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Mapping of rRNA Genes with Integrable Plasmids in *Bacillus subtilis*

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Integrable plasmids pGR102 and pWR103 containing ribosomal sequences from within the transcriptional units for 16S and 23S were used to transform *Bacillus subtilis*. To date, these plasmids integrated into 7 of 10 known *rrn* operons. Two such events occurred at unassigned operons, revealing a close linkage of the CAT gene of the plasmid to *pha-1* situated between *dal-1* and *purB33* for *rrnE* and to *thiA78* situated between *glyB133* and *tre-12* for *rrnD*. All seven integration events that led to the loss of unique ribosomal *BclI* fragments can now be assigned to known *rrn* operons.

In the genome of *Bacillus subtilis* there are as many as 10 rRNA gene sets clustered in three groups (5). A major group is composed of *rrnO* and *rrnA* located at the replication origin (24, 26, 35) and the closely situated (5, 26) repeats (*rrnI*, *rrnH*, *rrnG*) found near the attachment site of phage SPO2 (5). Minor clusters containing *rrnB* and *rrnC* have been mapped in the region between *thr-5* and *aroG* (5), and one cistron is believed to be located at the *ilvBC-leu* region (7, 11, 31). Strains of *B. subtilis* have been shown to contain only nine operons because of a deletion (11, 18) or to exhibit rearrangements involving ribosomal sequences (3, 11). Each transcriptional unit is arranged in the order 16S, 23S, and 5S rRNA genes, with two to three operons (including *rrnO* and *rrnA*) containing $tRNA^{Ala}$ and $tRNA^{Ile}$ genes in the abutment region between the 16S and 23S rDNA sequences (18, 33). We employed the integrative mapping method of Haldenwang et al. (13) to ascertain whether integrations can occur in operons, to locate various unmapped *rrn* genes on the chromosome, and to determine the physiological effects on growth and sporulation of such events in *B. subtilis*. In this study, the bifunctional plasmid pJH101 was used (10). The plasmid cannot replicate in *B. subtilis* because it does not contain an origin of replication for *B. subtilis*. If a region of homology with the *B. subtilis* chromosome is inserted in pJH101, the resultant plasmid gains the ability to transform competent cells and also, on occasion, integrates by Campbell-like recombination (10). We subcloned two similar but not identical ribosomal fragments from within the transcriptional unit for 16S and 23S which originated from the operons *rrnO* and *rrnI*. The resultant plasmids pGR102 and pWR103 can theoretically integrate into any of the 10 ribosomal operons by a Campbell-like insertion, leaving the entire plasmid with its antibiotic marker adjacent to the homologous region. We report here seven different integration events by these plasmids, two of which occur at unassigned operons (*rrnD* and *rrnE*), increasing the number from seven to nine known genomic locations in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains and plasmids used in this study are described in Table 1. Strains containing integrated plasmids were constructed by competent cell transformation, verified for integration by hybridization, and checked for site of integration by PBS1 transduction mapping. We designated the various transformants as the *B. subtilis* strain Ω plasmids, with Ω denoting plasmid integration. The plasmids with and without *B. subtilis* chromosomal insertions were gifts from K. Bott and K. Nagahari and were transformed into *Escherichia coli* HB101. Ribosomal or leucine fragments of *B. subtilis* were cloned into the ampicillin (Ap^r) or the tetracycline (Tc^r) determinants, respectively, of cloning vector pJH101. Plasmid construction resulted in the following integrable derivatives of pJH101: (i) pGR102 containing a 1.2-kilobase (kb) *Pst*I-*Eco*RI fragment from pMS102-B7 (24, 30), (ii) pWR103 containing a 1.0-kb *Pst*I-*Eco*RI fragment from pBC194 (33), and (iii) pER102 containing a 3.2-kb *Eco*RI-*Bam*HI fragment from RSF2124-*leu* of *B. subtilis* (22).

Culture methods, transformation, and transduction. Liquid *B. subtilis* cultures were grown in 2.5% veal infusion (Difco Laboratories, Detroit, Mich.)–0.5% yeast extract (Difco). Solid medium was TBAB (tryptose blood agar base [Difco]) supplemented with 5 μg of chloramphenicol per ml to maintain plasmid-bearing strains. *E. coli* cells containing plasmids were grown in LB broth supplemented with 100 or 15 μg of ampicillin or tetracycline per ml, respectively. Transformation was performed with frozen competent *B. subtilis* as described previously (28). PBS1 transductional mapping was carried out essentially as described previously (14). Selection of prototrophic recombinants in genetic analysis used Spizizen minimal medium (2) supplemented with 100 μg of the appropriate amino acid or nucleotide per ml (final concentration) with the exception of L-tryptophan (25 μg/ml), L-glycine (1,000 μg/ml), and thiamine B1 (1 μg/ml). Transductants for the *tre-12* marker were selected on modified minimal medium (8, 17) with 0.2% trehalose (filter sterilized) as the carbon source. For the selection of Pha^r and Pha^s TBAB plates were covered with phage SPO1 (2×10^9 phages per plate) (17), and for the detection of $FurB^r$ and $FurB^s$ minimal plates were supplemented with 40 μg of 5-fluorouracil and 40 μg of uracil per ml (38).

DNA manipulation. DNA preparations were carried out by a modification of the procedure of Marmur (20). Plasmid DNA was purified from *E. coli* cultures essentially by the procedure of Tanaka and Weisblum (6, 34). Chromosomal

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Source
Parental strains		
NCTC 3610	Prototroph	A. Sonenshein
168T	*trpC2*	K. Bott
GSY1269	*trpE26 ilvC1*	K. Bott
BD170	*trpC2 thr-5*	D. Dubnau
BD79	*leuB1 pheA1*	D. Dubnau
Kit 1 to kit 9	Mapping recipients	D. Dubnau (8)
IA122	*thiA78 glpK21 glyB133 tre-12*	BGSC[a]
IA150	*pha-1 catA hisA2*	BGSC
IA154	*met purB6 trpC2 furB gutB*	BGSC
IA84	*glyB133 metD1*	BGSC
Recombinant plasmids		
pJH101	Tc^r Ap^r Cm^r	K. Bott
pBC194	16S-23S rRNA Tc^r Ap^r	K. Bott
pMS102-B7	16S-23S rRNA Ap^r Km^r	K. Bott
RSF2124-*leu* of *B. subtilis*	*leu* Ap^r	K. Nagahari
pGR102	16S-23S rRNA Tc^r Cm^r	This study
pWR103	16S-23S rRNA Tc^r Cm^r	This study
pER102	*leu* Ap^r Cm^r	This study
Integrant strains[b]		
BD170ΩpGR-135	*trpC2 thr-5* Cm^r	This study
BD170ΩpGR-143	*trpC2 thr-5* Cm^r	This study
BD170ΩpGR-144	*trpC2 thr-5* Cm^r	This study
BD170ΩpGR-151	*trpC2 thr-5* Cm^r	This study
BD170ΩpGR-153	*trpC2 thr-5* Cm^r	This study
BD79ΩpGR-174	*leuB1 pheA1* Cm^r	This study
BD79ΩpGR-175	*leuB1 pheA1* Cm^r	This study
GSY1269ΩpGR-176	*trpE26 ilvC1* Cm^r	This study
GSY1269ΩpGR-177	*trpE26 ilvC1* Cm^r	This study
BD170ΩpWR-179	*trpC2 thr-5* Cm^r	This study
BD170ΩpWR-181	*trpC2 thr-5* Cm^r	This study
BD79ΩpWR-182	*leuB1 pheA1* Cm^r	This study
IA150ΩpGR-135	*pha-1 catA hisA1* Cm^r	This study
IA154ΩpGR-135	*met* $PurB^+$ *trpC2 furB gutB* Cm^r	This study
BD170ΩpER-115	*trpC2 thr-5* Cm^r	This study

[a] Bacillus Genetic Stock Center, Columbus, Ohio.
[b] Isolated as Cm^r transformants after plasmid transformation with pGR102 or pWR103.

DNA (5 to 10 μg) was digested individually or doubly with the appropriate restriction endonucleases (3 U of enzyme per μg of DNA for 12 h) by using the conditions for digestion recommended by the supplier (International Biotechnologies, Inc. [IBI]). DNA fragments were electrophoretically resolved in 0.6, 0.75, or 0.85% agarose gels; stained with ethidium bromide; and transferred to nitrocellulose filters for hybridization. Plasmid DNA digested with restriction endonucleases (IBI) was analyzed by gel electrophoresis on 0.8% agarose gels. Ligations at 2:1 or 4:1 ratio of target to vector DNAs (10 to 20 μg/ml) were done with T4 DNA ligase (IBI).

Nick translation and Southern blotting. ^{32}P-labeled plasmids (pJH101, pGR102, pWR103) or purified *Pst*I-*Eco*RI 16S-23S rDNA, *Eco*RI-*Hin*dIII 23S-5S rDNA fragments obtained by separation on low-melting agarose gels were prepared by nick translation (27) with [^{32}P]dATP and [^{32}P]dCTP (New England Nuclear, Corp., Boston, Mass). Labeled DNA was separated from unincorporated nucleotides on Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). DNA was routinely labeled to a specific activity of 1×10^8 to 3×10^8 cpm/μg. Gel blotting and DNA hybridization were carried out by the method of Southern (32), as modified by Ostapchuk et al. (25), with BA85 nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.).

RESULTS

Construction and transforming activity of integrative plasmids. The 5.7-kb *Bam*HI fragment from *rrnO*, cloned by Seiki et al. (30), which contained all the 16S and more than half of the 23S rDNA, was digested with *Pst*I followed by digestion with *Eco*RI to yield a suitable 1.2-kb fragment which includes 16S sequences, the large abutment region with two tRNA genes (*ile* and *ala*), and 155 base pairs of the 5′ end of 23S. A similar *Pst*I-*Eco*RI 1.0-kb fragment was obtained from plasmid pBC194 which contains the small abutment region without the tRNA genes of the operon *rrnI*. We reasoned that if the two tRNA genes play a role in the integration of the *rrn*-containing plasmids in the *B. subtilis* genome either by aiding the process or by limiting the integration to two to three operons, a similar DNA fragment without the tRNA genes should be examined. The large and the small *rrn* fragments were ligated into the *Pst*I and *Eco*RI sites of pJH101 to give plasmids pGR102 and pWR103, respectively. These plasmids, which were tetracycline-resistant, chloramphenicol-resistant, and ampicillin-sensitive, were transfected into *E. coli* HB101. The presence of the cloned *rrn* inserts in plasmids pGR102 and pWR103 was confirmed by restriction endonuclease digests of DNA purified on cesium chloride-ethidium bromide gradients and

TABLE 2. Transforming efficiencies of integrable plasmids containing homologous *leu* and *rrn* sequences or chromosomal DNA with integrated plasmids in *B. subtilis*

Plasmid and chromosomal DNA	Insert (kb) and termini or relevant genotype[a]	No. transformants per μg of DNA[b]	
		Leu$^+$	Cm^r
Constructed plasmids			
pJH101		0	0
pER102-*leu* of *B. subtilis*	3.2 (R-B)	9.2×10^6	7.5×10^4
pGR102-*rrn* of *B. subtilis*	1.2 (P-R)	0	2.5×10^1
pWR103-*rrn* of *B. subtilis*	1.0 (P-R)	0	4.5×10^1
Integrant strains			
BD170ΩpER-115[c]	*trpC2 thr-5* Cm^r	9.2×10^5	4.9×10^4
BD170ΩpGR-143	*trpC2 thr-5* Cm^r	6.0×10^5	8.1×10^4
BD170ΩpWR-179	*trpC2 thr-5* Cm^r	2.3×10^5	2.5×10^4

[a] Restriction sites are as follows: R, *Eco*RI; B, *Bam*HI; P, *Pst*I.
[b] The recipient strain was BD79(*leuB1 pheA1*).
[c] Among the Leu$^+$ transformants examined 2% were Cm^r; among the Cm^r transformants 97% were Leu$^+$ and 3% were Phe$^+$.

by hybridization to labeled ribosomal probes (data not shown).

We introduced pGR102 and pWR103 into RecE$^+$ strains of *B. subtilis* by transformation, and colonies resistant to 5 μg of chloramphenicol per ml were isolated at extremely low frequencies (Table 2). Plasmid pWR103 generated slightly more Cm^r transformants as compared with pGR102, which contained the larger abutment region (Table 2). Similar constructions of pJH101 with homologous *leuA*, *leuC*, *leuB* (pER102), or *pheA* fragments (10, 36) yielded higher frequencies of Cm^r transformants (Table 2). Once the plasmids were introduced into various *B. subtilis* strains like BD170 (*trpC2*, *thr-5*), BD79 (*leuB1*, *pheA1*), and GSY1269 (*trpE26*, *ilvC1*), the Cm^r determinant could be transferred to other strains by DNA-mediated transformation at reasonably high frequencies. Values of Cm^r transformants of 10^4 to 10^5 per μg of DNA were obtained, which is still 2- to 10-fold fewer recombinants than those obtained with ordinary chromosomal markers (Table 2).

Chromosomal integration of plasmids: Southern analysis. DNA was prepared from 18 integrant strains obtained by transformation with plasmids pGR102 and pWR103 to determine the chromosomal fragment involved in the integration process by Southern hybridizations. The integrants are designated with the symbol Ω, for example BD170ΩpGR-143 is strain BD170 (*trpC2*, *thr-5*) in which Ω denotes Cm^r from the integration of plasmid pGR102 and 143 is the number of the clone isolated initially. In theory integration of plasmids with *rrn* sequences into chromosomes by homologous recombination should result, at a minimum, in duplication of the region of homology (13) and sometimes in multiple repetitions of the region (1, 36, 37). The expected behavior of pGR102 during integration into the *B. subtilis* chromosome is shown in Fig. 1. The most convenient method was to restrict the chromosome at the *Bcl*I site, which is present once at the 3′ end of the 23S rDNA but not in the vector, and to compare the parental-type hybridization pattern with strains containing integrated plasmid. At least one *Bcl*I fragment should disappear, and a band larger than that in the parental type strain should appear. The expected new band should be composed of the plasmid plus the missing *Bcl*I *rrn* homolog, and bands corresponding to the insertion of multimeric forms should be observed. Double digestions with *Bcl*I-*Sal*I (pGR102 contains one *Sal*I site within the tetracycline gene, and no sites are found in the *rrn* sequences) release from the strains containing integrated plasmid one defined fragment of 7.6 or 7.8 kb (depending on the size of the abutment region of the operon involved) and one variable fragment with a minimum size of 2.5 kb (Fig. 1).

To confirm this model DNAs from the integrant strains, the recipient strains BD170 (*trpC2 thr-5*), BD79 (*leuB1 pheA1*), GSY1269 (*trpE26 ilvC1*), and NCTC 3610 were digested to completion with *Bcl*I or with *Bcl*I followed by digestion with *Sal*I, electrophoresed on agarose (0.75 or 0.85%), transferred to nitrocellulose, and probed with nick-translated pGR102 or the purified 1.2-kb *Pst*I-*Eco*RI ribosomal insert of pGR102 or pJH101. In Fig. 2a, b, and c we use Ω to denote plasmid integration along with the initials of the appropriate plasmid (i.e., GR or WR) and the number of the integrant strain but without the information on the original recipient that could be obtained from Table 1. Figure 2a and b illustrates the autoradiographic localization of *rrn* homologs in the *Bcl*I DNA digests numbering either 9 or 10 for the recipient strains and NCTC 3610, respectively. The largest *Bcl*I *rrn* homolog was 8.3 kb, and the smallest homologs appeared as doublets of 4.8 and 4.9 kb in strains NCTC 3610 and 168T (data not shown) and only as a single small fragment (4.9 kb) in the recipient strains (Fig. 2a and b). The faint band between 8.0 and 6.6 kb was visible only when probes with tRNA sequences were used (Fig. 2a and b). To increase the resolution of bands between 5.4 and 6.6 kb, a similar gel was electrophoresed for a longer period of time (Fig. 2c). The hybridization patterns obtained for the integrant strains revealed the disappearance of parental-type bands and the appearance of new larger bands, indicating that one, two, or three copies of pGR102 or pWR103 integrated in any given *rrn* operon. For example, in strain BD170ΩpGR-143, a 5.7-kb band was replaced by three new bands (11.5, 17.3, and 23.1 kb), the sizes of which correspond to the presence of a plasmid monomer, dimer, and trimer, respectively, inserted into the 5.7-kb homolog (Fig. 2b and c). In another strain, BD170ΩpGR-144, a 5.8-kb homolog was replaced by a single new band (11.6 kb) corresponding only to a monomer (Fig. 2a). Hybridization experiments with undigested integrant DNAs failed to show any trace of free plasmids when probed with radioactively labeled pGR102 (data not shown).

Results of Southern blot analysis of the *Bcl*I digests (Fig. 2) indicate that both plasmids (pGR102 and pWR103) can integrate into seven of the nine possible *rrn* homologs of the three recipients used. Of the 18 separate integrant DNA preparations, at least nine represented repeated occur-

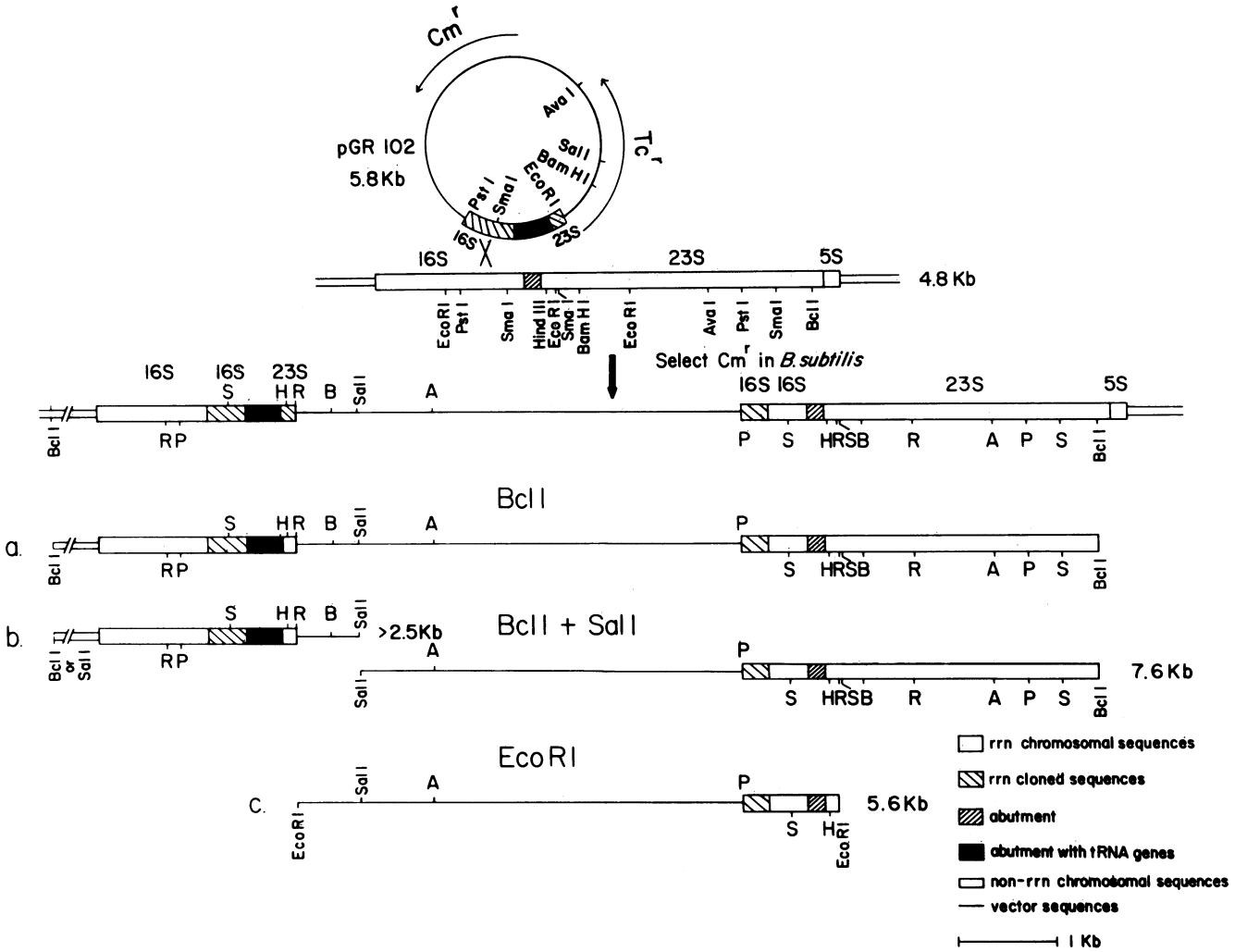

FIG. 1. Mechanism of integration of pGR102 into an *rrn* operon of *B. subtilis* containing the small abutment region. Only one integrated copy of the plasmid is shown, for simplicity, although the data indicate that two or three copies can exist. Integration of pGR102 into an *rrn* operon containing the large abutment region with the two tRNA genes would release a 7.8-kb fragment in a *Bcl*I-*Sal*I digestion and a 5.8-kb *Eco*RI fragment. The generalized restriction map of *B. subtilis rrn* operon was proposed by Stewart et al. (33). Restriction sites are as follows: *Ava*I (A), *Bam*HI (B), *Hin*dIII (H), *Pst*I (P), *Eco*RI (R), and *Sma*I (S).

rences. For example, DNA from strains BD170ΩpGR-135 and -153 revealed the displacement of the same 6.6-kb *Bcl*I fragment, or strains BD170ΩpGR-143 and BD170ΩpWR-181 showed the loss of the same 5.7-kb homolog (Fig. 2b and c). Two identical events leading to the simultaneous loss of two homologs (4.9 and 5.8 kb) occurred in strains BD170ΩpGR-151 and GSY1269ΩpGR-177, suggesting the deletion of one *rrn* operon. This unexpected class of transformants showed, in addition to the two lost *rrn* homologs, the presence of a single high-molecular-weight band (10.7 kb) equivalent to that of the integration of a monomer. No multimeric forms were observed in either preparation (Fig. 2a, b and c). The possible pathway of the deletion could be due to an interaction between a concatemer and two of the three closely situated repeats (*rrnI*, *rrnH*, *rrnG*).

Southern hybridizations of *Sal*I-*Bcl*I double digests probed with radioactively labeled pJH101 containing only vector sequences revealed, as predicted from the model (Fig. 1), the appearance of a new fragment with a uniform size of 7.6 or 7.8 kb (depending on the size of the abutment region of the operon involved) which was not present in the parental type DNA (Fig. 3a and b). For example, digests of strain BD170ΩpGR-143 and -135 released the 7.8- and the 7.6-kb fragments, respectively (Fig. 3a and b). More importantly, the double digestion produced a second variable band with sizes in the range of 3.0 to 6.1 kb (Fig. 3a and b). The appearance of this *Sal*I-*Bcl*I fragment is consistent with the proposed model of insertion of pGR102 or pWR103 into the chromosome of *B. subtilis* (Fig. 1) and allows for the estimation of size and the eventual isolation of the neighboring unique DNAs which terminate with a *Bcl*I site. For example, in strain BD170ΩpGR-151, the spacer sequence upstream from the 5′ end of 16S is 0.5 kb (3.0 minus 2.5 kb), while strains BD170ΩpGR-135 and -153 have longer unique regions of 1.3 kb (3.8 minus 2.5 kb). Another example is strain BD170ΩpWR-179 in which the spacer sequence upstream from the 5′ end of 16S is 3.6 kb (6.1 minus 2.5 kb), the largest seen in this study Fig. 3a and b). Since the probe used was the original cloning vector pJH101 which contains no homologous sequences, the control lanes of DNAs BD170

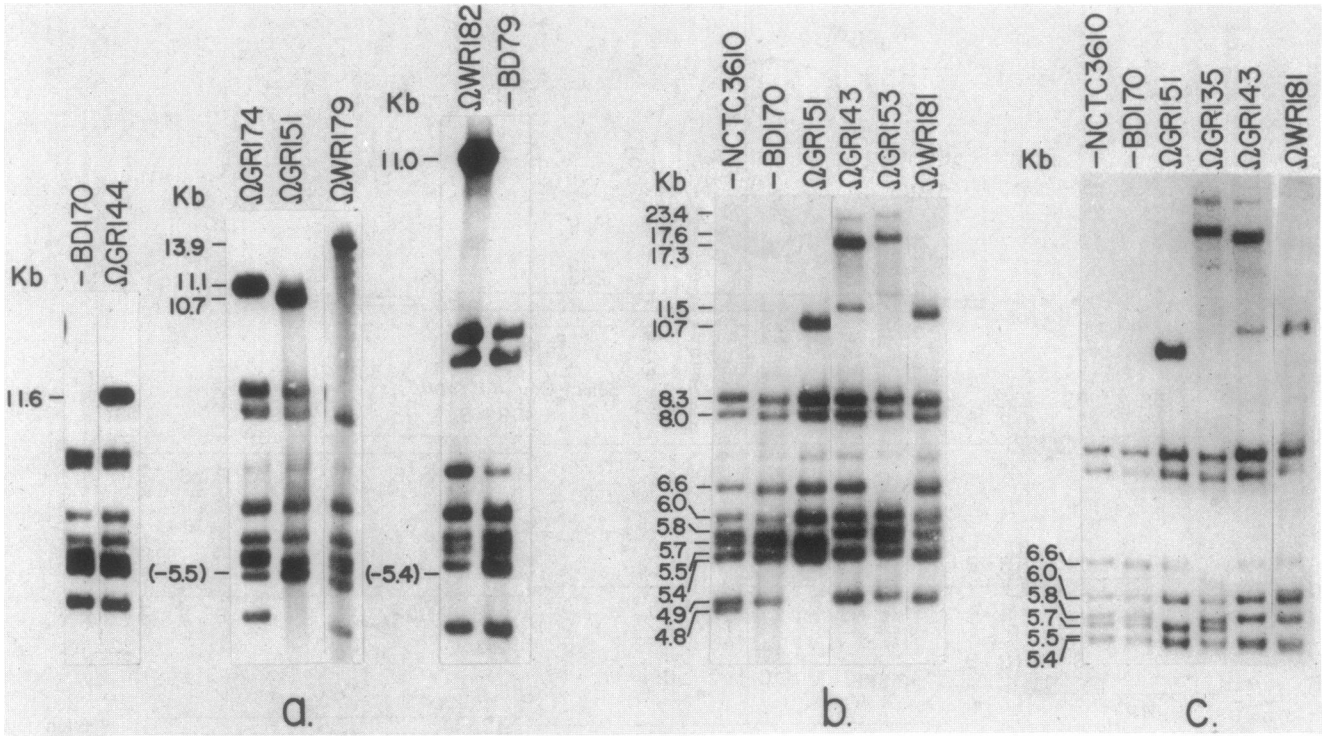

FIG. 2. Southern hybridization of total chromosomal DNAs of parental and integrant strains of *B. subtilis* with integration of either pGR102 or pWR103. A hyphen before a strain designation denotes that there is no integrated plasmid; Ω denotes plasmid integration of either pGR102 (GR) or pWR103 (WR) with the number of the integrant strain but without the original recipient (for example, ΩGR151 is BD170ΩpGR-151). Integrants are also described in Table 1. (a) Autoradiograms of *Bcl*I digests electrophoresed and probed as follows. Left panel, 0.85% agarose was probed with pWR103; center panel, 0.85% agarose was probed with pGR102; right panel, 0.75% agarose was probed with pWR103. (b) Autoradiograms of *Bcl*I digests electrophoresed on 0.75% agarose and probed with a labeled purified *Pst*I-*Eco*RI 16S 23S fragment from pGR102. (c) Similar autoradiograms of *Bcl*I digests electrophoresed on 0.75% agarose and allowed to run longer to increase the resolution of bands between 5.8 and 5.4 kb. DNA smaller than 5.4 kb was run off the gel. The probe was labeled pGR102.

and GSY1269 yielded no hybrid bands (Fig. 3a). Finally, it was possible to conclude from the hybridization patterns of the double digestions whether the integrants contained monomeric or multimeric forms of plasmid pGR102 or pWR103. A 5.8-kb band, which is the unit size of pGR102, could only be released on digestion with *Sal*I and *Bcl*I if the DNA harbored a multimer (Fig. 3a and b). Integrants containing a monomer did not release additional vector sequences, as was seen for strains BD170ΩpGR-151 and GSY1269ΩpGR-177 (Fig. 3a and b).

On restriction with *Eco*RI, the heterogeneity in the abutment region between the 3′ terminus of 16S rDNA and the 5′ terminus of 23S rDNA yields abutment fragments measuring 1.1 and 1.3 kb (12, 18). On integration of plasmid pGR102 or pWR103 three new patterns were seen on Southern blots of *Eco*RI-digested integrant DNAs probed with 16S and 23S rDNA or pGR102 (Fig. 4). In addition to the two internal *Eco*RI fragments (1.1 and 1.3 kb; data not shown), some DNAs displayed either a single band of 5.8 kb (e.g., BD170ΩpGR-143 and BD170ΩpWR-179) or a single band of 5.6 kb (e.g., BD170ΩpGR-144 and BD79ΩpWR-182), while others showed both bands (e.g., BD170ΩpGR-135 or BD170ΩpGR-153). The pattern shown in Fig. 4 indicates that the plasmids can integrate either into *rrn* operons with the large abutment region (5.8-kb band) or into those with the small abutment region (5.6-kb band). Parental strains (e.g., BD170, BD79, and GSY1269) did not reveal these hybrid bands and only exhibited the two internal fragments (data not shown; Fig. 4). Both bands were observed when a multimeric form of pGR102 was present at a *rrn* operon containing the small abutment region (Fig. 1 and 4). Since *rrnO* and *rrnA* have been characterized as those that contain the large abutment with two tRNA genes (5, 18, 24), and since the former has been sequenced along with the entire *oriC* region (21, 23), it was possible to assign the appropriate operon involved in the loss of two *Bcl*I homologs (8.3 and 5.7 kb, respectively; Fig. 2a and b). According to the published nucleotide sequence of the *oriC* region (21, 23), a predicted 8.3-kb *Bcl*I fragment should be produced on digestion with the enzyme, as was found for the integrant strain BD170ΩpWR-179 (Fig. 2c). One may conclude that the integration event for this strain occurred at *rrnO*, and for strains BD170ΩpGR-143 and BD170ΩpWR-181 the event occurred at *rrnA*. Genetic analyses described below verify this conclusion.

Genetic mapping of integrated plasmids. PBS1-transducing phages were prepared with integrant strains containing pGR102 or pWR103 as hosts. In all the transduction crosses with the nine mapping kit strains of Dedonder et al. (8), chloramphenicol was used initially as the selective marker to establish the gross chromosomal location followed by two- and three-factor crosses. Linkage was established either by direct replica plating or by patching the transductants on chloramphenicol-containing plates to test for cotransfer of the integrated plasmid with at least two unselected markers. The results (Table 3) indicate that the chromosomal locations of the integrated plasmids are limited to three or possibly four regions. The preferred sites of integration for

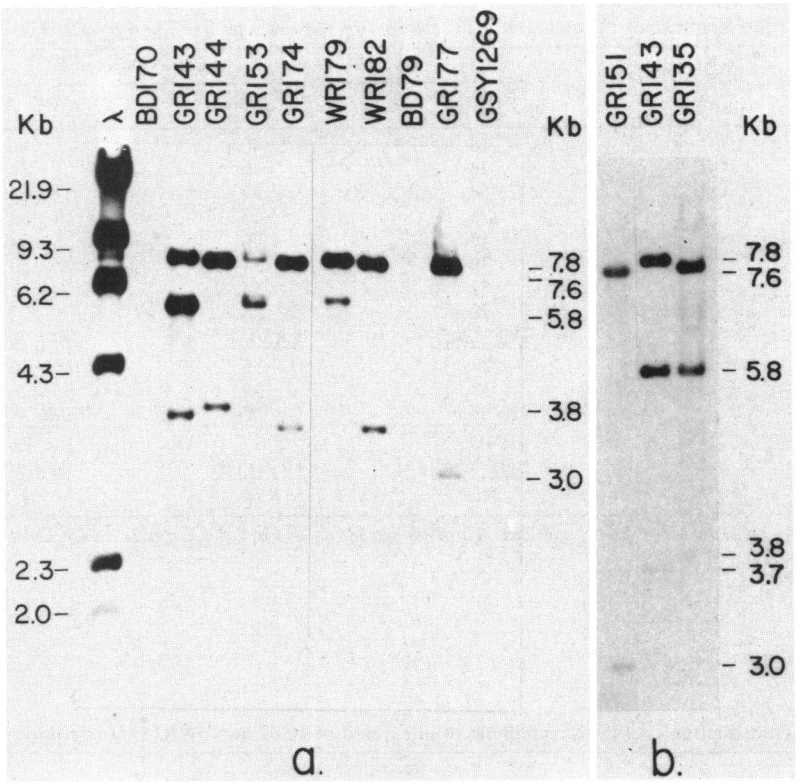

FIG. 3. (a) Autoradiograms of *BclI-SalI* digests electrophoresed on 0.85% agarose, and probed with labeled pJH101. (b) Autoradiograms of *BclI-SalI* digests electrophoresed on 0.75% agarose and probed with labeled pJH101 (Fig. 2 and Table 1).

the majority of the integrant strains were in the *purA16-cysA14* region. Of 12 strains, 8 exhibited a linkage between the Cm^r determinant of the integrated plasmids and the markers *purA16* and *cysA14* (Table 3). To date, among 60 integrant strains analyzed in transductional crosses, 53 have exhibited a linkage between the Cm^r determinant and the *purA16 cysA14* markers. Close linkages to *cysA14* were observed for strains BD170ΩpGR-151, BD170ΩpGR-144 and GSY1269ΩpGR-177. A close linkage to *purA16* was seen for strain BD170ΩpWR-181. The other strains revealed similar cotransduction frequencies (Table 3). This result is not unexpected in view of the fact that five operons (*rrnO*, *rrnA*, *rrnI*, *rrnH*, *rrnG*) have been previously mapped in that chromosomal region (5, 26).

Integrant strains BD170ΩpGR-135 and -153 showed linkage to two markers in strain kit 2, namely, to *dal-1* and *purB33* (Table 3). This is the first genetic proof for the existence of an unassigned ribosomal operon (*rrnE*) located in a region considerably removed from the major group of *rrn* genes. Another strain (BD79ΩpWR-182) containing the plasmid with a small abutment fragment revealed weak linkages to *glyB133* (29% cotransduction) and to *tre-12* (35% cotransduction) and none to *metC3*, the third marker of strain kit 3 (Table 3). This result also constitutes proof for the existence of another unassigned ribosomal operon (*rrnD*). Finally, DNA of integrant strain GSY1269ΩpGR-176 which showed the presence of vector sequences on Southern blots, but showed ambiguous results after digestion with *BclI*, revealed a close linkage of the Cm^r determinant to *leuA8* (Table 3). Among the 60 integrant strains mentioned above, 4 showed linkage to markers in kit 2 (*rrnE*), and 2 showed linkage to markers in kit 3 (*rrnD*). So far no integration events of pGR102 and pWR103 were observed in the chromosomal region containing *rrnB* and *rrnC* in which the Cm^r determinant would have shown linkage to the markers *aroG912* and *thr-5* of kit strains 7 and 8 (5).

To quantitate the linkage of the markers in the kit 1 strain and to establish the effect of the integrated vector on the recombination frequencies, we performed three factor crosses between the Cm^r determinant in phage PBS1 and the markers *purA16* and *cysA14*. Table 4 presents a sample of our crosses depicting two genetically similar strains BD170ΩpGR-144 and BD170ΩpWR-179 shown above to

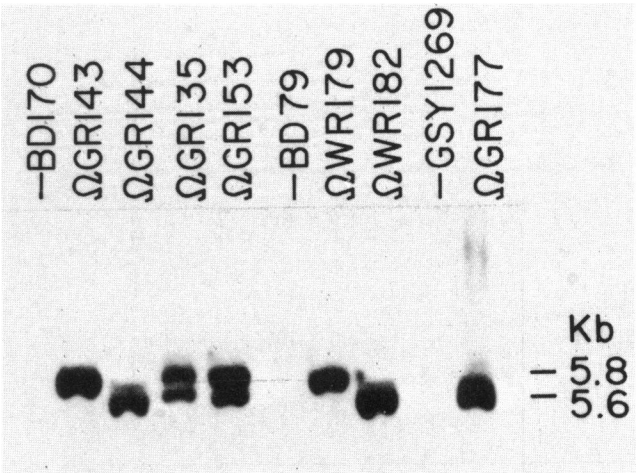

FIG. 4. Autoradiograms of *Eco*RI digests electrophoresed on 0.75% agarose and probed with labeled pGR102 (Fig. 2 and Table 1).

TABLE 3. Cotransduction frequencies of integrated Cm^r element of plasmids pGR102 and pWR103 to chromosomal markers

Integrant donor strain	Recipient kit strain[a]	Cotransduction %			
		Pur^+/Cm^r	Cm^r/Pur^+	Cys^+/Cm^r	Cm^r/Cys^+
BD170ΩpGR-151	1	18	40	96	90
GSY1269ΩpGR-177	1	30	40	84	80
BD170ΩpGR-143	1	54	57	61	57
BD170ΩpWR-181	1	76	10	83	14
BD170ΩpGR-144	1	49	36	94	97
BD79ΩpGR-174	1	79	51	89	59
BD79ΩpGR-175	1	51	54	67	56
BD170ΩpWR-179	1	78	28	72	9
		Dal^+/Cm^r	Cm^r/Dal^+	Pur^+/Cm^r	Cm^r/Pur^+
BD170ΩpGR-135	2	27	36	30	38
BD170ΩpGR-153	2	32	39	28	36
		Tre^+/Cm^r	Cm^r/Tre^+	Gly^+/Cm^r	Cm^r/Gly^+
BD79ΩpWR-182	3	ND[b]	35	26	29
		Leu^+/Cm^r	Cm^r/Leu^+	Aro^+/Cm^r	Cm^r/Aro^+
GSY1269ΩpGR-176	7	ND	74	ND	4

[a] Recipient strains are as follows: kit 1, *purA16, cysA14, trpC2*; kit 2, *aroI906, purB33 dal-1, trpC2*; kit 3, *tre-12, metC3, glyB133, trpC2*; kit 7, *leuA8, aroG912, ald-1, trpC2*.
[b] ND, Not determined.

TABLE 4. Transduction crosses for mapping of integrated pGR102 and pWR103 in the *purA cysA* region

Donor	Total no.[a]	Recombinant class[b]				Cotransfer	
		Cm^r	*purA*	*cysA*	No.	Type	%[c]
BD170ΩpGR-144	1.4×10^4	1	0	0	3	Cm^r/Pur^+	36
		1	1	0	6		
		1	0	1	181	Cm^r/Cys^+	97
		1	1	1	96		
	1.3×10^4	0	1	0	232	Pur^+/Cys^+	30
		1	1	0	113		
		0	1	1	20	Pur^+/Cm^r	49
		1	1	1	130		
	9.2×10^3	0	0	1	22	Cry^+/Pur^+	30
		0	1	1	18		
		1	0	1	416	Cys^+/Cm^r	94
		1	1	1	167		
BD170ΩpWR-179	1.8×10^4	1	0	0	200	Cm^r/Pur^+	28
		1	1	0	64		
		1	0	1	16	Cm^r/Cys^+	9
		1	1	1	20		
	1.5×10^4	0	1	0	67	Pur^+/Cys^+	21
		1	1	0	169		
		0	1	1	0	Pur^+/Cm^r	78
		1	1	1	64		
	1.9×10^4	0	0	1	82	Cys^+/Pur^+	17
		0	1	1	0		
		1	0	1	168	Cys^+/Cm^r	72
		1	1	1	51		
BD170	2.6×10^4		1	0	356	Pur^+/Cys^+	24
			1	1	113		
	2.0×10^4		0	1	375	Cys^+/Pur^+	25
			1	1	126		

[a] The recipient was kit 1 (*purA16 cysA14 trpC2*). The total number of transductants was determined.
[b] Donor and recipient phenotypes are indicated by 1 and 0, respectively.
[c] Number cotransferred per number tested.

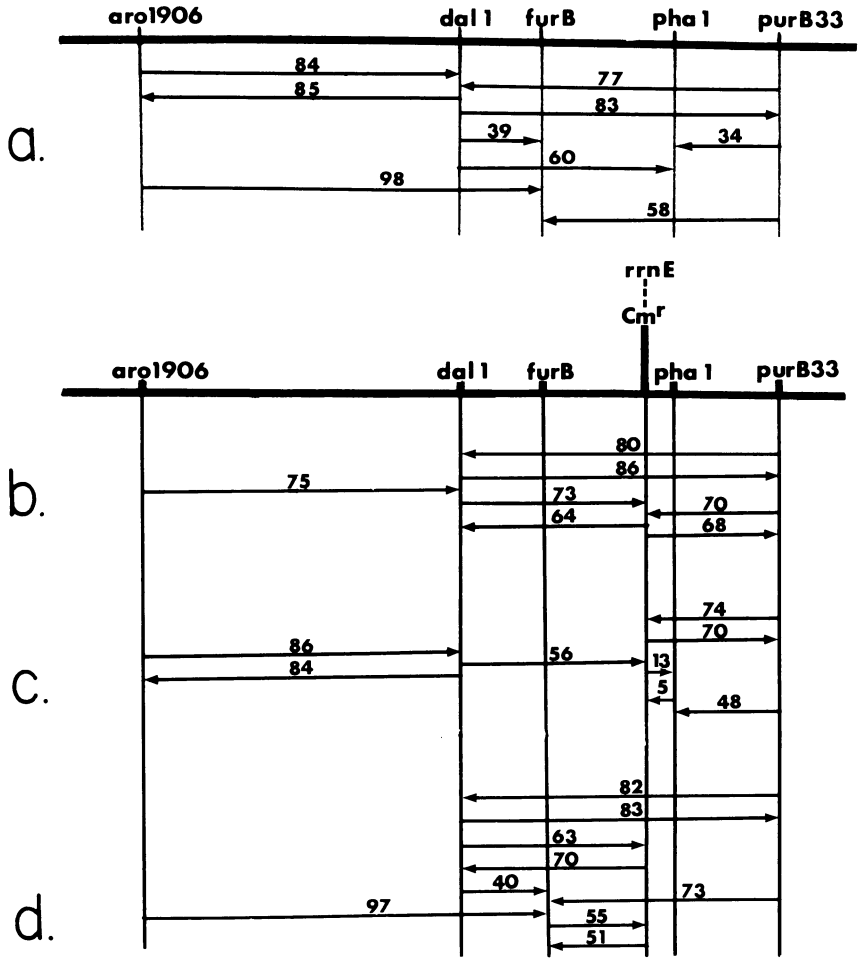

FIG. 5. Genetic map of the *aroI* to *purB* region containing the *rrnE* operon. The following donors were used: BD170 (*trpC2*, *thr-5*) (a); BD170 (*trpC2 thr-5*) ΩpGR-135 (b); IA150 (*pha-1 catA hisA2*) ΩpGR-135 (c); IA154 (*met trpC2 furB gutB*) ΩpGR-135 (d). Distances are expressed as the percentage of recombination in PBS1 transduction. The arrows point from selected to unselected markers.

have lost the 5.8- and 8.3-kb *Bcl*I fragments, respectively (Fig. 2a and b). In general the number of transductants per 10^9 PFU were consistently similar and relatively high (5×10^3 to 5×10^4 per ml). All three markers were transduced at comparable frequencies in most cases and were similar to those obtained with the parental strain BD170 (Table 4). We did not observe very low frequencies of transduction when chloramphenicol was used unselectively, as noted by others (29). Moreover, we did not detect the kind of asymmetry seen during transformation as with the integrant DNA BD170ΩpER-115 containing the plasmid pER102-*leu* of *B. subtilis* (Table 2) or a purified *phe-nic* segment reported previously (10, 36). In a representative experiment with our strain, Leu^+ transformants arose at a frequency of 9.2×10^5 per μg of DNA, and only 2% of them were simultaneously transformed for the Cm^r marker. Cm^r transformants arose at a reduced frequency of 4.9×10^4 per μg of DNA, but 97% of them were simultaneously transformed to Leu^+ (Table 2). On the other hand, among the transductants that were scored, no such extreme asymmetry in the cotransfer values were noted. The cotransfer values were comparable but not always equal in both directions of selection (Table 4). For example, the cotransfer values Cm^r/Pur^+ and Pur^+/Cm^r were similar (36 and 30%) for strain BD170ΩpGR-144 and quite different (28 and 78%) for strain BD170ΩpWR-179 (Table 4). As expected, the recombinant class Pur^+ Cys^+ Cm^s was invariably in the minority or not found at all if the gene order was *purA* (Cm^r = *rrnO*) *cysA* as compared with *purA cysA* (Cm^r = *rrnI*), indicating that a quadruple crossover event involving Cm^r is the most unlikely to occur (Table 4). The linkage relationship between *purA16* and *cysA14* was not noticeably affected by the presence of the plasmids pGR102 and pWR103. Certain unexplained biases associated with the Cm^r marker were noted, such as the high proportion of Pur^+ Cys^+ Cm^r in the first cross and a paucity of this group in the second cross. These biases which are always produced in transformation analysis presumably depend on the relative size of the heterologous and homologous portion of DNA and the extent to which homologous flanking of the insert is required to enable recombination with the recipient chromosome.

Genetic mapping of the *rrnE* and *rrnD* operons. Three integrant strains revealed the existence of two unassigned operons which we designated as *rrnE* and *rrnD*. The Cm^r determinant of strains BD170ΩpGR-135 and -153 showed linkage to *dal-1* and *purB33* (Table 3). Previous mapping data have shown that the *pha-1* marker is localized between the *purB* and *dal-1* loci (17) and the *furB* marker is 10% cotransduced with *purB6* (38). These findings enabled us to perform a series of two- and three-factor crosses which

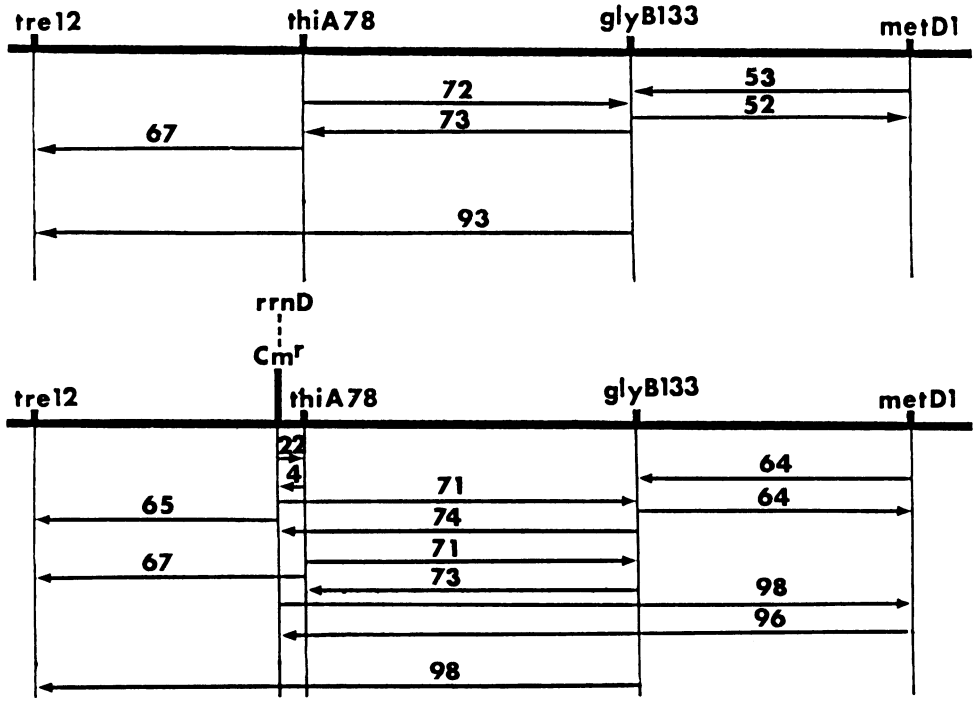

FIG. 6. Genetic map of the *tre* to *metD* region containing the *rrnD* operon. Distances are expressed as the percentage of recombination in PBS1 transduction. The arrows point from selected to unselected markers.

would establish the linkage relationships between the *rrnE* operon and nearby genes. Resistance to SPO1 phage (*pha-1*) or to 5-fluorouracil in the presence of uracil (*furB*) are nonselective markers in PBS1 transduction crosses. We constructed two donor strains by initially crossing the integrant strain BD170ΩpGR-135 with the recipient strains IA150 (*pha-1 hisA1 catA*) and IA154 (*met purB6 trpC2 furB gutB*) and selecting recombinants that were Cm^r *pha-1* or Cm^r *furB* PurB$^+$, respectively. Transductants were colony purified twice and used to prepare PBS1-transducing lysates which were designated as IA150ΩpGR-135 and IA154 PurB$^+$ ΩpGR-135, respectively (Table 1). These lysates were crossed with strain kit 2, and the *pha-1* and *furB* markers were either selectable or used as unselected markers among Pur$^+$, Dal$^+$, and Cm^r primary transductants. In the linkage map shown in Fig. 5, three integrant donors are compared with the parental strain BD170 (*trpC2 thr-5*). The Cm^r determinant is closely linked to *pha-1* (87 to 95% cotransduction) on one side and to *furB* (45 to 49% cotransduction) on the other side. The linkage relationship between Cm^r and either *purB33* or *dal-1* remained essentially unchanged for all three donor lysates, as did the linkage of *dal-1* to *purB33* (Fig. 5). The latter represents another example in which the insertion of a 5.8-kb plasmid (pGR102) does not affect the linkage relationship of the distant flanking markers. The linkage data show that the order is *aroI dal furB* (Cm^r = *rrnE*) *pha purB*.

The third integrant strain BD79ΩpWR-182, the Cm^r determinant of which showed linkage relationships to *tre-12* and *glyB133* (Table 3), was further examined for additional closer linkages to nearby genes. Two recipient strains, IA84 (*glyB133 metD1*) and IA122 (*thiA78 glpK21 glyB133 tre-12*), were transduced with PBS1 lysate made from strain BD79ΩpWR-182, and Cm^r was found to be tightly linked to *thiA78* (78% cotransduction) and weakly linked to *metD1* (2% cotransduction). Figure 6 summarizes the linkage data and shows that the order is *tre* (Cm^r = *rrnD*) *thiA glyB metD*. As mentioned above, the cotransfer of the integrated plasmid as followed by the Cm^r determinant varies with the direction of selection. Here, the percent cotransfer of Cm^r with the nearby marker *thiA* was lower (compare 78 with 96% cotransduction; Fig. 6).

DISCUSSION

Through integrative mapping with plasmids pGR102 and pWR103, we localized two additional previously unmapped operons, *rrnE* and *rrnD*, increasing the number to nine of known genomic locations. Both newly assigned operons are located with a major group of five operons (*rrnO*, *rrnA*, *rrnG*, *rrnH*, *rrnI*) in the map region between 0 and 90° (5, 26). The last operon, *rrnD*, was assigned to the map region between 60 and 75°, which is known for its low density of genetic markers and for which the task of accurate mapping is exceedingly difficult. Verification of the genomic assignments of *rrnD* and *rrnE* can be accomplished by excision or rescue of the integrated plasmid with the adjacent chromosomal sequences. To date, five new plasmids have been rescued from ligated *Bcl*I digests of integrant DNAs. For example, strain BD79ΩpWR-182 gave rise to the new plasmid pWR182 containing a 5.4-kb chromosomal insert with 0.8 kb of unique sequences 5′ to *rrnD*. Mapping data on transformants of this plasmid have shown the same location, in the region between *thiA* and *glyB* (R. Widom and E. Jarvis, unpublished data).

As in *E. coli* and *Salmonella typhimurium* (4, 16), at least seven rRNA genes of *B. subtilis* can be assigned to distinct *rrn* restriction fragments, each of which has a specific genetic locus. In the case of these enterobacteria, Southern analysis of *Bam*HI-*Pst*I genomic digests was used because neither restriction endonuclease cleaves within the seven *rrn* operons of *E. coli* (4). The available and complete DNA sequences or operons *rrnO*, *rrnA*, and *rrnB* (21, 23; K. Bott,

TABLE 5. Ribosomal *BclI* homolog loss and corresponding *rrn* operon assignment

Integrant strain	Missing *BclI* fragment(s) (kb)	Operon assignment	Genomic region
BD170ΩpWR-179	8.3	*rrnO*[a]	*purA-cysA*
BD170ΩpGR-143 BD170ΩpWR-181	5.7	*rrnA*[a]	*purA-cysA*
BD170ΩpGR-144	5.8	*rrnI*	*purA-cysA*
BD170ΩpGR-151 GSY1269ΩpGR-177	4.9 and 5.8	*rrnH*[b]	*purA-cysA*
BD79ΩpGR-174 BD79ΩpGR-175	5.5	*rrnG*	*purA-cysA*
BD170ΩpGR-135 BD170ΩpGR-153	6.6	*rrnE*	*dal-purB*
BD79ΩpWR-182	5.4	*rrnD*	*tre-12-glyB*
GSY1269ΩpGR-176		*rrnR*	*leuA-aroG*

[a] Operon with the large abutment containing two tRNA genes.
[b] The 4.9-kb fragment is assigned to *rrnH*.

personal communication) allowed us to choose the restriction endonuclease *BclI*, the recognition site of which at the 3′ end of 23S rDNA resolved 9 to 10 distinct *rrn* fragments, each of which can be assigned to specific genetic loci. Table 5 summarizes our identification of seven individual *rrn* operons with their corresponding *BclI* fragments. The assignment of *rrnE* and *rrnD* to the 6.6- and 5.4-kb *BclI rrn* homologs, respectively, is clear-cut since they occupy single genomic locations. Since operons *rrnO* and *rrnA* contain the large abutment with two tRNA genes, they were assigned to the 8.3- and 5.7-kb *BclI rrn* homologs, respectively, as predicted by the *EcoRI* Southern blot patterns (Fig. 4) and the known sequence of the *oriC* region (21, 23). The remaining three *BclI rrn* homologs (5.8, 5.5, and 4.9 kb) were assigned to the closely situated repeats in which the exact order of either *rrnI rrnH rrnG* or *rrnG rrnH rrnI* has not been established (5, 26). The unexpected disappearance of two *BclI rrn* homologs in strains BD170ΩpGR-151 and GSY1269ΩpGR-177 (data not shown) assisted in our assignment of the 4.9-kb *BclI rrn* homolog as the operon in the middle of the cluster, e.g., *rrnH*. The other two *BclI* fragments of 5.8 and 5.5 kb are either *rrnI* and *rrnG*, respectively, or vice versa, depending on the final orientation (5, 26), which will be established in a future study.

The presence of multimeric forms of the integrated plasmid pGR102 has been shown in this study. The relative proportion between the forms, i.e., monomer, dimer, and trimer, can vary among preparations. The dimeric form seemed most prevalent among the integrant strains containing multimers of the plasmid pGR102 (e.g., BD170ΩpGR-135, -153 and -143; Fig. 2b and c). Multiple copies of the inserted sequences such as *leu*, *phe*, and *amy* in *B. subtilis* have been observed by us and others (1, 36, 37). Integration into the chromosome by a Campbell-like mechanism of a plasmid bearing a cloned chromosomal segment gives rise to a nontandem duplication interrupted by the vector moiety of the plasmid (Fig. 1). The duplication should provide the basis for further amplification by unequal crossing over during chromosome replication or it can lead to the formation of deletions similar to that which occurred in strains BD170ΩpGR-151 and GSY1269ΩpGR-177.

We demonstrated here that ribosomal sequences from within the transcriptional unit that is devoid of neighboring spacer regions can be cloned and integrated into the chromosome of *B. subtilis*. These studies were prompted, in part, by the reports on the nonclonability in *B. subtilis* of a 2.5-kb fragment carrying rDNA sequences and a strong promoter (15) and by the inability of others to integrate a cloned DNA fragment from within the transcriptional unit of pure 16S and 23S sequences (5). The ability of our plasmids to recombine within operons may be due to the presence of the abutment region between 16S and 23S rDNA with or without the two tRNA genes. The extent to which the abutment regions facilitate the process of integration will be determined with new integrable clones containing pure 16S or 23S and 5S sequences, which we are currently constructing. It is possible that the cloned abutment region in pGR102 and pWR103, along with another newly constructed plasmid (pYR104) containing an *Eco*RI-*Hin*dIII 2.3-kb fragment carrying 23S and 5S DNA (Y. Setoguchi, unpublished data), provide enough processing signals so that the *rrn* operons are not inactivated by the integration and a certain amount of transcription and processing is permitted.

Results of our studies also show that due to the high multiplicity of *rrn* operons in *B. subtilis*, insertions into loci can occur without rendering the cells inviable. Many of these integrations were deleterious, judging by the extremely low frequencies of Cm^r transformants generated by these integrable plasmids. To date, integration of pGR102 into strains BD170 (*trpC2 thr-5*), BD79 (*leuB1 pheA1*), and GSY1269 (*trpE26 ilvC1*) (all have 9 instead of 10 *BclI rrn* homologs) resulted in strains with normal growth rates and sporulation for the first two recipients but not the last recipient. Integrant strains GSY1269ΩpGR-176 and -177 exhibited significant differences in growth rates in minimal medium and a decrease in sporulation efficiencies to 30 and 15%, respectively (W. Williamson, unpublished data). *B. subtilis* strains with the *trpE26* mutation (i.e., GSY1269) are known to possess a rearranged genome involving at least one unmapped *rrn* operon (11). Loughney et al. (19) reported that an *rrn* deletion strain of *B. subtilis* (168, *trpC2*) grew as well as the wild-type strain and sporulated with similar efficiency. Similarly, in *E. coli* deletion of one of the seven rRNA operons (*rrnE*) failed to show any decrease in growth rates and RNA synthesis (9). Construction of strains containing multiple ribosomal defects will elucidate how many and which *rrn* operons are dispensable in *B. subtilis* and the physiological effects on cell growth, sporulation, and rRNA synthesis of such defects.

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