A functional plasmid-borne rrn operon in soil isolates belonging to the genus *Paracoccus*

Anja Battermann, Claudia Disse-Krömker and Brigitte Dreiseikelmann

University of Bielefeld, Fakultät für Biologie, Mikrobiologie/Gentechnologie, Postfach 10015, 33501 Bielefeld, Germany

Plasmid analysis of isolates from a small *Paracoccus* population revealed that all 15 representatives carried at least one endogenous plasmid of 23 or 15 kb in size, in addition to further plasmids of different sizes. It was shown by restriction analysis and hybridization that the 23 and 15 kb plasmids from the different isolates were identical or very similar to each other. By partial sequencing of pOL18/23, one of the 23 kb plasmids, a complete rrn operon with the structural genes for 16S, 23S and 5S rRNA, two genes for tRNAleu and tRNAala within the spacer between the 16S and 23S rRNA genes, and a final tRNAfMet at the end of the operon were discovered. Expression of a green fluorescent protein gene (*gfp*) after insertion of a DNA fragment from the region upstream of the rRNA genes into a promoter-probe vector demonstrated that the rrn promoter region is functional. The rrn operon encoded by plasmid pOL18/23 is the first complete rrn operon sequenced from a strain of the genus *Paracoccus*, and only the second example of an rrn operon sequenced on a small plasmid.

INTRODUCTION

The genus *Paracoccus*, which, at the time of writing, includes 18 validly described species, belongs to the *Rhodobacter* group of the *ß-Proteobacteria* (Baj, 2000; see also http://www.bacterio.cict.fr/). Most studies on paracocci have focused on their versatile metabolism which is reflected by a high potential for adaptability to various carbon and energy sources. Little work has been done so far on the genetics of *Paracoccus* spp., but since the discovery of multiple chromosomes among the *ß-Proteobacteria* (Steinrücke & Ludwig, 1993; Jumas-Bilak et al., 1998; Mackenzie et al., 1999) interest in this field has increased. One member of this group of organisms with more than one chromosome is *Paracoccus denitrificans*, which contains three DNA species with sizes of 1·83, 1·16 and 0·67 Mb, respectively (Winterstein & Ludwig, 1998). The discovery of multiple DNA species within micro-organisms raised the question of the precise definition of the term ‘chromosome’ and of the distinction between plasmids and chromosomes. The main characteristics used for the definition of a chromosome are its size, its copy number and its essentiality, the latter of which is reflected by the presence of housekeeping genes such as the rrn operons.

Few publications have analysed plasmid abundance in members of the *ß-Proteobacteria*, but the available literature suggests that the various strains carry a large number of endogenous plasmids, including small and large plasmids up to megaplasmids. Analysis of natural strains of *Rhodobacter sphaeroides* has revealed that they have at least one and can carry up to six different plasmids (Fornari et al., 1984). Plasmid profiles of 53 isolates from a *Rhizobium leguminosarum* population demonstrated that 51 isolates contained between two and seven plasmids (Rigottier-Gois et al., 1998). Baj et al. (2000) have analysed the plasmid patterns of 11 species of the genus *Paracoccus* and have identified over 30 plasmids. Some of these plasmids have been characterized regarding their replication, but there have been no studies on further genes carried by the plasmids (Bartosik et al., 2002a, b, c). It has been suggested that the plasmids may contribute to the metabolic diversity of the *ß-Proteobacteria* and thus of the paracocci (Bartosik et al., 2002c).

In this study, we present data from a small population of *Paracoccus* spp., of which all representatives carry – among other plasmids – a 23 or 15 kb plasmid that encodes a functional rrn operon. Up until now, only one other example of a plasmid-encoded rRNA operon (pBM400, 53 kb) has been reported; this plasmid was isolated from *Bacillus megaterium* (Kunnimalaiyaan et al., 2001). On their chromosomes, most bacteria carry multiple rRNA operons with up to 15 copies. The copy number of rrn operons of the paracocci has yet to be determined. The closely related species *Rhodobacter sphaeroides* contains three copies of its rrn operons (Dryden & Kaplan, 1990). Although the expression of rRNA depends more on a complex regulation than on the gene dosage, it is assumed that multiple copies

Abbreviation: GFP, green fluorescent protein.

The GenBank accession number for the sequence reported in this article is AY312056.
of rrr operons are necessary for a rapid answer to changing physiological conditions. Condon et al. (1995a) have reported that Escherichia coli requires all of its seven rrr operons for optimal adaptation to changing physiological conditions. Klappenbach et al. (2000) have demonstrated a direct correlation in soil microcosms between the rRNA operon copy number and the time required by soil bacteria to form colonies in response to resource availability.

METHODS

Bacterial strains, plasmids and growth conditions. The 15 Paracoccus strains used in this study were members of a community of denitrifying bacteria consisting of 3000 isolates. The community was isolated as follows. A 0-7 g sample of an agricultural soil (Füsing #2, Schleswig-Holstein, Germany) was suspended in 2 ml of 30 mM phosphate buffer (pH 7-2). Dilutions of the soil suspension were plated onto a solid minimal medium containing maldose and nitrate. Incubation was done at 28°C under anaerobic conditions. Well-separated colonies were purified by streaking onto three different plates. Each plate contained 50 mM phosphate buffer (pH 7) or 0 5 TBY plates at 28°C under aerobic conditions. Isolation of the community was done in co-operation with the group of W. Wackernagel (Oldenburg, Germany). The isolates were named OL (isolated in Oldenburg) and BI (isolated in Bielefeld). Plasmids of the isolates were named pOL or pBI, respectively, because the genus or species of the strains have not yet been determined. E. coli DH5α supE44 ΔlacZΔM15 hisD17 recA1 endA1 gyrA96 thi-1 relA1 (Clontech) was used as a host for hybrid plasmids generated with the vector pUC18. E. coli S117.1 RP4-2-Tc::Mu-Km::Tn7 (Simon et al. 1983) was used for mobilization of pBBR1-GFP derivatives. The promoter- vector probe pBBR1-GFP was kindly provided by S. Köhler (Montpellier, France).

Mobilization of pBBR1-GFP. For mobilization of pBBR1-GFP derivatives, transformed E. coli S17.1 cells (CaCl2 transformation procedure) were used as a donor immediately after the expression period. Samples were spotted onto cellulose nitrate filters (1 cm diameter, pore size 0-45 μm; Sartorius) on 0 5 TBY plates together with exponential-phase cells of rifampicin-resistant P. denitrificans (DSM 4133) as a recipient. Mating took place for 15 h at 30°C. Thereafter, bacteria were washed off the filters and plated onto selective media containing rifampicin (100 μg ml–1), ampicillin (100 μg ml–1) and chloramphenicol (50 μg ml–1). Green fluorescent protein (GFP)-producing cells were monitored under UV light (365 nm).

DNA manipulations. Standard recombinant DNA procedures were used (Sambrook et al., 1989). Plasmid DNA was isolated according to the method of Birnboim & Doly (1979). For qualitative analysis, plasmids were isolated from the bacterial community and from the Paracoccus strains according to the method of Ramos-Gonzalez et al. (1991). Plasmid sizes were estimated by comparison of their electrophoretic mobilities with those of plasmids with known sizes (e.g. RP4, SAL, F). Total DNA was isolated with the High Pure PCR Template Preparation Kit (Roche). For Southern hybridization (Sambrook et al., 1989), probes were labelled with digoxigenin using the DIG DNA labelling and Detection Kit (Roche). Hybridization was performed at 62°C. Amplification of a 16S rDNA probe was performed with the primers 27F and 1385R (Lane, 1991), and amplification of a 23S rDNA probe was performed with primers P-23-1 (**GAGGGCCGATGGAGCTGTATCGT-3′** and P-23-2 (**CCTTAAAATGGTTGTTCCTCAAGCGCC-3′**). Amplification was done with Taq polymerase (Perkin Elmer) in a gradient-thermocycler (Stratagene).

DNA sequencing and analysis. A Sau3A fragment of about 10 kb in size of pOL18/23 was inserted into pUC18. DNA of the hybrid plasmid was isolated from E. coli DH5α and the nucleotide sequence of the insert was partially determined by primer walking. The nucleotide sequence was determined in the IIT Laboratory at the University of Bielefeld (Germany), analysed with the help of DNASTAR and CLONE software and compared to existing data with the BLAST program provided by NCBI (Altschul et al., 1990).

RESULTS AND DISCUSSION

Analysis of plasmids in a small Paracoccus population

We have isolated a community of denitrifying bacteria consisting of 3000 isolates from an agricultural soil. All members of the community were screened for large plasmids with the plasmid isolation method described by Ramos-Gonzalez et al. (1991). By using this method, one or more plasmids could be identified in 94 strains. All isolates of the community were analysed by amplified rDNA restriction analysis (ARDRA) with three enzymes, and the 16S rDNA sequences of representatives of the main ARDRA groups were sequenced (A. Battmann, C. Disse-Krönker & B. Dreiseikelmann, unpublished data).

Among the members of the bacterial community, a small ARDRA group of 15 isolates was identified that showed a strikingly high plasmid content. While the overall plasmid incidence of the community was about 3%, all isolates of this group carried at least one endogenous plasmid. The 15 strains were all Gram-negative and cocoid. 16S rDNA sequencing of all 15 isolates revealed that these strains represent a population belonging to the genus Paracoccus (data not shown).

The Paracoccus isolates carried a large number of different plasmids with sizes ranging from about 5 kb to larger than 100 kb (Fig. 1). Most of the individual isolates contained more than one plasmid. Twelve isolates carried a plasmid of about 23 kb in size. The remaining three isolates carried a plasmid of about 15 kb in size.

For comparison of the 23 and 15 kb plasmids, a restriction analysis was performed using the enzymes SacI, EcoRI, BamHI and HindIII. An example of the HindIII digestion of seven plasmids is shown in Fig. 2(a). The restriction patterns of the 23 and 15 kb plasmids, which are not shown in Fig. 2, were very similar. The pattern of DNA restriction fragments showed a significant degree of similarity between the 23 and 15 kb plasmids, especially among the DNA fragments smaller than 3 kb. The analysis of larger fragments was difficult due to contamination of the 23 kb plasmid DNA by other plasmids.

The plasmid preparation of pOL18/23 done according to the method of Birnboim & Doly (1979) was the only one that did not show DNA restriction fragments other than those of the 23 kb (or 15 kb) plasmid (Fig. 2a, lane 7) on
a stained agarose gel. The other plasmid of strain OL18 (Fig. 1, lane 13) is too large to be isolated in significant amounts by this method. The plasmid DNA of pOL18/23 was labelled with digoxigenin and hybridized against the plasmid preparations from the other isolates. An example of a Southern blot is shown in Fig. 2(b). The blot shows clearly that the pattern of HindIII digestion is identical for plasmids pOL18/23, pOL11/23, pBI237/23 and pBI161/23. Because the plasmids are all the same size the additional DNA fragments in the agarose gel were probably derived from the other plasmids of the isolates (Fig. 1). There are some minor differences in the digestion patterns of plasmids pBI1058 and pBI1099; for example, in the case of pOL1058 one restriction site for HindIII between DNA fragments g and e is missing (Fig. 2a). Notwithstanding a few differences in the digestion pattern, all HindIII DNA fragments hybridized with pOL18/23. A cross-hybridization with the other endogenous plasmids was not detected.

The restriction pattern and the hybridization showed that the 15 kb plasmid is a derivative of the 23 kb plasmid. There are several HindIII fragments of the same size as in the 23 kb plasmids and all fragments hybridized with pOL18/23. The same results were obtained for the plasmids of the isolates not shown in Fig. 2 and for restriction enzymes EcoRI and BamHI (data not shown).

Sequence analysis of the rrn operon of pOL18/23

Shotgun cloning of EcoRI and HindIII DNA fragments of pOL18/23 followed by sequencing with standard primers for inserts in pUC vectors revealed parts of 16S and 23S rRNA genes. This was unexpected as the only other example of a plasmid-borne rrn operon described in the literature is the 53 kb plasmid pBM400 from B. megaterium (Kunnimalaiyaan et al., 2001). The rRNA sequences from pOL18/23 were localized on a SalI fragment of about 10 kb in size, which was inserted into pUC18. The nucleotide sequence of the whole rrn operon was determined by primer-walking sequencing including the upstream regulatory region. We found a complete operon with the genes for 16S rRNA, tRNA^{Ile}, tRNA^{Ala}, 23S rRNA, 5S rRNA and tRNA^{fMet} (Fig. 3). Genes are annotated according to the rrn operons of Rhodobacter sphaeroides (Dryden & Kaplan, 1990, 1993), as a whole rrn operon of a Paracoccus species has not yet been described. Comparison of the sequences with those of the databases shows the highest degree of identity to the 16S rRNA gene of Paracoccus aminophilus (96 %), to the 23S rRNA gene of P. denitrificans (97 %) and to the 5S rRNA of Paracoccus versutus (97 %). The species and the localization of the tRNAs are identical to those found in the three chromosomal rrn operons of Rhodobacter...
sphaeroides, which all have the same tRNA genes in the same order (Dryden & Kaplan, 1990). At the end of the rrn operon of pOL18/23, we found a hairpin structure which could represent a rho-independent transcriptional terminator (Lesnik et al., 2001).

Hybridization with probes from the rRNA genes verified that the rrn operon is present on the 23 and 15 kb plasmids of all 15 Paracoccus isolates (data not shown). The HindIII DNA fragments e to i indicated in Fig. 2 are present in both plasmids and represent a large part of the rrn operon including the 16S rRNA gene, the genes for tRNAIle and tRNAAla and part of the 23S rRNA gene (compare to Fig. 3).

Analysis of the promoter region upstream of the rrn operon

The promoter sequences of the Paracoccus group have not been sufficiently studied and a reliable consensus sequence has not yet been achieved. Baker et al. (1998) have aligned the known promoters from the Rhodobacter group of the \(\alpha\)-Proteobacteria and classified them into three groups (A, B and C). The promoters of the three rrn operons of Rhodobacter sphaeroides belong to the group A promoters. All three operons each have only one promoter and two (rrnA, rrnB) or one (rrnC) Fis-binding sites (Dryden & Kaplan, 1993). The distance between the promoters and the start of the mature 16S rRNA is about 350 nt.

We found a sequence that resembles a group A promoter upstream of the 16S rRNA gene of pOL18/23. This possible promoter sequence (P1) is located 350 bp upstream from the mature 16S rRNA and has the sequence TTGCGG-N15-CGTAAATA (Fig. 4). At the 5' end of this possible promoter there is a sequence (TGTGGTTTTTGCCTCT) that resembles a Fis-binding site, but it does not match exactly the consensus sequence for Rhodobacter spp. (Condon et al., 1995b). Further upstream there is an AT-rich region which may correspond to an UP element described for E. coli rrn operons.

---

Fig. 3. Organization of the rRNA genes within the rrn operon of pOL18/23. The accession number of the 6260 bp region is AJ312056. Comparison with sequences within the databases was performed by using the BLAST program (Altschul et al., 1990). The lower-case letters indicate the HindIII fragments shown in Fig. 2. The arrows below the genes indicate the positions of the probes used for the Southern hybridizations documented in Fig. 5.

Fig. 4. Physical map of the BamHI DNA fragment upstream from the mature 16S rRNA gene. The solid boxes indicate hypothetical regulatory sequences deduced from the nucleotide sequence. These are UP for an UP element described for E. coli rrn operons (Condon et al., 1995b), P1 and P2 for group A-like promoters of the Rhodobacter group, a probable Fis-binding site immediately upstream of the –35 region of P1 (Baker et al., 1998; Dryden & Kaplan, 1993) and boxA (nucleotides 657–665) which is involved in anti-termination (Condon et al., 1995b). There are additional Sau3AI sites within the BamHI DNA fragment which have not been shown in this figure. The table shows the detailed positions and sequences of the hypothetical regulatory regions.
operons (Condon et al., 1995b). Immediately following the possible promoter there is a stretch of 13 bps with perfect identity to the corresponding region of the *rrn* operons of *Rhodobacter sphaeroides*. Within this region there is a transcriptional start site (Dryden & Kaplan, 1993).

In the leader and spacer regions of the *rrn* operons of many eubacteria and archaea there are two regions called boxA and boxB which are involved in anti-termination (Berg et al., 1989). BoxA is a conserved sequence, (C/T)GCTCTT(T/-)A, while boxB is not a consensus sequence but a region of hyphenated dyad symmetry. A sequence (TGCTTTTTTG, nucleotides 657–665) that may represent a boxA sequence is present in the leader of the 16S rRNA gene of pOL18/23. A second sequence resembling a boxA sequence (TGGCTT, nucleotides 3005–3013) is found upstream of the 23S rRNA gene. There is no evidence for a boxB in the vicinity of boxA. The boxA and Fis sequences of pOL18/23 are located at similar positions to those of *Rhodobacter sphaeroides*. Nevertheless, both sequences deviate significantly from the consensus sequences even more than those of *Rhodobacter sphaeroides*, so it is questionable if they are functional.

To demonstrate an active promoter in front of the *rrn* operon of pOL18/23 experimentally, the 948 bp *Bam*HI fragment was isolated, partially digested with Sau3AI and ligated with the promoter-probe vector pBBR1-GFP that had been digested with *Bgl*II (Ouahrani-Bettache et al., 1999). Insertion of a promoter into this site allows the simultaneous expression of the genes encoding GFP (*gfp*) and chloramphenicol acetyltransferase (*cat*). *E. coli* S17.1 was transformed by the CaCl₂ procedure and after the expression period the hybrid plasmids were immediately transferred from the transformed *E. coli* cells to *P. denitrificans* by conjugation. Restriction analysis of plasmid DNA from 12 green-fluorescing chloramphenicol-resistant clones revealed that seven plasmids contained the *Bam*HI fragment and five plasmids contained a partial Sau3A fragment from position 504 to 948 (Fig. 4). All inserts showed the same orientation. This orientation suggests that the promoter which allows expression of *gfp* and *cat* is the promoter of the *rrn* operon. Sequence determination of the inserts of one representative of the two species of hybrid plasmids verified the data from the restriction analysis.

The Sau3AI cut site at position 504 is located in the spacer between the −35 and −10 region of the possible promoter P1, which means that there must be a second active promoter downstream of promoter P1. A speculative candidate may be the region from position 564 to 587 with the sequence TTGACG-N₁₃-TAAAAT.

### Additional *rrn* operons of *Paracoccus* strain OL18

The small size of 23 kb strongly suggests that pOL18/23 is a plasmid. According to its definition, a plasmid does not carry essential genes but, as *rRNA* genes are essential genes, it was necessary to show that the *rrn* operon of pOL18/23 is not the only copy in the cell. Attempts to cure strain OL18 of plasmid pOL18/23 by growing the bacterium at a raised temperature, with SDS or novobiocin or by electroporation were not successful. Therefore, it was necessary to demonstrate the existence of chromosomal copies of the *rRNA* genes directly. The PCR product of the 16S *rRNA* gene was labelled with digoxigenin and hybridized against *EcoRI* DNA fragments of a plasmid preparation of pOL18/23 that had been purified by CsCl/ethidium bromide gradient centrifugation. The PCR product was also hybridized against a preparation of total DNA from strain OL18 (Fig. 5a). There is one *EcoRI* site within the 16S *rDNA* and no further site within the operon (Fig. 3). As expected, the probe hybridized with two *EcoRI* DNA fragments of the plasmid (Fig. 5a, lane 1). Hybridization with total DNA clearly shows three additional signals (a fourth signal may be immediately below the 1481 bp DNA fragment; Fig. 5a, lane 2). Amplification of the 16S *rRNA* using the total DNA of strain OL18 as template resulted in a single PCR product, suggesting that the chromosomal 16S *rRNA* gene does not differ significantly from the plasmid-encoded gene.

Digestion of the PCR product with several restriction enzymes (including *EcoRI*) confirmed this hypothesis (data not shown). In a further experiment, a labelled PCR product of part of the 23S *rDNA* (see Fig. 3) was hybridized against *Nrdl*-digested plasmid DNA and total DNA (Fig. 5b). The sole *Nrdl* site (at position 4691) of the *rrn* operon is located downstream of the amplified region. The size of the signal is about 15 kb when plasmid DNA is the template (Fig. 5b, lane 2).

**Fig. 5.** Hybridization of 16S and 23S *rDNA* with plasmid DNA of pOL18/23 and total DNA of *Paracoccus* strain OL18. (a) Southern blot of DNA after digestion with *EcoRI*. The probe was a digoxigenin-labelled PCR product derived from amplification of pOL18/23 with primers 27f and 1385r (Lane, 1991). (b) Southern blot of DNA after digestion with *Nrdl*. The probe was a digoxigenin-labelled PCR product from the amplification of part of the 23S *rDNA* from pOL18/23 with the primers P-23-1 and P-23-2 (see Fig. 3). Lanes: M, digoxigenin-labelled *EcoRI* HindIII; 1, pOL18/23 plasmid DNA; 2, total DNA of strain OL18.
lane 1). Hybridization with total DNA produced two additional signals with sizes of about 6 and 4-5 kb (Fig. 5b, lane 2). These results suggest that there are at least two additional chromosomal rRNA operons in strain OL18, assuming that the chromosomal rRNA genes have restriction sites for EcoRI and NruI identical to those used for this assay. The intensities of the signals from the plasmid are significantly higher than those from the chromosomal genes, indicating that the copy number of pOL18/23, which has not yet been determined, is probably greater than one.

Conclusions
We have demonstrated that all members of a small Paracoccus population contain a small plasmid (of about 23 or 15 kb in size) that carries a complete and functional rRNA operon. This is only the second example of a plasmid-borne rRNA operon. Investigations to show whether these plasmids are mobilizable are in progress. 16S rRNA sequences are often used as the sole criterion for various purposes. For example, to define a DNA species as a chromosome, the demonstration of an rRNA operon is the most dominant character used. A significant part of taxonomic decisions are also based on comparative 16S rRNA gene sequence analyses. Phylogenetic and evolutionary relationships are deduced from 16S rRNA gene sequence divergence. The presence of an rRNA operon on a multicopy and possibly mobilizable plasmid requires a more critical use of rRNA sequences as a sole criterion. More diverse approaches based on the analysis of a wider variety of loci and comparative analytical methods are necessary for more reliable taxonomic and phylogenetic decisions and also for the differentiation between chromosome and plasmid.

ACKNOWLEDGEMENTS
We wish to thank Claudia Disqué-Kochem for her engagement in the characterization of the bacterial community at the beginning of the project, Johannes Sikorski for the initial cultivation of the soil bacteria, Karl-Heinz Gartmann for helping us with sequence data analysis and Ulrike Harke for technical assistance. The project was started with a grant from the German BMBF.

REFERENCES

