JOURNAL OF BACTERIOLOGY, Feb. 1994, p. 1121–1127
0021-9193/94/$04.00+0

Vol. 176, No. 4

Cloning of a Gene Involved in rRNA Precursor Processing and 23S rRNA Cleavage in *Rhodobacter capsulatus*

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Received 24 September 1993/Accepted 10 December 1993

In *Rhodobacter capsulatus* wild-type strains, the 23S rRNA is cleaved into [16S] and [14S] rRNA molecules. Our data show that a region predicted to form a hairpin-loop structure is removed from the 23S rRNA during this processing step. We have analyzed the processing of rRNA in the wild type and in the mutant strain Fm65, which does not cleave the 23S rRNA. In addition to the lack of 23S rRNA processing, strain Fm65 shows impeded processing of a larger 5.6-kb rRNA precursor and slow maturation of 23S and 16S rRNAs from pre-23S and pre-16S rRNA species. Similar effects have also been described previously for *Escherichia coli* RNase III mutants. Processing of the 5.6-kb precursor was independent of protein synthesis, while the cleavage of 23S rRNA to generate 16S and 14S rRNA required protein synthesis. We identified a DNA fragment of the wild-type *R. capsulatus* chromosome that conferred normal processing of 5.6-kb rRNA and 23S rRNA when it was expressed in strain Fm65.

The bacterial 50S ribosomal subunit is generally composed of a 23S and a 5S rRNA molecule and ribosomal proteins. However, some bacterial species have ribosomes that do not contain an intact 23S rRNA species. In vivo fragmentation of 23S rRNA has been reported for some cyanobacteria (10), for *Agrobacterium tumefaciens* (38), for *Bdellovibrio bacteriovorus* (34), for *Salmonella* species (5, 43), and for the closely related bacteria *Rhodobacter sphaeroides* (33), *Rhodobacter capsulatus* (28), and *Paracoccus denitrificans* (29). The biological significance of 23S rRNA fragmentation is not understood.

Bacterial rRNA operons are transcribed into large precursor molecules often including tRNA sequences (23). These rRNA precursors are subsequently processed into the 23S, 16S, and 5S rRNA species. In *Escherichia coli*, two endoribonucleases that are involved in the processing of rRNA have been identified: RNase III and RNase E. RNase III cleaves double-stranded RNA regions with little sequence specificity (36, 37) and is responsible for the generation of 23S and 16S rRNA precursor molecules from the larger 30S precursor (4, 44). RNase III is also responsible for the excision of intervening sequences from the rRNA of *Salmonella* species, resulting in fragmentation of the 23S rRNA (5). The enzymes involved in the final maturation of 16S and 23S rRNA from pre-16S and pre-23S rRNA have not been characterized; however, these reactions are most likely catalyzed by exoribonucleases (23, 39). 5S rRNA is generated by cleavage of a 9S rRNA precursor that is catalyzed by the endoribonuclease RNase E (35). From comparison of different RNase E cleavage sites, the consensus sequence RAUUW (R = A or G; W = A or U) (14) has been postulated. RNase E cleaves single-stranded RNA regions with the maximal rate, when the cleavage site is preceded or followed by an mRNA hairpin-loop structure (14). No RNase has been isolated from *Rhodobacter* species until now, and none of the enzymes involved in rRNA maturation of *R. capsulatus* is known.

In *Rhodobacter* species, 23S rRNA is further processed to [16S] and [14S] rRNA species in vivo (33). The two *R. sphaeroides* chromosomes carry three *rrn* operons that have almost identical nucleotide sequences (12) and show strong homology to the *rrn* sequence of *R. capsulatus* (20), which is present in four copies on a single chromosome (15). The organization of the *R. sphaeroides rrn* operons is identical to the organization found in *E. coli*, with two tRNA genes in the 16S to 23S spacer region. An initiator methionine tRNA was identified immediately downstream of the 5S rRNA gene (12). Comparison of the secondary structures of the 23S rRNA molecules from *E. coli* and *Rhodobacter* species and the sizes of the cleavage products suggests that in *Rhodobacter* species, the cleavage of 23S rRNA occurs within an rRNA region forming a stem-loop structure that is present in *Rhodobacter* rRNA but is absent from *E. coli* rRNA (12, 18) (Fig. 1). The rRNA sequences forming these extra stem-loop structures in *R. sphaeroides* and *R. capsulatus* differ significantly from each other (Fig. 1).

Here, we describe the characterization of the *R. capsulatus* chemical mutant strain Fm65 (26), which was isolated because of its defect in bacteriochlorophyll synthesis. We found that strain Fm65 does not process the 23S rRNA. Our data indicate that altered processing of 23S rRNA of this strain is due to an independent mutation that results in reduced activity of an endoribonuclease. We have studied rRNA processing by pulse-chase experiments in the presence or absence of protein synthesis in a wild-type strain and in strain Fm65 and have isolated a DNA fragment from *R. capsulatus* that encodes a protein required for 23S rRNA cleavage.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. In this study, the *R. capsulatus* wild-type strain 37b4 (DSM938) and the mutant strain Fm65 (26), which was isolated after nitrosoguanidine mutagenesis because of its lack of bacteriochlorophyll, were used. *R. capsulatus* strains were grown in a minimal malate medium (11). Pulse-chase experiments were

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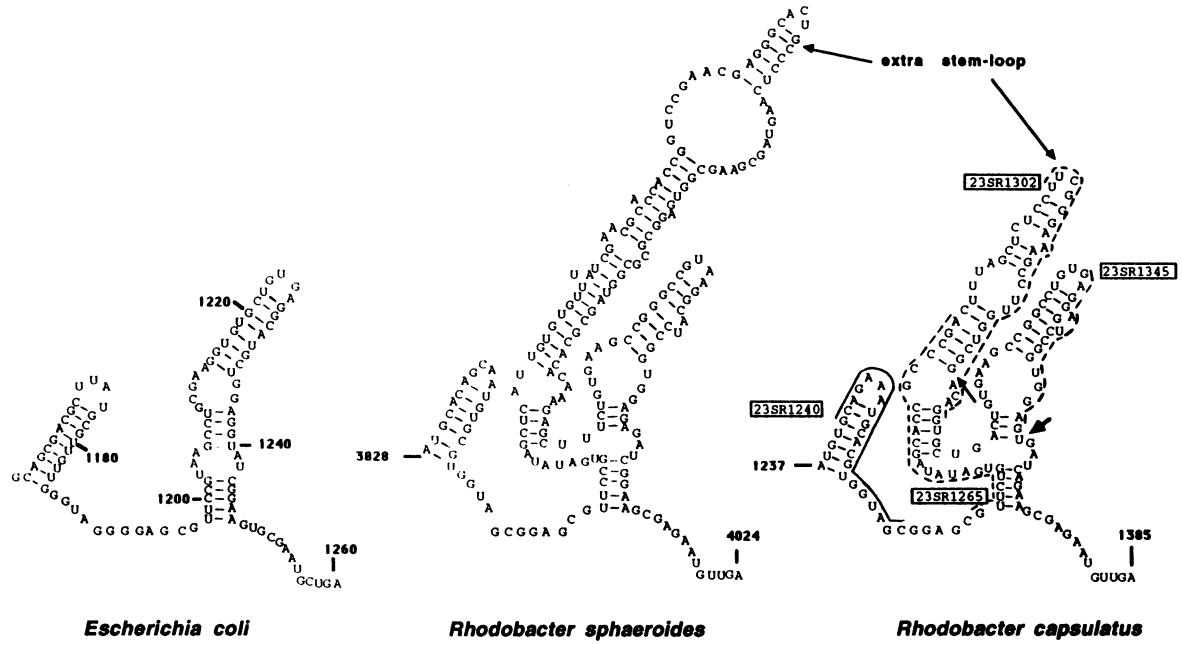

FIG. 1. Section of the 23S rRNA sequences and structures derived from *E. coli*, *R. sphaeroides*, and *R. capsulatus*. The numbering of the sequences is according to the data base. Lines drawn along the *R. capsulatus* rRNA sequence show primers used in this study. The primer that hybridized to the 14S rRNA of wild-type *R. capsulatus* is represented by a solid line. Primers that did not hybridize to RNA isolated from wild-type *R. capsulatus* are represented by dashed lines. The designations of the primers are shown in boxes close to their 3′ ends. The boldface arrow around position 1364 marks the approximate position of the major 5′ end of the wild-type 16S rRNA as mapped by primer extension analysis. The second arrow at position 1321 marks a minor 5′ end detected by the same technique.

also performed with *E. coli* BL321 (RNase III$^+$) and BL322 (RNase III$^-$) (40). The broad-host-range vector pRK290 (9) was transferred to *R. capsulatus* by triparental mating as described elsewhere (26). Tetracycline was used for cultivation of plasmid carrying strains at a final concentration of 2 μg/ml.

Isolation, amplification, and analysis of nucleic acids. Plasmid DNA was isolated from *E. coli* or *R. capsulatus* essentially as described by Birnboim and Doly (3). Chromosomal DNA from *R. capsulatus* was isolated according to the method described by Klug and Drews (26). RNA isolation, Northern (RNA) blotting, and hybridization were performed as described elsewhere (42). For half-life measurements, rifampin was added to the cultures at time zero at a final concentration of 200 μg/ml. Primer extension analysis of RNA 5′ ends has been described previously (25). The primers used for hybridizations or primer extension were 5′ CAGGAACCCTTGG ACTTTCG (23SR1425), 5′ ATCACTCCACCGGATCCCTC (23SR1345), 5′ CCTGTCCGACCAAGGCTTCCCGAA (23SR1302), 5′ TCGGGCGGTGCTATATCACAGAACG (23SR1265), and 5′ GCTACCACGTGCATTTCTGC (23SR 1240).

For construction of the *R. capsulatus* gene library, chromosomal DNA from strain B10 (31) was partially cut with *Eco*RI. *Eco*RI fragments from 1 to 20 kb were separated and isolated using low-melting-point agarose gels and were ligated to the broad-host-range vector pRK290 (9), which had been cut with *Eco*RI and treated with alkaline phosphatase (Boehringer, Mannheim, Germany). A total of 95% of the 4,000 clones that were pooled contained inserts with an average insert size of 7.5 kb.

Specific DNA fragments were amplified by PCR with chromosomal DNA from *R. capsulatus* Fm65 as a template essentially as described previously (24). The primers 23SR1345 and 23SR1240 were annealed to the DNA sequences corresponding to positions 1242 to 1262 and 1351 to 1372 of the rRNA as shown in Fig. 1 (sequence numbering refers to reference 20). The reaction was carried out with Vent-polymerase (Biolabs), and the buffer was supplied by the company. The reaction cycle was 40 s at 94°C, 1 min at 37°C, and 2 min at 72°C, and the reaction was run 30 times. The PCR fragments were cut with *Eco*RI and *Bam*HI, cloned into the corresponding sites of pGEM3Zf(−) (Promega), and sequenced with Sequenase (U.S. Biochemicals) using SP6 or T7 primers.

For Southern hybridization, the *E. coli rnc*-specific DNA fragment was radioactively labeled with [^{32}P]ATP using a nick translation kit (Boehringer) according to the supplier's instructions. A total of 3.5 × 10^6 cpm was used in a 22-ml hybridization mix containing 7× SSPE (20× SSPE is 3 M NaCl, 20 mM EDTA, and 200 mM NaH_2PO_4 × $7H_2O$ [pH 7.0]) and 0.1% sodium dodecyl sulfate (SDS). Hybridization was carried out at 37°C overnight. The filter was then washed for 30 min in 0.1% SSC (20× SSC is 3 M NaCl and 300 mM sodium citrate [pH 7.2])–0.5% SDS at 37°C.

In vivo labeling of RNA. A total of 6 to 15 μCi of [^{14}C]uridine (50 μCi/ml) was added to a 14-ml culture of *R. capsulatus* or *E. coli* in logarithmic growth phase. After a pulse of 1 min, unlabeled uridine was added to a final concentration of 1.5 mM, and samples of 2 ml were withdrawn at several time points. To inhibit protein synthesis, chloramphenicol was added to the cultures 20 min before the pulse at a final concentration of 200 μg/ml. The procedure for RNA isolation was the same as that used for unlabeled cultures. Formaldehyde gels were soaked in Amplify (Amersham) for 30 min prior to vacuum drying and exposure. Quantification was

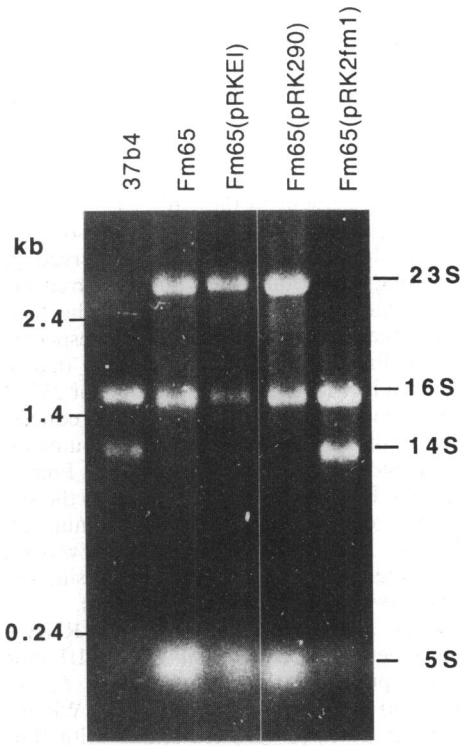

FIG. 2. Ethidium bromide-stained formaldehyde gel (1% agarose) loaded with 7 μg of total RNA isolated from different *R. capsulatus* strains per lane. The positions of marker bands (not shown) are indicated on the left.

performed by using a laser densitometer (PhosphorImager; Molecular Dynamics).

RESULTS

Lack of bacteriochlorophyll synthesis and altered rRNA processing in strain Fm65 are due to independent mutations. Strain Fm65 (26) is a chemical mutant of *R. capsulatus* 37b4 and excretes a bacteriochlorophyll precursor (absorbance maximum, 626 nm). Because of its lack of bacteriochlorophyll, Fm65 cannot form photosynthetic complexes. When total RNA isolated from strain Fm65 was run on a formaldehyde gel and stained with ethidium bromide, its rRNA pattern differed significantly from the rRNA pattern found in wild-type strains of *R. capsulatus* (Fig. 2). Like *E. coli*, strain Fm65 contains rRNA species of 3.3 kb (23S), 1.6 kb (16S), and 0.11 kb (5S) (Fig. 2). When RNA was isolated from wild-type strain 37b4, most of the 23S rRNA was cleaved to 1.6-kb (16S) and 1.3-kb (14S) rRNA species (Fig. 2). We found that strain Fm65 not only differed from the *R. capsulatus* wild-type strain with regard to bacteriochlorophyll synthesis and rRNA processing but also exhibited a chemotrophic growth rate (doubling time [semiaerobic incubation], 210 ± 10 min) that was significantly lower than the growth rate measured for wild-type cells (doubling time [semiaerobic incubation], 135 ± 5 min).

To determine whether the lack of bacteriochlorophyll and altered rRNA processing in Fm65 were due to the same mutation, we conjugationally transferred plasmids of a partial *Eco*RI gene bank of *R. capsulatus* wild-type B10 into strain Fm65. We selected colonies of the characteristic wild-type color, red. All 12 colonies that were tested carried plasmids with 4.5-kb *Eco*RI inserts. By cross-hybridization and restriction mapping, this *Eco*RI fragment was shown to be identical to the *Eco*I fragment (41) of plasmid pRPS404 (32) that carries a cluster of photosynthesis genes of *R. capsulatus* (data not shown). The *Eco*I fragment of pRPS404 carries the *bchF* gene encoding 2-vinyl bacteriochlorophillide hydratase and the *bchBN* genes encoding subunits of protochlorophyllide reductase (6). The pRK290 (9) derivative carrying the *Eco*I fragment was named pRKEI.

Strain Fm65(pRKEI) showed the same altered rRNA pattern (23S and 16S) (Fig. 2) and the same doubling time (215 ± 5 min) as its parental strain, Fm65. Thus, lack of bacteriochlorophyll synthesis and altered rRNA processing are due to independent mutations present on the chromosome of strain Fm65.

Determination of the 23S rRNA processing region in *R. capsulatus*. rRNA sequences of *R. capsulatus* and *R. sphaeroides* are highly homologous (12, 20), and a very similar structural organization of *Rhodobacter* rRNA and *E. coli* rRNA has been proposed elsewhere (18). However, there are stretches of about 110 nucleotides in the rRNA sequence of *R. sphaeroides* and of about 65 nucleotides in the rRNA sequence of *R. capsulatus* that have no corresponding nucleotides in the *E. coli* rRNA sequence. These nucleotides form an extra stem-loop structure (18) (Fig. 1). On the basis of the sizes of cleavage products and hybridization to specific oligonucleotides, Dryden and Kaplan proposed that cleavage of the *R. sphaeroides* 23S rRNA occurs in this region (12). To define the region of 23S rRNA processing in *R. capsulatus* more precisely, we hybridized total RNA isolated from the *R. capsulatus* strains 37b4 (wild type) and Fm65 (no 23S rRNA processing) to different oligonucleotides (Fig. 1) and performed primer extension analysis.

The oligonucleotide 23SR1240 (Fig. 1) showed hybridization to the 23S rRNA band of strain Fm65 and to the [14S] rRNA band of strain 37b4, whereas oligonucleotide 23SR1425 (data not shown) showed hybridization to the 23S rRNA band of strain Fm65 and the 16S rRNA of strain 37b4 (data not shown). This proves that the site of processing of 23S rRNA from *R. capsulatus* is localized between the sequences detected by these two oligonucleotides. When oligonucleotide 23SR 1345, 23SR1302, or 23SR1265 (Fig. 1) was used, only the 23S rRNA band of strain Fm65 showed strong hybridization (data not shown). We conclude that most of the rRNA sequence homologous to these oligonucleotides has been removed from the 23S rRNA during processing into [14S] and [16S] rRNA molecules. To determine the cleavage or processing sites more precisely, we performed a primer extension analysis with oligonucleotide 23SR1425 as the primer and total RNA isolated from wild-type strain 37b4 as the template. The major 5′ end detected with RNA isolated from strain 37b4 is indicated by the boldface arrow in Fig. 1. This 5′ end was not detected with RNA isolated from strain Fm65. In addition, a faint 5′ end was found with RNA isolated from strain 37b4 that maps within the extra stem-loop of the *R. capsulatus* 23S rRNA (indicated by a weak arrow in Fig. 1). This 5′ end was not detectable with RNA isolated from strain Fm65.

The sequence of the extra stem-loop of the 23S rRNA is identical in strain Fm65 and the wild-type strain 37b4. The higher stability of 23S rRNA in strain Fm65 could be due either to altered rRNA sequence or to altered RNase activity. Since the *R. capsulatus* chromosome carries four copies of the *rrn* operon (15), it is unlikely that a mutated rRNA sequence is responsible for the lack of 23S rRNA processing. To completely exclude this possibility of an altered sequence at the 23S rRNA cleavage site, we amplified the DNA sequence that corresponds to the extra stem-loop of *R. capsulatus* 23S

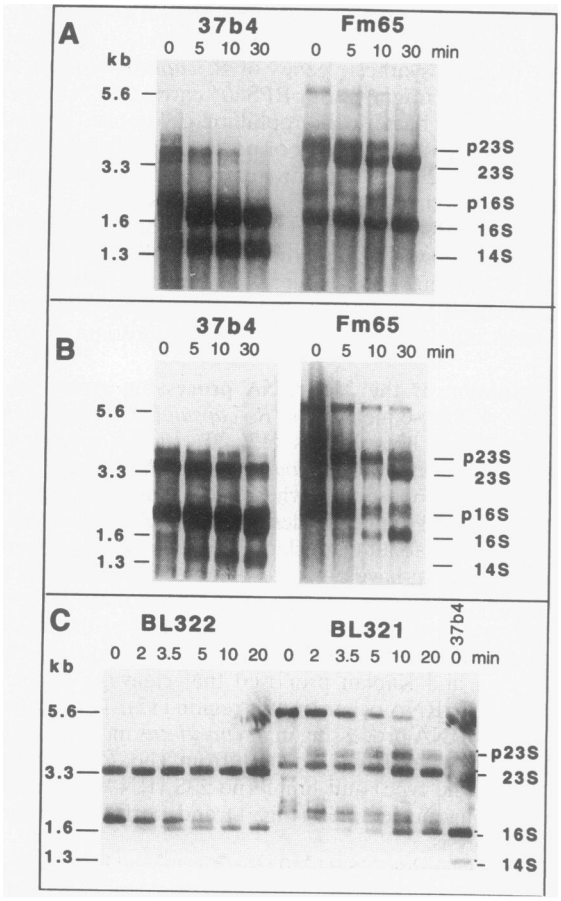

FIG. 3. Autoradiographs of Northern blots of total RNAs from *R. capsulatus* (A and B) and *E. coli* (C) labeled in vivo with [^{14}C]uridine. (A and C) Pulse-chase labeling in the absence of chloramphenicol; (B) pulse-chase labeling in the presence of chloramphenicol.

rRNA using primers 23SR1240 and 23SR1345 (Fig. 1). In *R. sphaeroides*, all three rRNA operons have almost identical sequences (12). Therefore, the primers allow amplification from all rRNA operons. The amplified DNA segments of 130 nucleotides were cloned into pGEM3zf(−) (Promega), and 10 of these clones were sequenced in both directions by using T7 or SP6 specific primers. The sequences of all 10 inserts were identical to the rRNA sequence published previously (20). We conclude that the lack of 23S rRNA processing in strain Fm65 is not due to alteration of the sequence of the extra stem-loop of the *R. capsulatus* 23S rRNA.

In vivo studies of rRNA processing in wild-type *R. capsulatus* and mutant strain Fm65. When total RNA from the wild-type strain of *R. capsulatus* is analyzed on formaldehyde gels, almost no 23S rRNA can be detected and no larger precursor molecules are visible (Fig. 2). We performed in vivo pulse-chase experiments with strain 37b4 and strain Fm65 using [^{14}C]uridine to detect rRNA precursor molecules and to monitor the processing of these precursors. With RNA isolated from strain 37b4 directly after the pulse, most of the radioactivity was present in diffuse bands covering RNA sizes of 3.3 to 3.4 kb and of 1.8 to 2.0 kb. In addition, faint bands of 3.5, 1.7, and 1.4 kb were visible (Fig. 3A). This suggests that the putative primary rRNA precursor is processed to 23S rRNA and to [14S] rRNA and [16S] rRNA precursors during the 1-min pulse. The 3.3-kb (23S) and the 1.8- to 2.0-kb bands disappeared almost completely within the first 10 min of the chase. At the same time, the intensities of a 1.6-kb (16S rRNA) and a 1.3-kb (14S rRNA) band increased. After 30 min of chase, no 3.3-kb band was detectable, and twice as much 1.6-kb rRNA as 1.3-kb RNA was present (Fig. 3A).

In strain Fm65, we detected bands of about 5.6, 3.4, 3.3, 2.1, and 1.7 kb immediately after the pulse, with most of the radioactivity being present in the 5.6-, 3.4-, and 1.7-kb bands (Fig. 3A). The presence of a 5.6-kb precursor rRNA in *R. capsulatus* was thus proven, while the observed pattern of rRNA processing during the 1-min pulse differed significantly in wild-type strain 37b4 and strain Fm65. The fact that the larger rRNA precursors of 5.6 and 3.4 kb, respectively, were more stable in Fm65 than in 37b4 indicates that an RNase activity responsible for the cleavage not only of 23S rRNA but also of the even larger precursor molecules is reduced in strain Fm65. After 10 min of chase, significant amounts of 5.6- and 3.4-kb RNA could still be isolated from strain Fm65. After 30 min of chase, the RNA pattern was identical to the steady-state pattern, as observed by ethidium bromide staining. During the 30 min of chase, the size of the 1.7-kb band was reduced to about 1.6 kb (Fig. 3A), indicating the processing of pre-16S-rRNA precursors as described for *E. coli* (16).

Since impeded processing of the primary rRNA transcript has also been described for *E. coli* RNase III mutants, we performed the pulse-chase experiment with *E. coli* BL322 (RNaseIII$^+$) and BL321 (RNaseIII$^-$) (40). When we compared the rRNA patterns of these strains with those for *R. capsulatus* strains, we found striking similarities between BL321 (Fig. 3C) and Fm65 (Fig. 3A). Both strains showed the 5.6-kb precursor and slow turnover of a pre-23S rRNA to mature 23S rRNA (Fig. 3C).

Processing of 23S rRNA in *R. capsulatus* requires protein synthesis. In *E. coli*, the processing of the primary rRNA precursor molecule takes place in the absence of protein synthesis, whereas the maturation of 16S rRNA requires the binding of ribosomal proteins (23). To monitor rRNA processing in *R. capsulatus* in the absence of protein synthesis, we performed pulse-chase experiments with strain 37b4 and strain Fm65 after the addition of chloramphenicol.

The RNA isolated from each strain directly after the pulse showed the same pattern of bands as the RNA isolated directly after the pulse in the absence of chloramphenicol (Fig. 3B and A). This finding suggests that the processing of the 5.6-kb precursor molecule is independent of protein synthesis in *R. capsulatus*. The fact that the 5.6-kb band visible in RNA preparations from strain Fm65 disappeared with the same kinetics in the presence or absence of chloramphenicol (Fig. 4A) supports this view. However, the processing reactions following the initial 5.6-kb precursor processing were much slower in the presence of chloramphenicol in both strain 37b4 and Fm65. Strain 37b4 showed significant amounts of 23S rRNA even after 30 min of chase (Fig. 3B and 4B). In the presence of chloramphenicol, the shortening of putative 16S rRNA precursor (1.7 kb) to its mature size (1.6 kb) was much slower in strains 37b4 and Fm65 (Fig. 3A and B). This suggests that both 23S rRNA cleavage and 16S rRNA maturation require protein synthesis in *R. capsulatus*.

Isolation of a DNA fragment from *R. capsulatus* that complements 23S rRNA processing in *trans*. Strain Fm65(pRKEI), as well as the parental strain Fm65, shows the same low growth rate compared with the wild-type strain, although it carries the wild-type bacteriochlorophyll genes. To determine whether the reduced growth rate of both strains is a result of the altered rRNA processing, we transferred the plasmids of an *R. capsulatus* gene library into strain Fm65. About 1 in 400 transcon-

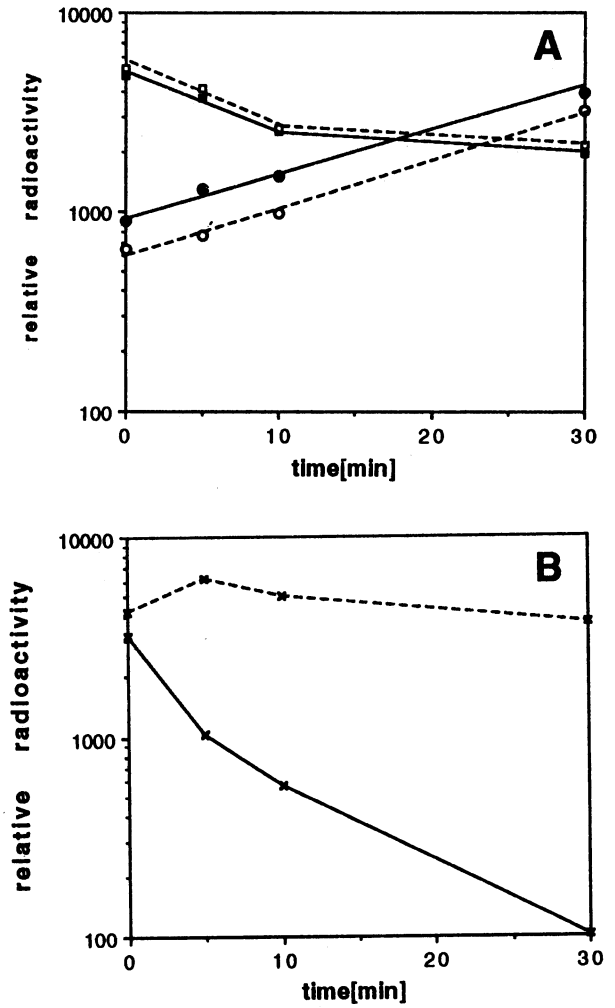

FIG. 4. Quantification of bands visible on autoradiographs shown in Fig. 3 using a laser densitometer. (A) Relative radioactivities of the 5.6-kb rRNA band of strain Fm65 in the absence (■) and presence (□) of chloramphenicol and of the 23S rRNA band in the absence (●) and presence (○) of chloramphenicol; (B) relative radioactivities of the 23S rRNA band of wild-type 37b4 in the absence (——) and presence (– –) of chloramphenicol.

jugants showed a significantly higher growth rate. We isolated the plasmids of 12 clones with growth rates similar to that of wild-type cells. All 12 plasmids carried a 6.5-kb *Eco*RI fragment, and three of the plasmids carried additional *Eco*RI fragments with smaller sizes (data not shown). When rRNA was isolated from six of these clones and analyzed on formaldehyde gels, all six clones showed processing of 23S rRNA to [16S] and [14S] rRNA species (data not shown). Plasmid pRK2fm1, isolated from 1 of the 12 clones, was transferred into *E. coli* by transformation, and the resulting *E. coli* clone was mated to strain Fm65. As a control plasmid, pRK290 lacking any *R. capsulatus*-specific sequences was transferred into strain Fm65. The transconjugants carrying plasmid pRK2fm1 showed significantly faster growth (doubling time, [semiaerobic cultivation], 130 ± 5 min) than the Fm65 (pRK290) transconjugants (doubling time, 210 ± 5 min). Total RNA isolated from Fm65(pRK290) and from Fm65 (pRK2fm1) was analyzed on formaldehyde gels. RNA isolated from Fm65(pRK290) showed 23S rRNA and 16S rRNA bands identical to the pattern found in strain Fm65 (Fig. 2). RNA isolated from Fm65(pRK2fm1) showed 16S rRNA and 14S rRNA bands identical to those for the RNA isolated from wild-type strain 37b4 (Fig. 2). These data prove that the expression of pRK2fm1 restores the wild-type RNase activities in strain Fm65 and that the reduced growth rate of this strain is due to the rRNA processing defect. We have subcloned several fragments of plasmid pRK2fm1 into the conjugative plasmid pRK415 (21) and have analyzed the rRNA patterns of Fm65-derived clones carrying the various plasmids. A 4-kb *Hin*dIII fragment of pRK2fm1 was sufficient to restore 23S rRNA cleavage.

The pulse-chase experiments had suggested that an enzyme resembling *E. coli* RNase III may be affected by the mutation in strain Fm65. Therefore, we hybridized the *rnc* gene (encoding RNase III) of *E. coli* (isolated from plasmid pTD101 [8, 30]) with plasmid pRK2fm1. A very weak hybridization signal suggested only very limited homology of the *R. capsulatus* sequence cloned in pRK2fm1 with the *E. coli rnc* gene (data not shown).

We also tried to test whether the *E. coli rnc* gene could functionally complement the mutation of the *R. capsulatus* strain Fm65. Since most *E. coli* promoters are not recognized by *R. capsulatus* polymerases, we cloned the *E. coli rnc* gene (0.9-kb *Pst*I-*Bam*HI fragment of plasmid pTD101) behind the *lac* promoter of plasmid pRK415 (21), creating plasmid pRK4rne, which was transferred into *Rhodobacter* species by conjugation. Genes cloned behind the *lac* promoter can be expressed in *R. capsulatus* (35a). RNA isolated from strain Fm65(pRK4rne) showed the same pattern as RNA isolated from strain Fm65 (data not shown).

DISCUSSION

Analysis of the chemical mutant *R. capsulatus* Fm65 revealed that it carries at least two independent mutations. Lack of bacteriochlorophyll synthesis could be restored by expression of the *bchFBN* genes in *trans*. Cleavage of 23S rRNA and wild-type growth rates were restored by transferring plasmid pRK2fm1 into strain Fm65. Strain Fm65 served as a tool for the study of rRNA processing in *R. capsulatus* because of its lack of 23S rRNA cleavage.

Our pulse-chase data show that the initial steps in rRNA processing in *R. capsulatus* are very similar to those in *E. coli* (23). By using pulse-chase labeling, the processing of pre-16S rRNA molecules to mature 16S rRNA in both the wild type and the Fm65 strain could be monitored. The presence of a 5.6-kb rRNA precursor was observed only in strain Fm65 but not in wild-type cells. This resembles the very rapid processing of the primary rRNA transcript in *E. coli* that takes place even before transcription of the entire operon is completed (1, 22). The mutation present in strain Fm65 resulted in a remarkable slowdown of the processing of the 5.6-kb rRNA precursor and in the inhibition of the cleavage of 23S rRNA, suggesting that both processes are catalyzed by the same enzymatic activity. A similar slowdown of the processing of the primary rRNA transcript was observed in *E. coli* RNase III mutants. The processing of the primary rRNA transcript was therefore attributed to the action of RNase III (see references 4 and 44 and the present work), which is known to cut RNA in double-stranded regions (36).

Normal amounts of 5S rRNA were detected in strain Fm65, suggesting that enzymes involved in the formation of 5S rRNA were not affected by this mutation. 5S rRNA formation in *E. coli* requires endoribonuclease E (17), which cuts single-stranded rRNA regions (14). Our data suggest that an enzy-

matic activity resembling that of RNase III catalyzes both the processing of the 5.6-kb rRNA precursor and the cleavage of the 23S rRNA of *R. capsulatus*. This is in agreement with earlier findings that attributed the removal of intervening sequences from the 23S rRNA in *Salmonella* species to RNase III activity (5). A reduction in the activity of an RNase III-like enzyme could also account for the prolonged growth rate in strain Fm65, since the same effect is observed in *E. coli* strains with reduced RNase III activity (13). However, we cannot exclude the possibility that the mutation in strain Fm65 affects a gene that is required for the activity of two different RNA-processing enzymes.

It was proposed that the cleavage of the 23S rRNA of *R. sphaeroides* occurs at an rRNA sequence not present in *E. coli* which can potentially form a stem-loop structure (Fig. 1) (12). It is evident from our hybridization experiments that the [16S] and [14S] rRNA species in wild-type cells do not originate from just a single cleavage at a specific site but rather are the products of the precise removal of a short stretch of rRNA from the 23S rRNA. We suggest that processing of 23S rRNA occurs either by single-site cleavage and subsequent degradation, reducing the size of the cleavage products, or by at least two distinct endonucleolytic cuts. This assumption is supported by the primer extension data showing that the majority of the 5′ ends of the [16S] rRNA of *R. capsulatus* maps around position 1363, whereas a minor part of [16S] rRNA molecules extends to position 1320 (Fig. 1). The mechanism of 23S rRNA processing in *R. capsulatus* appears to resemble that of the 23S rRNA processing in *Salmonella typhimurium*, in which an intervening secondary-structure element was shown to be removed by staggered RNase III cuts (5). The extra stem-loop structures of *R. capsulatus* and *R. sphaeroides* have strong similarity to secondary structures that are recognized by RNase III in *E. coli*, i.e., stems of less than 20 consecutive bp that contain unpaired bases forming internal loops (7). A sequence that fits the consensus sequence proposed for RNase III cleavage in *E. coli* by Krinke and Wulff (27) is not present within these *Rhodobacter* stem-loops. However, even in *E. coli*, functional RNase III sites can differ significantly from this consensus sequence (7). There is also no significant sequence homology to the secondary structures that are removed from 23S rRNA in *Salmonella* species. One of the 23S rRNA intervening sequences identified in *S. typhimurium* and *Salmonella arizonae* is in a position similar although not identical to that of the *Rhodobacter* intervening sequences described here.

Addition of chloramphenicol, an inhibitor of procaryotic protein synthesis, had different effects on the various steps of rRNA processing in *R. capsulatus*. As reported for *E. coli* (2, 19), the processing of the 5.6-kb rRNA precursor was also very fast in the absence of protein synthesis, whereas the maturation of 16S rRNA was clearly slowed down. The cleavage of 23S rRNA, on the other hand, was clearly dependent on protein synthesis. This is in agreement with earlier results showing that the cleavage of 23S rRNA in *R. capsulatus* takes place only while the molecule is part of a 45S ribonucleoprotein precursor particle (33). The difference in response to protein synthesis does not invalidate our hypothesis that processing of the 5.6-kb rRNA and cleavage of 23S rRNA are catalyzed by the same enzyme. It is conceivable that binding of ribosomal proteins is required for a structural organization of the 23S rRNA necessary for subsequent recognition by the processing endonuclease. This may also be the reason for the lower processing rate of 23S rRNA compared with the cleavage rate of the primary rRNA transcript. This aspect makes the prediction of potential RNase cleavage sites based on sequence analysis and RNA secondary-structure analysis very difficult. Fragmentation of 23S rRNA in *Salmonella* species was reported to occur in the presence of chloramphenicol as well as in vitro (5), indicating that protein binding is not a prerequisite for subsequent rRNA cleavage. Thus, 23S rRNA fragmentation in *Salmonella* species and *Rhodobacter* species is due to mechanisms that resemble each other but that are not identical. To our knowledge, there are no other examples cited in the literature for a protein synthesis-dependent cleavage of 23S rRNA by an endonucleolytic activity. Our results support the hypothesis of Burgin et al. (5) that the 23S rRNA intervening sequences were independently acquired by different bacterial species relatively recently in evolution.

The effect of impeded rRNA processing on the growth rate of *R. capsulatus* enabled us to isolate a DNA fragment from wild-type *R. capsulatus* that restores 23S rRNA processing when it is expressed in strain Fm65. Most likely, this DNA segment carries the putative RNase III gene, and sequencing will allow us to compare the encoded protein with RNases identified in *E. coli*. In addition, we will be able to recombine a mutated RNase gene into a wild-type strain and to study the effect of the mutation in a defined genetic background instead of using the chemical mutant Fm65. These future experiments will provide more insights not only into the biological relevance of 23S rRNA processing in *R. capsulatus* but also into more general aspects of bacterial rRNA processing and its regulation.

ACKNOWLEDGMENTS

We thank P. March and M. Inouye for plasmid pTD101, W. Studier for *E. coli* BL321 and BL322, R. Gutell for providing the *R. capsulatus* rRNA secondary structure, and Andreas Jäger for technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft (Kl 563/4-1), by the Bundesministerium für Forschung und Technologie, and by the Fonds der Chemischen Industrie.

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