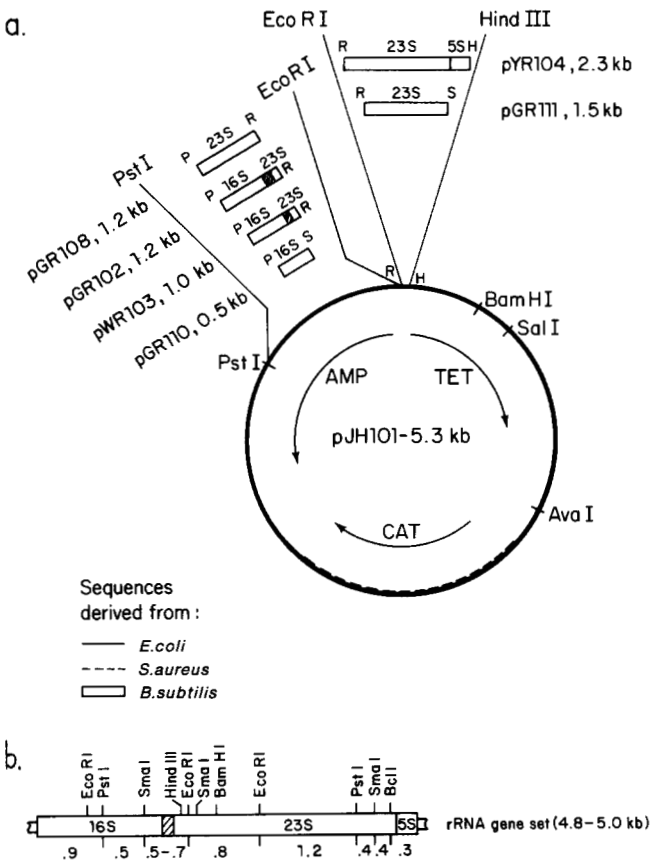

FIGURE 1a.—Restriction endonuclease map of plasmid pJH101 (FERRARI *et al.* 1983) and various integrable derivatives containing *rrn* sequences of *B. subtilis*. Abbreviations: TET, tetracycline resistance; CAT, chloramphenicol acetyl transferase; AMP, ampicillin resistance; kb, kilobases. The letters H, P, R and S indicate *Hin*dIII, *Pst*I, *Eco*RI and *Sma*I restriction sites, respectively. b, Generalized restriction map of a *B. subtilis* rRNA gene set as proposed by STEWART, WILSON and BOTT (1982). The hatched area represents the abutment region between 16S and 23S rDNA with or without tRNA genes.

0.5% NaCl, 10 mM $MgSO_4$ and 0.2% maltose (YEHLE and DOI 1967) that had been spread with 10^7 to 10^8 plaque-forming units of SPO2c phage, a clear plaque mutant of SPO2 as described by SMITH and SMITH (1973). Lysogenic colonies (recipient phenotype) grew, while nonlysogenic colonies (donor) showed no growth on these plates. The recipients 1A241, RJ25 and the donors 168T-37, 168T-79, 168T-80 and 168T-88 (Table 1) were lysogenized with phage SPO2 lysates obtained by induction of the lysogenic strain IS25 (SPO2) with 0.5 μg mitomycin C per ml (Sigma) according to SMITH and SMITH (1973).

DNA manipulation: DNA preparations were carried out as described previously (LAFAUCI *et al.* 1986). Plasmid DNA was purified from *E. coli* culture essentially by the procedure of TANAKA and WEISBLUM (1975). Chromosomal DNA was restricted for Southern hybridizations as described previously (LAFAUCI *et al.* 1986). The construction of pGR102 and pWR103 was described previously (LAFAUCI *et al.* 1986) (Figure 1a). The other plasmids containing pure 23S, 23S–5S, or 16S sequences were constructed as follows: (1) pYR104 contains a 2.3-kb *Eco*RI-*Hin*dIII 23S–5S fragment from p12E2 (STEWART, WILSON and BOTT 1982) cloned into the *Eco*RI-*Hin*dIII sites of pJH101 by ligating with T4 DNA ligase (New England Biolabs, Beverly, Massachusetts) restricted DNAs at a 2:1 target to vector ratio; (2) pGR108 contains the 1.2-kb *Pst*I-*Eco*RI 23S fragment from pYR104 inserted into the *Pst*I-*Eco*RI sites of pJH101 as above yielding a plasmid with pure 23S sequences in the opposite orientation with respect to the CAT gene of the vector; (3) pGR102 was restricted with *Eco*RI-*Sma*I; the *Eco*RI recessed end was filled with Klenow enzyme (IBI Inc., New Haven, Connecticut) followed by blunt-end ligation according to MANIATIS, FRITSCH and SAMBROOK (1982) to yield pGR110 containing *Pst*I-*Sma*I pure 16S sequences; and (4) pYR104 was restricted with *Hin*dIII-*Sma*I and treated as pGR102 above to yield pGR111 containing *Eco*RI-*Sma*I pure 23S sequences (LAFAUCI 1987) (Figure 1, a and b).

DNA labeling and Southern blotting: ^{32}P-labeled plasmids or purified rDNA fragments were used as hybridization probes. Fragments were separated on agarose gels and the appropriate bands electrophoresed onto a DEAE membrane (Schleicher & Schuell, Keene, New Hampshire). The probes were prepared by nick translation (RIGBY *et al.* 1977) as described previously (LAFAUCI *et al.* 1986) or with the random primer extension kit (Boehringer Mannheim Biochemicals, Indianapolis, Indiana). DNA digested with *Bcl*I was transferred to nitrocellulose filters from 0.75% agarose gels and hybridized according to the method of Southern (OSTAPCHAK, ANILIONIS and RILEY 1980; SOUTHERN 1975) as described previously (LAFAUCI *et al.* 1986).

RESULTS

Distribution of restriction fragments containing the *rrn* operons in *B. subtilis*: The enzyme *Bcl*I restricts only once in the operon, 79 bp before the end of the 23S rDNA. Its cutting site upstream from the 5′ end of the 16S is variable (LAFAUCI *et al.* 1986) (Figure 1b). Southern hybridizations of *Bcl*I digests of genomic DNA from *B. subtilis* strains NCTC3610 or 168T (Table 1) yielded 10 distinct fragments when probed with any rDNA fragment 5′ to the internal *Bcl*I site (LAFAUCI *et al.* 1986) (Figure 2). Among the 10 *Bcl*I *rrn* homologs, the largest was 8.3 kb and the smallest was 4.8 kb (Figure 2). Genomic assignments of the *Bcl*I fragments were done through the integrative mapping procedure (HALDENWANG *et al.* 1980) and transductional crosses.

The six constructed plasmids containing *rrn* sequences (Figure 1a) can theoretically integrate into any of the 10 ribosomal operons by a Campbell-like mechanism, placing the antibiotic marker adjacent to the homologous region and leading to a shift of a single *Bcl*I homolog. The higher molecular weight band corresponds to the size of the plasmid plus the missing *Bcl*I *rrn* homolog (LAFAUCI *et al.* 1986). For example, as shown in Figure 2, the smallest 4.8-kb *Bcl*I homolog of integrant strain 168T-37 disappeared with the concomitant appearance of a new larger band of 10.5 kb, or the largest 8.3-kb *Bcl*I homolog of integrant strain SB25-35 disappeared with the appearance of a 13.5-kb fragment. On several occasions bands corresponding to the presence of dimers and trimers of the plasmid were observed (LAFAUCI *et al.* 1986) (168T-80 in Figure 2). Integrants have been

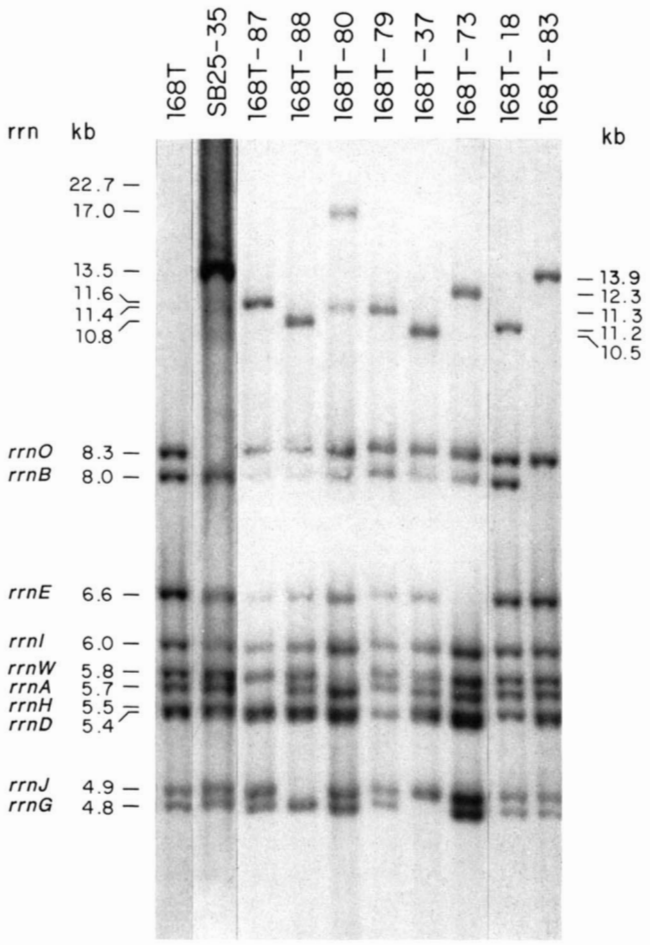

FIGURE 2.—Southern hybridizations of total chromosomal DNAs of integrant strains of *B. subtilis* 168T and *rrn* operon assignments of *Bcl*I homologs. The lanes from left to right display insertions into single rRNA operons according to their map position starting at the origin with *rrnO* and ending with *rrnB* as shown in Figure 4. The *Bcl*I digests were electrophoresed on 0.75% agarose gels for 5–7 days at 15–20 mA and probed with labeled *Eco*RI-*Pst*I 23S fragment purified from pYR104 (Figure 1). For additional strain information see Table 1.

isolated in which each of the *Bcl*I *rrn* homologs was individually lost, with the exception of the 6.0-kb *Bcl*I fragment for which no integrant has been found to date (Figure 2).

Strains with inserted recombinant plasmids were used as donors in transductional crosses to genetically assign the individual *Bcl*I *rrn* homologs (LAFAUCI *et al.* 1986) (see below). Table 2 summarizes the relevant chromosomal location of 10 *rrn* operons 9 of which have been given *Bcl*I homolog assignments reported earlier (LAFAUCI *et al.* 1986) and completed in this paper. Similarly, Figure 2 illustrates the autoradiographic localization of *rrn* homologs in *Bcl*I digests with their known genomic assignment.

Three of our previous assignments (the 4.9-, 5.5- and 5.8-kb *Bcl*I homologs) were in error (LAFAUCI *et al.* 1986) and the correct ones are presented in Table 2. The two sets of closely spaced *rrn* operon clusters (*rrnJ-rrnW* and *rrnI-rrnH-rrnG*) at positions 10° and 14° present a problem in the assignment of *Bcl*I homologs. Since these operons are arranged in tandem with intergenic spaces of not more than 1.2 kb (CHOW and DAVIDSON 1973; WAWROUSEK and HANSEN 1983; WIDOM *et al.* 1988), several *Bcl*I homologs contain sequences of two neighboring *rrn* gene sets. Figure 3 presents the structure of both clusters with respect to the location of *Bcl*I and *Eco*RI sites and the intergenic spacers identified by restriction analysis (WIDOM *et al.* 1988). The 5.8-kb *Bcl*I fragment contains mostly *rrnW*, the 3′ end of *rrnJ* and the *rrnJ-rrnW* intergenic spacer. By our convention, we assigned the 5.8-kb fragment to *rrnW*, since most of this fragment (4.6 kb) comes from *rrnW*. Similar assignments have been made for the 5.5- and 4.8-kb fragments. The 5.5-kb *Bcl*I fragment assigned to *rrnH* also contains the 3′ end of *rrnI* and the *rrnI-rrnH* spacer. The 4.8-kb fragment assigned to *rrnG* also contains the 3′ end of *rrnH* and the *rrnH-rrnG* spacer (Figure 3). Strains such as BD170 and its derivatives constructed by DUBNAU *et al.* (1967) showed 9 *rrn* gene sets; they are missing the 4.8-kb *Bcl*I fragment (*rrnG*), while strains such as CU420 constructed by ZAHLER's laboratory (WARD and ZAHLER 1973) are missing the 5.8-kb *Bcl*I homolog (*rrnW*) (WIDOM *et al.* 1988). Table 1 provides information on the number of *rrn* operons found in various common laboratory strains of *B. subtilis*. Restriction site polymorphisms involving ribosomal sequences observed in *Bcl*I digests of DNA from other *B. subtilis* strains such as 166 or 168W will be reported separately (E. D. JARVIS and R. RUDNER, manuscript in preparation).

Genetic mapping of unassigned *Bcl*I *rrn* homologs: Three *Bcl*I *rrn* homologs, 4.8, 6.0 and 8.0 kb, were not correlated to a genetic locus at the time of publication of our last communication (LAFAUCI *et al.* 1986). Through the introduction of new recipients with 10 *rrn* operons and new integrable plasmids we hoped to isolate strains with insertions into these three operons. In this effort we isolated 190 integrant strains which were used as hosts for PBS1 transducing phages and 80 preparations of DNA were made for Southern analyses. Transductional crosses were carried out with the nine mapping kit strains of *B. subtilis* (DEDONDER *et al.* 1977). The relevant strains for mapping *rrn* operons were kit 1, 2, 3, 7 and 8 (Figure 4). Six Cm^r transformants of recipients with 10 *rrn* operons (*i.e.*, 168T or SB25) revealed an integration event into the 4.8-kb fragment and mapped between *cysA* and *aroI* (kit strains 1 and 2; see below). We assigned the 4.8-kb *Bcl*I homolog to *rrnG* because it has been correlated with the 2.9-kb *Eco*RI fragment which contains the intergenic space of *rrnH-rrnG*. Similarly the 5.5-kb *Bcl*I homolog was assigned to *rrnH* because it has been correlated with the 3.6-kb

TABLE 2

Chromosomal position of the *rrn* operons in *B. subtilis* and the corresponding *BclI* homologs

Operon	Chromosomal position (degrees)[a]	*BclI* fragment size (kb)	Reference for genetic mapping
rrnO	001[b]	8.3	HENCKES *et al.* (1982)
rrnA	006	5.7	WILSON, HOCH and BOTT (1981)
rrnJ	010	4.9	This study
rrnW	010	5.8	This study
rrnI	014	6.0[c]	BOTT, STEWART and ANDERSON (1984) and WIDOM *et al.* (1988)
rrnH	014	5.5	BOTT, STEWART and ANDERSON (1984) and this study
rrnG	014	4.8	BOTT, STEWART and ANDERSON (1984) and this study
rrnE	044	6.6	LAFAUCI *et al.* (1986)
rrnD	070	5.4	LAFAUCI *et al.* (1986)
rrnB	280	8.0	BOTT, STEWART and ANDERSON (1984)

[a] On a scale of 360° the markers *cysA* and *rpsE* are at 011 and 012, respectively (PIGGOT and HOCH 1985) (Figure 4). We calculated the position of the first cluster *rrnJ, rrnW* at 9.5° and the second cluster *rrnH, rrnG* at 13.9° which were adjusted to 010 and 014, respectively.
[b] The actual value is 1.3° based on the sequence of 10 kb the origin region (MORIYA, OGASAWARA and YOSHIKAWA 1985).
[c] Based on indirect evidence of CHOW and DAVIDSON (1973) and WIDOM *et al.* (1988).

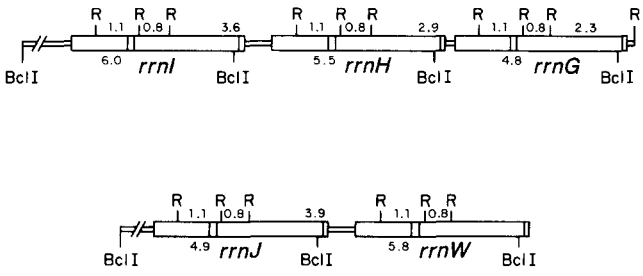

FIGURE 3.—Structure of the *rrn* operon clusters in *B. subtilis*. The triplet (*rrnI-rrnH-rrnG*) is located between *att*SPO2 and *glpT6* whereas the doublet (*rrnJ-rrnW*) is located between *purA* and *cysA* (Figure 4). *Bcl*I and *Eco*RI (R) fragment sizes shown are those identified by restriction analysis (WIDOM *et al.* 1988).

*Eco*RI fragment which contains the intergenic space of *rrnI-rrnH* (Figure 3) (WIDOM *et al.* 1988).

Six integrants were isolated among 80 DNA preparations which exhibited a loss of the 8.0-kb *Bcl*I homolog and mapped between *aroG* and *thrA*. We assigned them to *rrnB* based on mapping data by BOTT, STEWART and ANDERSON (1984). Our linkage relationships of the Cm^r determinant to *aroG932* (kit 7) and to *thr-5* (kit 8) were 41% and 37% cotransduction, respectively. When the structural genes were selected initially, the cotransduction values were 55% (Aro^+Cm^r) and 63% (Thr^+Cm^r). Operon *rrnC* was originally assigned by BOTT, STEWART AND ANDERSON (1984) as a second operon located near *rrnB* between *aroG* and *thrA* but all six integrants from that genomic location exhibited the loss of a single *Bcl*I homolog (8.0 kb) and the linkage data were consistent with the presence of only one operon, namely *rrnB*.

Integrants with insertions into the 4.9- and 5.8-kb *Bcl*I homologs showed a close linkage relationship of the Cm^r to *cysA14*. They represent new assignments and were designated *rrnJ* and *rrnW*, respectively (see below). Finally, no transformants were isolated which showed an integration event into the 6.0-kb *rrn* homolog (Table 2) which is most probably *rrnI*, the third operon in the triplet and the last known operon without an insertion (BOTT, STEWART and ANDERSON 1984; CHOW and DAVIDSON 1973).

Genetic mapping of the closely spaced operon clusters containing *rrnJ-rrnW* and *rrnH-rrnG*: Transductional crosses with the mapping kit strains 1 and 2 revealed that among those integrants which showed a linkage to *cysA14* there was a distinct assortment into three genetically similar groups. The integrants were identified on Southern blots to be those with losses of the *Bcl*I homologs 8.3 or 5.7 kb (*rrnO* or *rrnA*, respectively), those with losses of *Bcl*I homologs 4.9 or 5.8 kb (*rrnJ* or *rrnW*, respectively) or those with losses of 5.5 or 4.8 kb (*rrnH* or *rrnG*, respectively) (Figure 2). Two- and three-factor transduction crosses were performed using recipient strains that include *spc-2* (*rpsE2*), *glpT6*, *lin-2* and *amyE* known to be located in that region (PIGGOT and HOCH 1985; ZEIGLER and DEAN 1985). Table 3 summarizes the cotransduction frequencies of the integrated Cm^r element of the plasmid to these genes obtained with representative PBS1 phage donors of each group. The linkage relationship to the integrated Cm^r element are in a gradient making the initial genomic assignment of these operons easy (Table 3 and Figure 4). The cotransduction values obtained for members of each of the clusters were relatively similar and at times equal (Table 3). The values presented in Table 3 suggest a very close proximity of the first cluster, *rrnJ-rrnW*, to *cysA14* either upstream or downstream to this marker.

The gene order of *rrnJ-rrnW* with respect to *cysA14* was established from typical three-factor transductional crosses. Table 4 presents a sample of our crosses depicting the linkage relationships of the markers *purA16*, *cysA14* and the Cm^r element integrated either

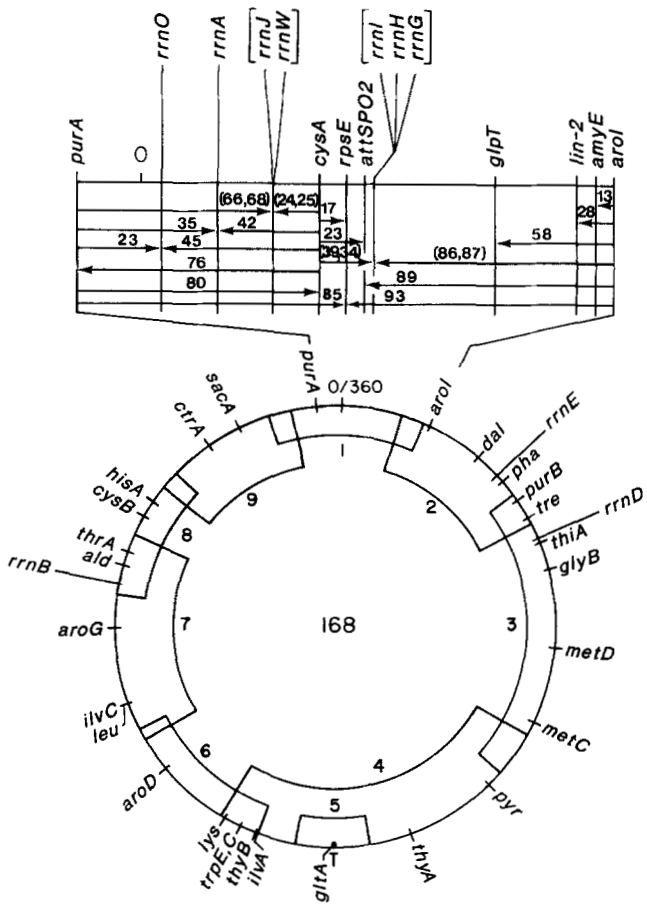

FIGURE 4.—Genetic map of *B. subtilis* with the positions of all 10 rRNA genes. The basic reference for the known loci is taken from the mapping kit strains of DEDONDER *et al.* (1977), which are designated 1–9. Included is the transduction map of the *cysA14-arol906* region; the marker order and map distances were determined from three-factor crosses. Map distances are defined as 100-frequency of cotransduction with PBS1. The arrows point from selected to unselected markers. The linkage values in parenthesis for the paired operons are given individually according to the indicated position with no values given for *rrnI*.

into *rrnJ* (donor strain 168T-88) or into *rrnW* (donor strain 168T-80). The *cysA14* mutation is known to contain multiple nonreverting mutational sites involving sensitivity to cysteine, excretion of hydrogen sulfide which is toxic, methionine requirement and sporulation defects (HARFORD and SUEOKA 1970; KANE, GOODE and WAINSCOTT 1975; PIGGOT 1973). In genetic analyses the *cysA14* behaves as a double mutation and could upset the results of three-factor crosses. When the selection is first carried out on enriched plates like LB broth containing chloramphenicol followed by replica plating to minimal medium, the expected three recombinant classes could be easily distinguishable by their morphology and color. As shown in Table 4, Cys^+ recombinant colonies were white or pink which were clearly different from the Pur^+Cys^- class that appeared flat and transparent. Although upon initial selection of Pur^+ or Cys^+ colonies on minimal medium the numbers were unequal with a preponderance of the former, the recombinant classes could easily be predicted from their morphology prior to replica plating (Table 4). The total absence of the quadruple recombinant class, namely $Pur^+Cm^sCys^+$, and the presence of the $Pur^+Cm^rCys^-$ and $Pur^-Cm^rCys^+$ classes established the gene order of the first cluster to be: *purA16-(rrnJ-rrnW)-cysA14*. Similarly, the recombinant class $Cm^rCys^-Spc^r$ was undetectable in other three-factor crosses involving either the recipient strain 1A241 (Table 1) or when the donors (168T-88 and 80) were constructed by transformation to contain the *rpsE2* marker (data not shown). The total genetic data provided evidence in favor of the most probable gene order: *purA16-(rrnJ-rrnW)-cysA14-rpsE2*. Finally, the exact gene order of the first cluster (*rrnJ-rrnW*) relative to *rrnO* and *rrnA* was easily established from the linkage gradients to these and other markers such as *glpT6*, *lin-2* and *amyE* (Table 3 and Figure 4).

The linkage relationship of the SPO2 attachment site relative to the two ribosomal clusters was determined by transductional crosses between lysogenic recipients and nonlysogenic Cm^r donors as described by SMITH and SMITH (1973). To map *att*SPO2, two *B. subtilis* strains carrying either *cysA14* (strain 1A241) or *arol906* (strain RJ25) each bearing one or two antibiotic resistance markers were lysogenized with SPO2, and used as recipients (Table 1). Control

TABLE 3

Cotransduction frequencies of integrated Cm^r element of plasmids with *rrn* inserts to genomic markers from crosses involving nonlysogens (−) and lysogenic (+) donors

Operon assignment	Missing *Bcl*I homolog (kb)	Donor strain	*Cm/Pur*	− + *Cm / Cys*		− + *Cm / Spc*		*Cm/Spo2*	− + *Cm / Glp*		*Cm/Lin*	*Cm/Amy*	*Cm/Aro*
rrnO	8.3	SB25-35	61	41		33		30	6		0	0	0
rrnA	5.7	168T-87	46	51		46		45	10		0	0	0
rrnJ	4.9	168T-88	39	80	76	58	58	71	31	9	9	3	0
rrnW	5.8	168T-80	41	75	82	60	49	68	27	8	10	3	0
rrnH	5.5	168T-79	5	67	41	73	53	88	48	47	32	28	12
rrnG	4.8	168T-37	7	64	39	76	46	87	42	46	28	35	12

The recipient strains were: kit 1 (*trpC2, purA16, cysA14*); 1A241 (*cysA14, rpsE2*) and its SPO2 lysogen; RJ4 (*trpC2, amyE, lin-2, arol906*); RJ13 (*trpC2, lin-2, glpT6, arol906*); RJ25 (*trpC2, rpsE2, glpT6, arol906*) and its SPO2 lysogen.

TABLE 4

Transduction crosses for mapping integrated plasmids into *rrnJ* and *rrnW* in the *purA-cysA* region

Donor	Total no.[a]	Recombinant class[b] *purA*	*cysA*	Cm^r	Morphology[c]	No.[d]	Cotransfer Type	%[e]
168T-88		0	0	1		0		
rrnJ		1	0	1	Weak	18	Cm^r/Pur$^+$	39
		0	1	1	White	54		
	1.5×10^4	1	1	1	Pink	17	Cm^r/Cys$^+$	80
		1	0	0		117		
		1	0	1	Weak	25	Pur$^+$/Cm^r	34
		1	1	0		0*		
	2.0×10^4	1	1	1	Pink	34	Pur$^+$/Cys$^+$	19
		0	1	0		52		
		0	1	1	White	83	Cys$^+$/Cm^r	71
		1	1	0		0*		
	8.4×10^3	1	1	1	Pink	44	Cys$^+$/Pur	25
	Gene order: *purA rrnJ cysA*							
168T-80		0	0	1		4		
rrnW		1	0	1	Weak	18	Cm^r/Pur$^+$	41
		0	1	1	White	48		
	1.4×10^4	1	1	1	Pink	18	Cm^r/Cys$^+$	75
		1	0	0		85		
		1	0	1	Weak	19	Pur$^+$/Cm^r	32
		1	1	0		0*		
	1.8×10^4	1	1	1	Pink	21	Pur$^+$/Cys$^+$	17
		0	1	0		33		
		0	1	1	White	46	Cys$^+$/Cm^r	75
		1	1	0		0*		
	8.3×10^3	1	1	1	Pink	53	Cys$^+$/Pur$^+$	41
	Gene order: *purA rrnW cysA*							

[a] The recipient was Kit 1 *purA16, cysA14, trpC2.* The total number of transductants determined.

[b] Donor and recipient phenotypes are indicated by 1 and 0, respectively.

[c] Colony morphology and color on minimal plates; weak = flat and transparent; white = normal and opaque; pink = dense, pigmented and typical spore-forming.

[d] No. = number of transductants tested.

[e] Number of cotransferred per number tested.

* The quadruple crossover class which is undetectable according to the gene order indicated in the table.

crosses with 168T as donor showed that *att*SPO2 was 23% linked to *cysA14* and 17% to *rpsE2* as was reported by others (IRIE, OKAMOTO and FUJITA 1986; SMITH and SMITH 1973) and weakly linked to *aroI* (89%). The suggested gene order was: *cysA-rpsE-attSPO2-glpT-aroI* (Figure 4). Transductional crosses with the six nonlysogenic integrant strains as donors summarized in Table 3 clearly demonstrate a similar gradient relationship of the *rrn* operons to *att*SPO2. Operons *rrnH,rrnG* showed the tightest linkage of 88 and 87% cotransduction, respectively, followed by 71 and 68% for *rrnJ,rrnW* and finally 45% to *rrnA* and 30% to *rrnO* (Table 3).

The influence of a 39-kb prophage on the linkage relationships in the chromosomal segment delimited by the *cysA14* and *glpT6* mutations was examined in crosses using SPO2 lysogens both as donors and recipients as described by IRIE, OKAMOTO and FUJITA (1986). The cotransduction values are presented as separate underlined columns in Table 3. It is evident that linkages of the Cm^r determinants to *glpT6* were unaltered for the second cluster of *rrnH, rrnG* while a decrease of the cotransduction values from 31 to 9% and from 27 to 8% was observed for *rrnJ* and *rrnW,* respectively. On the other hand, the presence of the prophage altered linkages between the Cm^r determinant inserted into *rrnH,rrnG* and *cysA* or *rpsE2.* Decreases in cotransduction values from 67 to 41% for *rrnH* and from 64 to 39% for *rrnG* between Cm^r and *cysA14* were recorded. Similar decreases from 73 to 53% for *rrnH* and from 76 to 46% for *rrnG* between Cm^r and *rpsE2* were recorded (Table 3).

In conclusion, the results of the cotransduction gradients seen in crosses with and without the SPO2 prophage and the three-factor crosses places seven *rrn* operons in the following order: *purA-rrnO-rrnA-(rrnJ-rrnW)-cysA-rpsE-att*SPO2-(*rrnI-rrnH-rrnG*)-*glpT-aroI.* The brackets denote our uncertainty regarding the gene order relative to the origin. The genetic map of *B. subtilis* with the position of all 10 rRNA genes including *rrnI* and a detailed transduction map is presented in Figure 4 and will be discussed below.

Differential integration frequencies of *rrn* operons: In general, the integrable plasmids (Figure 1a) grown in *E. coli* strain HB101 transformed *B. subtilis* RecE$^+$ recipients (*i.e.*, 168T, SB25) (Table 1) at relatively low efficiencies (10^1–10^3 Cm^r transformants per μg plasmid). The same plasmids grown in *recB, recC, sbcB* or *sbcA* mutants of *E. coli* (COHEN and CLARK 1986; Table 1) were enriched in linear multimeric forms and gave a 10–100-fold increase in transforming efficiencies (data not shown). The frequency of integration of the six integrable plasmids is summarized in Table 5. It represents studies of 190 integrants which arose by plasmid transformation into three recipients (168T, SB25, E88) containing 10 *rrn* operons and two recipients (BD170 and BD79) containing nine *rrn* operons. Transductional crosses and Southern hybridizations verified the genomic assignment of the integrants with the exception of three ambiguous occurrences (Table 5). They exhibited a close linkage of the Cm^r determinant to *aroI* and *dal-1* (18 and 48% cotransduction, respectively) and none to *purB* (Table 5). These three integrants did not reveal a specific loss of a *Bcl*I homolog and so they remain unidentified.

A bias for integration near the replication origin of the chromosome in the region between *purA16* and *aroI906* was evident (Table 5). In view of the fact that seven operons are clustered at the region between the

TABLE 5

Frequency of integration of six hybrid plasmids containing *rrn* sequences at seven regions of the *B. subtilis* genome

Gene order	Operon assignment	Number of integrant recipients with 9 or 10 *rrn* operons transformed with plasmids[a]: *pGR102, pWR103* 10	*pGR102, pWR103* 9	*pYR104, pGR108 pGR110, pGR111* 10	*pYR104, pGR108 pGR110, pGR111* 9	Percent expected 10	Percent observed[b] 10	P^c 10
purA, Cm^r, cysA	*rrnO rrnA*	29	23	18	4	20	35	<0.01
purA, Cm^r, cysA	*rrnJ rrnW*	32	16	9	1	20	31	<0.01
cysA, Cm^r, aroI	*rrnI*							
	rrnH rrnG	26	3	2	1	30	21	0.1–0.05
aroI, Cm^r, dal-1		2	0	1	0		2	
dal-1, Cm^r, purB	*rrnE*	2	3	0	1	10	1.5	<0.01
tre-12, Cm^r, glyB	*rrnD*	5	3	2	1	10	5	0.1–0.05
aroG, Cm^r, thrA	*rrnB*	3	0	3	0	10	4.5	0.05–0.02
Total (190):		99	48	35	8	100	100	

[a] Strains with 10 *rrn* operons were: 168T, SB25 and E88. Strains with 9 *rrn* operons were: BD170 and BD79 (for further information see Table 1).

[b] Calculated only from recipient strains with 10 *rrn* operons; total = 134.

[c] Values of P for individual operons calculated from Chi square values using one degree of freedom.

origin and 15° on the map (Figure 4) the expected integration frequency should have been 70%, or 20% for each pair of operons (*rrnO,rrnA* and *rrnJ,rrnW*) and 30% for the cluster of three. Instead, we observed that 87% of the events occurred in that region (Table 5). The greatest bias of integration events occurred in operons close to the origin of replication (*i.e.*, *rrnO,rrnA* followed by *rrnJ,rrnW*). The more distant the operons from the origin (*i.e.*, *rrnE*, *rrnD*, *rrnB*) the more infrequent were the integration events (1.5–5.0%; Table 5). The type of plasmid used whether its *rrn* insert contained the abutment region between 16S and 23S (pGR102, pWR103) or 23S and all of 5S rDNA (pYR104) or merely "pure" 16S or 23S sequences in either orientation with respect to the CAT gene (pGR110, pGR108, pGR111) did not alter the bias pattern significantly, although some variations were noted (Figure 1 and Table 5). For example, a slight enhancement of integrations into *rrnO* and *rrnA* (18/35) occurred with plasmids containing pure ribosomal sequences compared with those containing the abutment region (26/99). Moreover, plasmid preparations enriched for multimeric forms increased the frequency of integration events in general but did not alter the observed pattern of insertions (data not shown). The values of a chi square determined for the observed series of integrations in strains with 10 operons corresponded to P values far below 0.01 in three cases (*rrnO*, *rrnA*; *rrnJ-rrnW* and *rrnE*; Table 5). Higher P values of 0.1–0.02 were determined for the other cases (Table 5). Consequently we can say that the bias for integration near the origin or the sparsity at other genomic locations (*rrnE*, *rrnB*) is statistically significant. The departure from random expectation is strongly evident (Table 5). The same bias for integration near the origin of the chromosome (*rrnO*, *rrnA*, *rrnJ*, *rrnW*) occurred in strains containing nine operons (Tables 1 and 5). These strains which lack an operon equivalent in the second cluster (Figure 3 and WIDOM *et al.* 1988) exhibited as expected a paucity of integration events into this location (Table 5).

DISCUSSION

The technique of integrative mapping with six vectors carrying chromosomal rDNA sequences in recombination-proficient *B. subtilis* has been successfully used to complete the mapping of its rRNA operons. The linkage map of *B. subtilis* with respect to the location of all 10 *rrn* operons presented in Figure 4 highlights the following changes from previously published linkage maps (PIGGOT and HOCH 1985; ZEIGLER and DEAN 1985): (1) The absence of *rrnC* from the *aroG-thrA* segment; (2) the absence of *rrnR* from the *ilvBC-leu* region; (3) the location of the cluster of three *rrnI-rrnH-rrnG* downstream of *att*SPO2; and (4) the discovery of a new pair of closely spaced operons *rrnJ-rrnW* located upstream of *cysA14*.

The transduction data reported by BOTT, STEWART and ANDERSON (1984) for *rrnC* are not that different from the values reported here for *rrnB* in crosses with kit 7 and 8 as recipients. All integrants from that genomic location were associated only with a single *Bcl*I homolog the 8.0 kb (Figure 2 and Table 2). The second proposed operon, *rrnR*, has been assumed to be associated with a large cluster of tRNA genes (*trnD* also called *trnR*) (VOLD 1985; WAWROUSEK, NARASIMHAN and HANSEN 1984). It was tentatively given a genomic location at about position 225 near *leuA8*

(VOLD 1985). In our studies a total of 300 integrants were used as donors in transductional crosses and none revealed linkage between an integrated Cm^r element and *leuA8* or the *aroG932* markers of kit 7 (DEDONDER *et al.* 1987). One case was reported by us previously (LAFAUCI *et al.* 1986), where integrant strain GSY1269 Ω pGR-176 revealed a close linkage of the Cm^r determinant to *leuA8* (74% cotransduction) but its DNA gave a normal *BclI* pattern. The recipient strain (*i.e.*, GSY1269) is known to possess a rearranged genome (ANAGNOSTOPOULOS and TROWSDALE 1976) which could account for the unexplained finding. One may conclude that both the physical and genetic evidence for the presence of *rrnR* is unavailable. The tRNA gene cluster may be distal to 23S rDNA sequences of either *rrnW*, *rrnE* or *rrnD* whose 3′ sequences have not yet been identified.

Our main contribution to the mapping of rRNA genes in *B. subtilis* is finding the doublet (*rrnJ-rrnW*) which increases the number of operons found between the origin and 70° to a total of 9 out of 10 (Figure 4). In the region between 0 and 15°, an area with a high density of genetic markers, seven rRNA genes are found clustered with a unique arrangement of well separated sets, of which two are composed of tandemly repeated operons. Our genetic data placed one of these tandemly spaced sets, *rrnJ-rrnW*, at 10° on the map between *purA* and *cysA* downstream of *rrnA*. The second cluster composed of three closely spaced operons, *rrnI-rrnH-rrnG*, corresponds to some of the heteroduplex structures reported by CHOW and DAVIDSON (1973), later mapped by BOTT, STEWART and ANDERSON (1984). In their EM studies, the SPO2 prophage sequence of 39 kb is 6.2 kb from a rDNA triplet containing two intergenic spacers of 0.3 and 0.6 kb. They also characterized a doublet with a spacer of 1.0 kb (CHOW and DAVIDSON 1973) which we identified here as *rrnJ-rrnW* (Figures 3 and 4).

We verified the location of the three closely situated repeats *rrnI-rrnH-rrnG* downstream from the attachment site of the phage SPO2 and not near the *spoOH* gene as reported in the revised genetic map of ZEIGLER and DEAN (1985). The third operon in the cluster of three, *rrnI*, predicted by the heteroduplex mapping has had no insertions so far (out of 80 integrant DNAs examined). Restriction analysis of rescued DNA from integrants into *rrnH* provided additional evidence for the existence of *rrnI* in the cluster (WIDOM *et al.* 1988). Operon *rrnI* is expected to be assigned to the 6.0-kb *BclI* fragment, the last unidentified homolog (Table 2). To date, no deletion events occurred between *rrnI* and *rrnH* as observed for the other tandems *rrnH-rrnG* and *rrnJ-rrnW* (WIDOM *et al.* 1988). The cluster of six tRNA genes [*trnB* (VOLD 1985; WAWROUSEK and HANSEN 1983), also called *trnH* (PIGGOT and HOCH 1985)] reported to be located in the space between *rrnI* and *rrnH* and the possible existence of a single promoter region for all the operons could explain the lack of viable integrants or deletions involving *rrnI*. The other cluster, *rrnJ-rrnW* is expected to contain a tRNA gene cluster in its intergenic spacer (VOLD 1985; WAWROUSEK and HANSEN 1983). However their higher integration frequencies (Table 5) may indicate a fundamentally different relationship to this tRNA gene compared with the ones downstream of *rrnI*.

We report the existence of "hot spots" of plasmid integrations found in operons located near the origin of replication (*rrnO, rrnA, rrnJ, rrnW*) and "cold spots" for those further away (*rrnE, rrnB*; see Table 5). This observation may depend on the extent of chromosome branching (OISHI, YOSHIKAWA and SUEOKA 1964). Although competent *B. subtilis* are less likely to have multiple replication forks as reported from marker ratio analyses (DOOLEY, HADDEN and NESTER 1971), this possibility must be further examined experimentally in strains with initiation-defective mutations (*i.e.*, *dna-1* or *dnaB19*) (WINSTON and SUEOKA 1980). Alternatively, the relationship of tRNA gene clusters to certain *rrn* operons may render them "cold spots" or "lethal spots" for plasmid integration. Those tRNA gene sets found upstream of rRNA gene clusters (*i.e.*, *rrnI* or *rrnB*) may be more sensitive to gene disruption if they are part of a large transcription unit (VOLD 1985; WAWROUSEK, NARASIMHAN and HANSEN 1984). The linked tRNA gene clusters could increase intrastrand stem and loop structures which would hinder synapsis between the rDNA inserts and the resident operon. It is possible that *trrnD* (*trnR*) will be remapped near *rrnE* or *rrnD*, two operons with infrequent integration events (Table 5). Analyses of plasmid rescued from specific operons (*i.e.*, *rrnG, rrnH, rrnW, rrnJ, rrnE, rrnD*) which include unique spacer sequences 5′ and 3′ to the site of integration (R. L. WIDOM and E. D. JARVIS, experiments in progress) will provide information on structural and functional organization of linked rRNA and tRNA gene clusters.

In conclusion, the high density of *rrn* operons near the origin of replication found in *B. subtilis* as well as in *E. coli* and *S. typhimurium* (NOMURA, GOURSE and BAUGHMAN 1984; LEHNER, HARVEY and HILL 1984) may imply a functional role in regulation of stable RNA synthesis. The activity of a fused *lacZ* gene to individual ribosomal promoters has already revealed that operons nearer to the origin are expressed at much higher levels than the ones further away from it (R. L. WIDOM and A.-M. WHITE, unpublished results). Therefore in *Bacillus*, and perhaps in other prokaryotes, to support the high rate of rRNA synthesis in rapidly dividing cells, an increase in the redundancy of rRNA operons (from 10 to 17) is

required and could be accomplished by the reinitiation of chromosomal replication.

We dedicate this paper to the memory of INGA R. RICHTER and STEFFEN R. BUCHHOLZ who were members of our research group and died recently in an automobile accident (8/6/88).

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