Localization of rRNA Synthesis in *Bacillus subtilis*: Characterization of Loci Involved in Transcription Focus Formation

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In *Bacillus subtilis*, RNA polymerase becomes concentrated into regions of the nucleoid called transcription foci. With green fluorescent protein-tagged RNA polymerase, these structures are only observed at higher growth rates and have been shown to represent the sites of rRNA synthesis. There are 10 rRNA (*rrn*) operons distributed around nearly half of the chromosome. In this study we analyzed the *rrn* composition of transcription foci with fluorescently tagged loci and showed that they comprise the origin-proximal operon *rrnO* but not the more dispersed *rrnE* or *rrnD*. This suggests that transcription foci comprise only the seven origin-proximal operons *rrnO*, *rrnA*, *rrnJ*, *rrnW*, *rrnI*, *rrnH*, and *rrnG*. These results have important implications for our understanding of microbial chromosome structure.

As the growth rate of a bacterial cell increases, so does the cellular demand for ribosomes (16). As a result, transcription of rRNA operons (*rms*) increases with growth rate, so that at very high rates, nearly 80% of the cellular RNA is rRNA (5). The localization of RNA polymerase in the gram-positive bacterium *Bacillus subtilis* has recently been described (25). RNA polymerase was found to lie primarily within the central region of the nucleoid and to concentrate into subnucleoid regions termed transcription foci at higher growth rates (25) (Fig. 1A).

On induction of the stringent response, transcription foci rapidly disappeared, suggesting that they represent the sites of rRNA synthesis within the cell. The stringent response occurs when ribosomes encounter uncharged tRNA molecules and is a starvation response that results in the production of the alarmone (p)ppGpp (6, 16). There is mounting evidence that (p)ppGpp exerts its pleiotropic effects on gene expression through direct interaction with RNA polymerase (7). In *Escherichia coli*, ribosomal genes have promoters which are sensitive to (p)ppGpp levels (11). When these levels rise within the cell, there is a concomitant downregulation of rRNA (*rrn*) transcription (16). It has also been long established that due to the strength of *rrn* promoters, there is a disproportionate loading of RNA polymerase onto *rrn* genes (14). Therefore, transcription foci in *B. subtilis* were interpreted to represent the sites of *rrn* transcription within the cell and presented a dynamic illustration of transcriptional activity in vivo. Transcription foci are only rarely observed at slow growth rates (25). This is consistent with the observation that ribosome number is directly related to growth rate, and at low rates, the cell requires relatively few ribosomes. Therefore, at low growth rates, the level of *rrn* transcription would also be relatively low (9, 16).

There are 10 *rrn* operons in the *B. subtilis* chromosome (21). The number and chromosomal location of *rms* appear to be

directly related to the growth rate of organisms (1). For example, the slow-growing *Mycobacterium tuberculosis* (doubling time, about 24 h) contains a single *rrn* operon located 1.5 Mb away from the origin region (about 120° on a 360° circular chromosomal map [8]), whereas rapidly growing organisms like *Escherichia coli* (doubling time, ≤20 min) contain seven operons, all in the origin half of the chromosome (within 90° on both sides of *oriC* on a circular map [4]). *Vibrio natriegens*, which can grow with a doubling time of around 10 min, has at least 13 *rrn* operons per genome (1).

The 10 *rms* in *B. subtilis* are arranged in four groups (Fig. 1B). The first group contains seven *rrn* operons arranged in four clusters within 175 kb of the origin region. The remaining three operons are located at 54° (*rrnE*), 81° (*rrnD*), and 275° (*rrnB*). These origin-distal *rms* are associated with large numbers of tRNA (*trn*) genes (21). Therefore, these operons are likely to be highly transcribed and important within the cell. It is known that the number of *rms* in *B. subtilis* can vary due to deletion and recombination events, but this normally occurs in the *rrnI-H-G* and *rrnJ-W* clusters close to *oriC* (15, 37).

Transcription foci appear to be well-defined structures with a very regular appearance within the nucleoid (see Fig. 1A). Given that transcription foci represent the sites of *rrn* synthesis, we wanted to investigate their genetic composition. Do they contain all or just some of the *rrn* operons? If so, which ones? The clustering of origin-distal *rms* into transcription foci would represent significant chromosomal reorganization during rapid growth. If this clustering occurs, is it also observed at low growth rates, when transcription foci are not observed due to the low level of loading of RNA polymerase onto *rms*? If transcription foci contain origin-distal *rms*, does incorporation occur following replication of the locus, in a cell cycle-dependent manner, or as a single-step event following duplication of all *rms*? Previous studies have elegantly shown that the linear arrangement of genes on the chromosomes appears to be spatially retained in vivo (35). Therefore, any clustering of loci into transcription foci would most likely be specific for highly transcribed genes such as the *rms* and imply the formation of

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FIG. 1. Transcription focus appearance and *rrm* distribution in the *Bacillus subtilis* chromosome. Panel A shows representative cells of strain 1048 carrying an *rpoC-gfp* fusion and grown in CH medium, displaying transcription foci (arrows). Scale bar, 5 μ m. Panel B shows the locations of *rrm* operons and the control *yvfS* locus in the *B. subtilis* chromosome. Numbers represent coordinates in degrees on the 360-degree map of the chromosome. *oriC* and *terC* represent the origin and terminus of chromosome replication, respectively. Arrows indicate the direction of movement of replication forks.

structures akin to eukaryotic nucleoli where such clustering of rRNA genes does occur (33).

In this study, *rrms* were individually labeled with the system developed by Webb et al. (36), in which a green fluorescent protein (GFP)-LacI fusion binds to tandem repeats of the *lacO* site inserted at the desired chromosomal locus. These fusions were colocalized with origin regions with a Spo0J-cyan fluorescent protein (CFP) fusion. Spo0J foci have previously been shown to colocalize with transcription foci (25), and so it could also be used as a marker for *rrm* clustering at low growth rates, when transcription foci are not observed. A strong correlation between origin regions and the origin-proximal *rrmO* was ob-

served at both low and medium growth rates. However, no evidence for clustering was observed for origin-distal *rrms* at either growth rate. These results indicate that transcription foci represent the loading of RNA polymerase onto the seven origin-proximal *rrms* and that all 10 *rrms* in *B. subtilis* do not aggregate to form a bacterial nucleolus.

In addition, we have shown that the *rrn* loci tested all had functional, active promoters, indicating that lack of clustering of origin-distal loci was not due to any lack of transcription of those genes. No change in colocalization frequencies was observed on induction of the stringent response. These results confirm that the cell is required to maintain the linear order of genes on the chromosome and that complex chromosome remodeling does not occur as a normal part of the cell cycle. The maintenance of chromosome structure throughout the cell cycle has important implications for our understanding of chromosome segregation.

MATERIALS AND METHODS

Bacterial strains and media. All cloning was carried out in *Escherichia coli* DH5 α (Gibco-BRL). The *Bacillus subtilis* strains used and constructed in this study are listed in Table 1. Transformation of *B. subtilis* was carried out by the method of Anagnostopoulos and Spizizen (2), as modified by Jenkinson (20). *B. subtilis* transformants were selected on nutrient agar containing the appropriate antibiotic (chloramphenicol, 5 μ g/ml; spectinomycin, 50 μ g/ml; kanamycin, 5 μ g/ml). Double-crossover integrants into the *amyE* locus were selected as described previously (24). S medium, used for slow growth (doubling time, 70 min at 37°C), and CH medium, used for medium growth (doubling time, 45 min at 37°C), have been described previously (34).

DNA manipulations. Cloning steps were performed as detailed before (32). Oligonucleotide primers used for PCR are detailed in Table 2, and details of plasmid construction are given in Table 1.

Cell cycle modeling. Detailed cell cycle parameters for *B. subtilis* grown in S and CH media have been determined (34). Cell length measurements were made with MetaMorph (version 4.6; Universal Imaging Corporation) and used to determine the most likely stage of the cell cycle for each individual cell measured with the cell cycle models of Sharpe et al. (34). The point in the cell cycle at which each locus would be duplicated was calculated and placed onto the models so that cells could be classified as having *oriC*-locus ratios of either 1:1 or 2:1. Cells in which the ratio was 1:1 were classed as cells in which colocalization was most likely to occur if transcription foci contained clustered loci.

***rrn-bgaB* fusions and assays.** *rrn* promoter activity was determined with strains 1285-O5, 1285-DN1, and 1285-EN1 (Table 1), kindly provided by F. Kawamura (Rikkyo [St. Paul's] University, Japan). These strains utilize the thermostable β -galactosidase gene from *Bacillus stearothermophilus* (19). Assays were carried out at 62°C as described previously (19, 29).

Induction of stringent response. Cultures in CH medium were grown to mid-exponential phase (A_{600} 0.4) and then split into two. To one half, arginine hydroxamate was added to a final concentration of 500 μ g/ml (25) to induce the stringent response. The other half of the culture was used as a positive control. For β -galactosidase assays, samples were taken at 10-min intervals and assayed as detailed above. For microscopic analysis, cultures were placed onto an agarose slide 10 min after the addition of arginine hydroxamate to the culture, and images were acquired for the following 20 min. Images were processed as detailed below.

Microscopy, image acquisition, and analysis. Cultures were grown in S and CH media as detailed previously (34). Image data for all strains were collected from cells grown to mid-exponential phase in S and CH media at 30°C and 25°C, respectively. Strains required the addition of 0.2% xylose in S medium and 0.1% xylose in CH medium for the efficient expression of *gfp-lacI*. Cells were viewed on 1.2% agarose slides (17). All images were acquired with a Zeiss Axioskop 2 fluorescence microscope and Photometrics Quantix 1401E cooled charge-coupled device camera. GFP fluorescence was visualized with filter set 41018, and CFP fluorescence was visualized with filter set 31044v2 (Chroma Technology, Brattleboro, Vt.). Image analysis was performed with MetaMorph software (version 4.6; Universal Imaging Corporation), and figures were prepared for publication with Adobe Photoshop.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or construction
B. subtilis		
168	<i>trpC2</i>	C. Anagnostopoulos
1501	<i>trpC2 chr::pSG1517 (spo0J-gfp cat)</i>	13
1063	<i>trpC amyE::(spec P_{xyr}-gfp-lacI)</i>	168 transformed with pSG1189
1064	<i>trpC2 amyE::(spec P_{xyr}-gfp-lacI) chr::pSG1196 (rmD-lacO cat)</i>	1063 transformed with pSG1196
1067	<i>trpC2 chr pSG1197 (spo0J-cfp kan)</i>	168 transformed with pSG1197
105	<i>trpC2 amyE::(spec P_{xyr}-gfp-lacI) chr::pSG1196 (rmD-lacO cat) chr::pSG1197 (spo0J-cfp kan)</i>	1064 transformed with chromosomal DNA of 1067
106	<i>trpC2 amyE::(spec P_{xyr}-gfp-lacI) chr::pSG1201 (rmO-lacO cat)</i>	1063 transformed with pSG1201
107	<i>trpC2 amyE::(spec P_{xyr}-gfp-lacI) chr::pNG105 (rmE-lacO cat)</i>	1063 transformed with pNG105
108	<i>trpC2 amyE::(spec P_{xyr}-gfp-lacI) chr::pNG106 (yvfS-lacO cat)</i>	1063 transformed with pNG106
109	<i>trpC2 amyE::(spec P_{xyr}-gfp-lacI) chr::pSG1201 (rmO-lacO) chr::pSG1197 (spo0J-cfp kan)</i>	106 transformed with chromosomal DNA of 1067
110	<i>trpC2 amyE::(spec P_{xyr}-gfp-lacI) chr::pNG105 (rmE-lacO) chr::pSG1197 (spo0J-cfp kan)</i>	107 transformed with chromosomal DNA of 1067
111	<i>trpC2 amyE::(spec P_{xyr}-gfp-lacI) chr::pNG106 (yvfS-lacO) chr::pSG1197 (spo0J-cfp kan)</i>	108 transformed with chromosomal DNA of 1067
1285-05	<i>trpC2 lys1 aprEΔ3 nprR2 nprE18 amyE::P_{rmO}-bgaB cat</i>	F. Kawamura (Rikkyo [St. Paul's] University, Japan)
1285-DN1	<i>trpC2 lys1 aprEΔ3 nprR2 nprE18 amyE::P_{rmD}-bgaB cat</i>	F. Kawamura
1285-EN1	<i>trpC2 lys1 aprEΔ3 nprR2 nprE18 amyE::P_{rmE}-bgaB cat</i>	F. Kawamura
Plasmids		
pAT12	<i>bla lacO cat</i>	35
pSG1170	<i>bla P_{spac}-gfpUV P_{pen}-lacI cat</i>	24
pSG1729	<i>bla amyE3' spc P_{xyr}-gfp amyE5'</i>	24
pSG1192	<i>bla amyE3' spc P_{xyr}-cfp' amyE5'</i>	12
pSG1189	<i>bla amyE3' spc P_{xyr}-gfp-lacI amyE5'</i>	<i>Bam</i> HI- <i>Xho</i> I digest of <i>lacI</i> (Δ C-terminal 11 amino acids) PCR product from pSG1170 into pSG1729 cut with <i>Bam</i> HI- <i>Xho</i> I
pSG1195	<i>bla amyE3' spc P_{xyr}-spo0J-cfp amyE5'</i>	PCR <i>spo0J</i> from strain 1510 DNA, cut with <i>Xho</i> I- <i>Nco</i> I and insert into <i>Xho</i> I- <i>Nco</i> I-cut pSG1192
pSG1196	<i>bla rmD-lacO cat</i>	PCR of region immediately downstream of <i>rmD</i> cut with <i>Xho</i> I- <i>Bam</i> HI into <i>Xho</i> I- <i>Bam</i> HI-cut pAT12
pSG1197	<i>bla spo0J-cfp kan</i>	<i>Acc</i> 651- <i>Xba</i> I-digested <i>spo0J-cfp</i> into <i>Acc</i> 651- <i>Xba</i> I pUK19
pSG1201	<i>bla rmO-lacO cat</i>	PCR of region immediately downstream of <i>rmO</i> operon cut with <i>Xho</i> I- <i>Bam</i> HI-cut pAT12
pNG105	<i>bla rmE-lacO cat</i>	PCR of region immediately downstream of <i>rmE</i> operon, cut with <i>Xho</i> I- <i>Bam</i> HI into <i>Xho</i> I- <i>Bam</i> HI-cut pAT12.
pNG106	<i>bla yvfS-lacO cat</i>	PCR of region immediately downstream of <i>yvfS</i> gene cut with <i>Xho</i> I- <i>Bam</i> HI into <i>Xho</i> I- <i>Bam</i> HI-cut pAT12

RESULTS

Strain construction and determination of signal crossover.

Since *rrn* clustering could happen at both slow and fast growth rates but transcription foci are only visible at higher growth rates, it was not possible to colocalize *rrn* loci directly with RNA polymerase. Instead, *rrn* loci were colocalized with a Spo0J fusion that marked the origin regions of the cell (see below) and has been shown previously to colocalize with transcription foci (25). Dual-labeled strains containing Spo0J-CFP and GFP-LacI fusions were constructed. The GFP-LacI fusion was able to bind to *lacO* sequences inserted at a variety of places in the chromosome (Table 1). The *lac* operator inserts contain 256 tandem repeats of *lacO* (31), and so if these sites are saturated with GFP-LacI, they could represent a significant block to an advancing DNA replication fork, slowing the natural growth rate. Strains 105, 109, 110, and 111 containing Spo0J-CFP, GFP-LacI, and the *lac* operators downstream of *rmD*, *rmO*, *rmE*, and *yvfS*, respectively (Table 1), were grown

in S and CH media in the presence of 0.2 and 0.1% (wt/vol) xylose (see below), and their growth rates and cell size distributions were compared with that of the wild-type strain 168. These were found to be identical to 168 (not shown). Therefore, under the growth conditions used in this study, it is reasonable to assume that the cell cycle parameters of the labeled strains will be the same as that of the wild-type control strain.

Fluorescent protein fusions have quite broad excitation and emission spectra (18), and so considerable fluorescence cross-over would be expected to occur between the cyan and green fluorescent fusion proteins. If this occurred, it would not be possible to assign signal definitively to one fusion or the other when observing fluorescence in either the cyan or green channel. Nevertheless, since the fluorescence fusions used in this study were relatively weak, the small percentage of signal that crossed over into another channel represented a very small proportion of the total amount of signal present in that chan-

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence
<i>lacI</i> forward	5'-GATCGGATCCACGTGAAACCAGTAACGTTA-3'
<i>lacI</i> reverse	5'-AACCTCCTCGAGCAGCTGCATTAATGAATC-3'
<i>rmD</i> forward	5'-CTTACTCAACTCGAGGGTCTAGGATCTCTT-3'
<i>rmD</i> reverse	5'-CTTACGTATATCTGTGGATCCAAAAGAAAA-3'
<i>rmE</i> forward	5'-CCATTAATGCTCGAGAAACGATCATGT-3'
<i>rmE</i> reverse	5'-GCTTCAACGGATCCAATTGAAACACA-3'
<i>rmO</i> forward	5'-GAATGATGTCTCGAGTGTATCTAGTTTTG-3'
<i>rmO</i> reverse	5'-TTATAAGGATCCGCATATTCTCCCTTAGA-3'
<i>yvfS</i> forward	5'-GTGTAATACCTCGAGAAGGCAATATCT-3'
<i>yvfS</i> reverse	5'-GTG CTT GAT GGA TCC AGT CAC GGC TTC-3'
<i>spo0J</i> forward	— ^a
<i>spo0J</i> reverse	5'-TTCTCCTTTACTACCATGGGCAGGAATTC-3'

^a Glaser et al. (13).

nel. Strains containing just *spo0J-cfp* (1067; Table 1) or *rmD-lacO* and *gfp-lacI* (1064; Table 1) fusions were grown, and images were obtained in the green and cyan fluorescence channels. Strain 1064, containing a xylose-inducible copy of *gfp-lacI*, was grown in both S and CH media containing different levels of xylose to determine the optimum levels required for good fluorescence in the green channel and minimal crossover into the cyan channel. Strains grown in the presence of 0.2% (wt/vol) xylose in S and 0.1% (wt/vol) xylose in CH medium were found to display good fluorescence in the green channel with virtually no crossover into the cyan channel (not shown). The weakly fluorescent Spo0J-CFP fusion strain 1067 gave no observable crossover into the green channel (not shown).

Dual-labeled strains were constructed, and cyan and green fluorescence was observed. In strain 105 (Spo0J-CFP *rmD-lacO* GFP-LacI; Table 1), the *oriC-rmD* ratio will be 2:1 for nearly half a cell cycle, as *rmD* is distal to the origin (Fig. 1B; see below), and so around half the cell population should contain readily distinguishable cyan and green foci. This was observed and is illustrated in Fig. 2A. Spo0J-CFP foci have been pseudocolored red, and GFP-LacI foci have been pseudocolored green. The image overlay in Fig. 2A shows a single cell containing two Spo0J foci (red) and a single LacI focus (green), with no observable overlap between the signals. This is confirmed by the line scan of the cells shown directly below the overlay. The peak corresponding to GFP-LacI is mutually exclusive to the Spo0J-CFP peaks, confirming that there is essentially no signal crossover between the fusions and that it is possible to localize these green and cyan fluorescent protein fusions in the same cells with confidence.

Colocalization of *oriC* with *rrns*. Once the level of signal crossover had been determined, it was important to establish how images would be scored to ascertain which *rrns* cluster into transcription foci. A Spo0J-CFP fusion was used to mark the location of the origin region as it binds to multiple sites and condenses approximately 400 kb of the chromosome around *oriC* that also encompasses part of the *rmO-rmG* operon cluster (23, 27, 28). Thus, there should be a strong correlation between the subcellular localization of the *oriC* region and the *rmO-rmG* cluster.

Image overlays of Spo0J-CFP with different GFP-decorated *rrns* are shown in Fig. 2. In Fig. 2A, an image is shown containing two Spo0J foci and a single *rmD* focus. The line scan shown below the image clearly shows that the signals are mutually exclusive (the peak of one signal corresponds to the

FIG. 2. Representative images and line scans of dual-labeled cells. Panels A, B, and D show single cells, whereas a pair of cells is shown in panel C. Line scans through the cells are shown below the images. Cells from strain 105 (*rmD*) are shown in panels A and C, from strain 110 (*rmE*) in panel B, and from strain 109 (*rmO*) in panel D. Spo0J-CFP foci have been pseudocolored red, whereas GFP-LacI foci have been pseudocolored green. These color assignments have been conserved in the line scans below the images. Bar, 2 μ m.

trough of the other) and as such would be scored as noncoincident signals. Figures 2B, C, and D shows cells containing equal numbers of Spo0J and *rrn* foci. In Fig. 2B (*rmE*) and 2C (*rmD*), the foci lie either adjacent to or between each other. The line scans below the images show that peaks either do not coincide (they are offset from each other; Fig. 2B) or are mutually exclusive (Fig. 2C) and so would also be scored as noncoincident signals. In Fig. 2D (*rmO*), the signals are perfectly coincident, and this was confirmed by the line scan. Only these signal patterns were scored as coincident signals.

It was also necessary to take into account the stage of the cell cycle for individual cells being examined. If chromosome replication was under way and the origin regions duplicated but replication forks had not yet moved through an *rrn* locus (e.g., Fig. 2A), the ratio of *oriC* to *rrn* would be 2:1. Therefore, the maximum possible level of signal colocalization for that locus would be 50%. Detailed cell cycle models have been produced describing the growth of *B. subtilis* under a range of defined conditions in previous studies (34). Use was made of these models to assign the cell cycle stage of individual cells in this

FIG. 3. Cell cycle models for *B. subtilis*. The figures shown in both panels A and B are derived from those developed previously (34). S and CH represent S and CH medium, respectively. The letters i, bi, s, b, and t above the cartoon cells correspond to initiation of chromosome replication, binucleation, chromosome segregation, cell birth, and termination of the chromosome replication phases of the cell cycle, respectively. In panel A, the points in the cell cycle at which replication forks move through *rrnO*, *rrnE*, *yvfS*, and *rrnD* are labeled and marked by arrows under the figures. In panel B, the light grey boxes highlight the cell length distributions in which the *oriC-rrnD* ratio is 2:1, and the dark grey boxes highlight the cell length distributions in which the *oriC-rrnD* ratio is 1:1. Cell length is indicated on the scales below the figures. Adapted from reference 34 with permission.

study. Cells were grown in S medium, in which transcription foci are not observed, and CH medium, in which they are. Cell cycle models for cultures grown in these media are shown in Fig. 3. The points at which individual loci are duplicated were calculated and mapped onto the models (Fig. 3A). For example, *rrnD* is situated at 81° on the chromosome and so will not be duplicated until the replication cycle is nearly 50% complete (forks situated at 90° and 270° on a circular map have duplicated 50% of the chromosome). Therefore, the *oriC-rrnD* ratio will be 2:1 for nearly half a cell cycle.

The cell size distribution for this part of the cell cycle is indicated by the light grey boxes in Fig. 3B. Since the *oriC-rrnD* ratio is 2:1 in this portion of the cell cycle, the maximum level of colocalization of signals would be 50%. When replication

forks pass through the *rrnD* locus, the ratio of signals will become 1:1, and so up to 100% colocalization of signals could be expected in this size range (dark grey boxes, Fig. 3B). In addition to *rrnO*, *rrnE*, and *rrnD*, the colocalization frequency of the control locus *yvfS* with *oriC* was also determined. *yvfS* is the last gene in an operon containing a proposed ABC transporter, is located at 299° on the *B. subtilis* chromosome, and is approximately equivalent to the location of *rrnE* but on the opposite arm of the chromosome (Fig. 1B). Therefore, if *rrn* locus clustering was found to occur, we could determine whether it was specific to those loci or a general feature of the origin half of the chromosome.

At least 400 cells of each strain in both S and CH media were scored to determine the level of colocalization of signals, and the results are presented in Table 3. The theoretical size range in which transcription foci could contain each locus (*oriC*-locus ratio of 1:1) was determined from the cell cycle models shown in Fig. 3 and detailed in reference 34 and is presented in the left-hand column for each of the media used. The following two columns present the percentage signal colocalization observed in cells that fell into the predicted size range in which transcription foci could be observed for that strain (in range), or that fell outside the predicted size range (ex range).

Strain 109 (Table 1), in which *rrnO* is labeled, lies so close to *oriC* (9.8 kb) and within the region of the chromosome that contains utilized Spo0J binding sites (28) that all of the cells were considered to lie within the range in which the locus would be able to form transcription foci. In both S and CH media, a high level of colocalization of *rrnO* and Spo0J signals was observed (74.4% and 75.5%, respectively; Table 3). This level of colocalization is in close agreement with the level of Spo0J and transcription focus colocalization previously measured with a strain carrying RNA polymerase-GFPuv and Spo0J-GFP fusions (79%) (25). Therefore, it is highly likely that *rrnO* is a component of transcription foci in vivo.

For the remaining strains used in this study, if a specific locus was incorporated into a transcription focus, the frequency of colocalization should be much higher for cells that fall into the in range category than those falling into the ex range category. The level of colocalization in the ex range category would represent the random colocalization of signals observed due to the orientation of cells on the slide, the resolving limitations of the light microscope, and the movement of DNA-protein-fluorescent protein complexes in the cell due to random diffusion. In addition, if transcription foci represent the exclusive clustering of *rrn* operons, the frequency of in range colocalization should be much higher for *rrn* loci than for the control *yvfS* locus. Results for colocalization of the loci are listed in Table 3. For all of the remaining loci, there was no significant difference in the level of colocalization of signal between cells falling into the in range and ex range classes. In addition, the levels of colocalization observed were similar for the control *yvfS* locus and the *rrn* loci. Overall, these results indicate that *rrnE* and *rrnD* are not specifically recruited into transcription foci in either S or CH medium.

Nevertheless, some differences in colocalization frequency between the media were observed. The level of colocalization was higher and less variable in CH medium than in S medium (Table 3). This may be due to the DNA content of the cells at higher growth rates. Under the conditions used, the large ma-

TABLE 3. Colocalization of *oriC* regions with *rm* loci

Marker	S medium			CH medium			Frequency of colocalization in CH medium + arginine hydroxamate (500 µg/ml) (%)	
	Theoretical transcription focus size range (µm)	Frequency of colocalization (%)		Theoretical transcription focus size range (µm)	Frequency of colocalization (%)			
		In range	Ex range		In range	Ex range	In range	Ex range
<i>rmO</i>	All	74.4		All	75.5		75	
<i>rmE</i>	1.70–2.80	46.9	43.4	2.85–4.80	39.4	34.3	ND ^a	ND
<i>rmD</i>	1.95–2.80	33.3	20.5	3.35–4.80	50.2	36.4	38	35
<i>yvfS</i>	1.75–2.80	24.8	18.1	3.05–4.80	34.7	40.0	ND	ND

^a ND, not determined.

jority of cells growing in S medium contained two origins and one terminus (34) (Fig. 3). By contrast, the large majority of cells grown in CH medium contained four origins and one or two terminus regions. The increase in the number of loci per cell in CH medium might simply lead to a higher chance of random colocalization being observed in cells, accounting for the differences noted in Table 3.

***rm* promoter activity.** Our colocalization studies indicated that *rmE* and *rmD* were not recruited into transcription foci. However, it was not known whether these operons were actually being transcribed under the experimental conditions used, and very little data are available on the transcriptional activity of *rm* promoters in *B. subtilis*. Therefore, we decided to check the promoter activity of *rmO*, *rmE*, and *rmD* in S and CH media. β -Galactosidase gene (*lacZ*) fusions to *rm* promoters were kindly donated by F. Kawamura (Rikkyo [St. Paul's] University, Japan) and are listed in Table 1. The strains were grown in both S and CH media, and assays were performed as detailed in Materials and Methods. The results of the assays are shown in Fig. 4A.

It is clear that all the promoters were active in both media. As expected, the level of activity was approximately two- to fourfold greater in CH medium than in S medium. This would be consistent with the requirement for greater numbers of ribosomes for rapidly growing cells in rich media (5, 16). There were other slight variations with the level of activity of the promoters in different media. The level of expression of *rmD* was approximately two-thirds that of *rmO* and *rmE* in S medium, while the level of *rmE* expression was about 50% greater than that of *rmO* and *rmD* in CH medium (Fig. 4A). However, these differences are relatively modest, and quite large standard deviations were obtained during the assays ($n = 5$; Fig. 4A). Nevertheless, it is clear that all the *rm* promoters tested showed considerable and similar levels of activity, indicating that they would all be transcribed under the experimental conditions used to determine transcription focus composition.

Colocalization patterns on induction of stringent response. Transcription foci were shown to be the sites of *rm* transcription, as transcription foci disappear on induction of the stringent response and subsequent downregulation of *rm* transcription (25). In order to ensure that all of the *rm* promoters used in this study functioned correctly and were under stringent control, they were tested as detailed in Materials and Methods. Within 10 min of the addition of the stringent response inducer arginine hydroxamate, there was a significant decrease in the level of transcription from *P_{rmD}* (Fig. 4B). Similar results were

also obtained for *P_{rmO}* and *P_{rmE}* (not shown). The low level of expression from the promoters in the presence of arginine hydroxamate continued throughout the time course and showed that they behaved in the expected manner on induction of the stringent response.

Although there was no evidence to suggest clustering of *rm*s outside of the origin region into transcription foci, an additional check was performed to determine if any changes in colocalization of labeled loci were observed upon induction of the stringent response. Colocalization frequencies of CFP and GFP signals in strains 109 (*rmO*) and 105 (*rmD*) (Table 1) were determined as detailed in Materials and Methods, and the results are presented in Table 3. The level of colocalization of both *rmO* and *rmD* with Spo0J was similar to that observed in CH medium during normal exponential growth (compare *rmO* and *rmD* colocalization data in CH medium in Table 3). The in range level of *rmD* colocalization was slightly lower than that observed in normal growing cells, and this may be due to a slight reduction in the number of *rmD* loci on induction of the stringent response due to the activity of Ster sites (see Discussion).

Ster sites are located approximately 200 kb either side of *oriC* and act as reversible replication termination sites on induction of the stringent response (3). The *rmD* operon is located nearly 1 Mb from *oriC*, and so on activation of Ster sites, replication forks will not be able to move beyond 200 kb of *oriC* and the *rmD* locus will not be duplicated. Therefore, the proportion of cells with an *oriC*-*rmD* ratio of 1:1 will drop on induction of the stringent response. Since the *rmO* operon lies within 10 kb of *oriC*, the proportion of cells with an *oriC*-*rmO* ratio of 1:1 should not be affected on induction of the stringent response. Since image acquisition was carried out within 30 min of induction of the stringent response, Ster termination would have had only a modest effect on the ratios of loci in our samples. Our results confirm that it is extremely unlikely that there is any chromosomal refolding involved in clustering of distantly located *rm* operons to form transcription foci. Rather, the formation of transcription foci is most likely to be exclusively due to the increased loading of RNA polymerase onto the seven *rm* operons clustered adjacent to *oriC*.

DISCUSSION

In this work, we characterized the structural composition and nature of transcription foci in *Bacillus subtilis*. In addition to the increased level of loading of RNA polymerase onto *rm*

FIG. 4. Panel A shows *rrn* promoter activity measured as β -galactosidase activity in both CH (dark grey) and S (pale grey) media. Error bars indicate standard deviations. The promoter fusions are indicated on the x axis. β -galactosidase activity (Miller units) is shown on the y axis. Panel B shows a plot of a time course assay for *rrnD* promoter activity in CH medium in the presence (grey diamonds) and absence (black squares) of arginine hydroxamate (RX).

genes at high growth rates (14), it was also possible that some or all of the 10 *rms* distributed around nearly half of the *B. subtilis* chromosome could be involved in the formation of these structures. We analyzed the level of clustering of several *rrn* operons at both slow growth rates, when transcription foci are not observed, and high growth rates, when they are (Table 3). In addition, we also analyzed any dynamic changes in transcription focus structure that occurred on induction of the stringent response and subsequent downregulation of *rrn* expression (Table 3).

Our results clearly show that *rrnO* located adjacent to *oriC* is involved in transcription focus formation. Likewise, there is no evidence to suggest that *rrnE* and *rrnD* are involved in the formation of transcription foci (Table 3). By inference, it is also extremely unlikely that the remaining origin-distant operon, *rrnB*, would be involved in transcription focus formation either.

We also determined the transcriptional activity of the pro-

motors for all the *rrn* operons used in this study in order to determine their activity under our experimental conditions. All the promoters were active to a similar level, indicating that all of the operons were being transcribed. This result indicates that while *oriC*-distal *rrn* operons are transcribed, transcriptional activity does not require translocation to a nucleolus-like subregion of the bacterial nucleoid dedicated to *rrn* transcription and ribosome assembly. It should be noted that transcriptional activity of *rrn* promoters was determined from β -galactosidase gene fusions at the *amyE* locus rather than from the chromosomal location. Therefore, our results reflect a direct comparison of the transcriptional activity of these promoters in the cell independent of gene dosage effects. The level of expression of the strains used in this study was similar to that of constructs with β -galactosidase fusions inserted at the chromosomal locus of the *rrn* operons (R. Rudner, New York University, New York, N.Y., personal communication). Nevertheless, it was important to check that these promoter sequences behaved as would be expected for sequences located at their normal chromosomal sites. On induction of the stringent response, the activity of all the promoters was significantly reduced, indicating that P_{rrn} activity was attenuated, as would be expected. No significant change in colocalization frequency was observed for labeled loci on induction of the stringent response, confirming that transcription focus disappearance is not due to disaggregation of clustered *rrn* loci and is due to the reduced level of loading of RNA polymerase onto the seven origin-proximal *rrn* operons.

Therefore, we conclude that transcription foci comprise the seven *rrn* operons that are located within 175 kb of *oriC*, *rrnO*, *rrnA*, *rrnJ*, *rrnW*, *rrnI*, *rrnH*, and *rrnG*. Rather than specifically clustering, we suggest that transcription foci purely represent the additional loading of RNA polymerase that occurs at *rms* when cells are growing rapidly (14, 16). Although we have shown that all the *rms* tested in this study are transcribed at similar levels, we cannot detect additional foci that represent RNA polymerase loading onto the more dispersed sites *rrnE* and *rrnD* at high growth rates with any confidence (not shown). If we assume that the rate of transcription from each *rrn* promoter is approximately equal, the proportion of RNA polymerase involved in transcription of the three origin-distal loci at high growth rates represents not much more than 10% of the level of RNA polymerase involved in total *rrn* transcription when gene dosage is taken into account, and so it is unlikely that we would be able to detect specific signals above the background RNA polymerase level.

Our results help confirm an emerging model of chromosome structure and organization within living bacterial cells. While it had been assumed for years that there must be some level of structural organization of bacterial chromosomes, it is only relatively recently that we have been able to start probing these delicate structures in vivo (26). In both *E. coli* and *B. subtilis*, a number of studies have shown that origin regions tend to be oriented towards the cell poles, whereas terminus regions tend to localize to the cell center around the site where the new division septum will form. This orientation appears to be important both with respect to providing a polarity for chromosome segregation (13, 36) and for the faithful decatenation of chromosomes on completion of DNA replication (10, 22). More detailed studies on chromosome structure in *E. coli*

indicate that genes in the origin half of the chromosome cluster into an origin domain, whereas those in the terminal half cluster into a terminus domain (30). Other studies in *B. subtilis* imply that the linear order of genes observed in the chromosomal sequence is preserved in vivo (35). Therefore, a gene that lies closer to *oriC* on the linear DNA sequence of the chromosome will also lie closer to it in situ than a more distant gene.

Despite these data, it was still possible that certain sequences, such as those involved in the coordinated large-scale production of rRNA, might prove exceptions to this rule and be involved in the formation of a higher-order structure that increased the efficiency of rRNA synthesis. Our results suggest that this is not the case and are most consistent with the individual *rm* genes' retaining their positions relative to other genes within the linear order of the chromosome. This, in turn, suggests that maintenance of chromosome structure is important throughout the cell cycle and at different growth rates and is likely to ensure accurate segregation of daughter nucleoids upon cell division.

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