Analysis of Inverted Repeat DNA in the Genome of 
*Rhodomicrobium vannielii*

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The DNA of *Rhodomicrobium vannielii* was analysed for the presence of inverted repeat sequences (IR DNA) by S1 nuclease digestion. Approximately 7% of chromosomal DNA was found to be IR DNA which comprised two size classes. The large IR DNA was heterogeneous and contained species in the size range 100–700 bp. The smaller size class contained species of 17 and 27 bp. Both size classes of IR DNA hybridized to many chromosomal restriction fragments, suggesting that these IR DNA sequences are dispersed throughout the genome. Hybridization studies also indicated sequence homology between the two classes of IR DNA and suggested that the 17 and 27 bp IR DNA sequences may exist in clusters.

INTRODUCTION

*Rhodomicrobium vannielii* is a member of the *Rhodospirillaceae* and exhibits a polymorphic cell cycle (Whittenbury & Dow, 1977). Under anaerobic, photoheterotrophic conditions the 'complex cycle' of growth is usually followed, in which the culture consists of large numbers of small, ovoid cells which are joined by stalks or prosthecae to form ramifying multicellular arrays. Late in the exponential phase of complex cycle growth peritrichously flagellate 'swarmer cells' are released from these arrays. These cells have a strictly limited metabolic capability: they are reproductively inactive, exhibit no DNA or rRNA synthesis, and have reduced protein synthesis (Dow et al., 1983). Under favourable environmental conditions swarmer cells can become reproductively mature by an obligate developmental process (see Whittenbury & Dow, 1977) which culminates in cell division and which takes approximately 6 h under laboratory conditions.

The genome of *R. vannielii* contains a relatively high proportion of rapidly renaturing sequences, consisting mainly of inverted repeat sequences (Potts et al., 1980). Sequences of this type have been implicated in a variety of roles, from DNA transposition to termination of transcription. The abundance of inverted repeat sequences in *R. vannielii* may be related to its ability to initiate and maintain a pathway of cellular differentiation. Two other species of bacteria which exhibit differentiation, *Bacillus subtilis* and *Caulobacter crescentus*, also have abundant inverted repeat sequence DNA (Galloway & Rudner, 1979; Wood et al., 1976); in contrast *Escherichia coli* has 0.5–1.0% IR DNA (Kato et al., 1974). The population of inverted repeat sequences in the genome of *R. vannielii* may, then, include elements which are involved in the control of the cell cycle and particularly in swarmer cell differentiation. This paper describes the isolation and characterization of the inverted repeat DNA of *R. vannielii* and the determination of the patterns of its hybridization to chromosomal DNA.

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*Abbreviation:* IR DNA, inverted repeat DNA.
of obtained by digestion of denatured and rapidly renatured chromosomal DNA with the single-strand specific 
an aerobic liquid batch culture in the pyruvate-malate medium (PM medium) of Whittenbury & Dow (1977) at 30°C 
with illumination of approximately 2000 lx. Swarmer cells were selected from liquid culture by filtration 
according to the method of Whittenbury & Dow (1977) and their differentiation was initiated by reillumination.
Preparation of chromosomal DNA. Cells, from complex cultures or swarmer cell preparations, were harvested by 
centrifugation at 23000 g for 20 min. Cell pellets were washed once in TES buffer (50 mM-Tris/ HCl pH 8.0, 50 mM-NaCl, 50 mM-Na2EDTA) and resuspended in TES buffer containing 25% (w/v) sucrose. After passage through a 
French pressure cell at 1000 lbf in-1 (6.9 MPA), lysozyme (Sigma) was added to 1 mg ml-1 and the cell suspension 
incubated at 37°C for 10 min. This was followed by addition of Na2EDTA to 50 mM, transfer to ice and addition 
of sodium N-lauroylsarcosinate (Koch-Light Laboratories) to 3.5% (w/v). The lysate was then made 1 M with 
respect to sodium perchlorate and incubated for 10 min at 65°C. It was then deproteinized twice with 
phenol/chloroform/isoamyl alcohol (25: 24: 1, by wt). S1-resistant DNA was then precipitated at 
up to 6 h at 37°C. after which the reaction was stopped by addition of Na,EDTA to 30 mM and extraction with an 
equal volume of phenol/chloroform/isoamyl alcohol (25: 24: 1, by wt). S1-resistant DNA was then precipitated at
−20°C by addition of 2 vols ethanol in the presence of 100 μg glycogen as carrier. The abundance of inverted repeat sequences was determined by assaying trichloroacetic acid (TCA)
precipitable DNA throughout S1 digestion of denatured and rapidly renatured chromosomal DNA with the single-strand specific 
nuclease S1 as follows. DNA at a concentration of 1 mg ml-1 in water was denatured by incubating at 100°C for 
10 min, followed immediately by rapid chilling on ice, yielding OC3r renatured DNA. When cool an equal volume of 
× 2 concentrated S1 buffer (0.6 M-NaCl, 0.05 M-sodium acetate, 0.003 M-ZnCl2, pH 4.6) was added to the OC3r 
renatured DNA and S1 nuclease (BRL; 1 unit per μg DNA) was then added. Digestion was allowed to continue for 
up to 6 h at 37°C, after which the reaction was stopped by addition of Na2EDTA to 30 mM and extraction with an 
equal volume of phenol/chloroform/isoamyl alcohol (25: 24: 1, by wt). S1-resistant DNA was then precipitated at
−20°C by addition of 2 vols ethanol in the presence of 100 μg glycogen as carrier.
The abundance of inverted repeat sequences was determined by assaying trichloroacetic acid (TCA)
precipitable DNA throughout S1 digestion of denatured and OC3r renatured DNA labelled with 32P in vivo 
as described by Russell (1984). Nuclease S1 digestion of 32P-labelled DNA (107–109 c.p.m. per assay) was done as 
described above. Duplicate 5 μl samples were taken at various times during digestion and assayed as follows. 
Samples were added to 500 μl calf thymus DNA (200 μg ml-1) in TE buffer on ice and were immediately made 
10% (w/v) with respect to TCA by addition of a 100% (w/v) solution. Samples were kept on ice for 30 min before 
collecting acid-precipitable material by filtration on 25 mm glass microfibre filter discs (Whatman GF/C). The filters were washed with 3 × 1 ml 5% TCA and 1 ml ethanol, air-dried at 30°C and assayed by scintillation 
counting in 5 ml Beckman cocktail EP.
Restriction endonuclease digestions and electrophoresis of DNA. Restriction enzymes were used as directed by the 
suppliers. DNA fragments were electrophoresed in horizontal 0.7–3.0% agarose or vertical 5% polyacrylamide 
slab gels.
Hybridization. DNA fragments in agarose gels were transferred to nitrocellulose membranes (Schleicher & 
Schüll BA85) by the method of Southern (1975). Transfer buffer was 20 × SSC (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0) except in the case of the 3% agarose gel in Fig. 3 where 10 × SSC was used. 
Hybridization fragments immobilized on nitrocellulose was done by a modification of the method of Denhardt (1966). Filters were initially prehybridized in 0.02% (w/v) Ficoll, 0.02% polyvinylpyrrolidone and 0.02% 
bovine serum albumin (BSA) in 3 × SSC at 68°C. Further prehybridization was done in the same buffer with 50 μg 
denatured calf thymus DNA ml-1 (CT-DNA, Sigma) and 0.1% (w/v) sodium dodecyl sulphate (SDS) at 68°C for 
1 h. Hybridization was done in a small volume (1-3 ml) of final prehybridization solution containing 106– 
108 c.p.m. denatured, nick-translated (Rigby et al., 1977) probe DNA for at least 16 h at 68°C. Hybridized filters 
were washed twice in hybridization solution at 68°C for 5 min, then in 0.1 × SSC, 0.1% SDS, 50 μg CT-DNA ml-1 
twice for 15 min at 68°C. Washed filters were allowed to dry thoroughly before autoradiography. This method was not used with the 17/27 bp 1R DNA probe because of the size of the fragments. Instead, filters were 
hybridized by the method of Wallace et al. (1981) in 0.9 M-NaCl, 0.09 M-Tris/HCl pH 7.5, 0.006 M-Na2EDTA, 
0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, 0.5% SDS, 50 μM-ATP, 10% (w/v) dextran sulphate at 37°C 
with 106 c.p.m. 17/27 bp IR DNA, 5’ end labelled with [γ-32P]ATP as described by Maniatis et al. (1982).
RESULTS
Previous work used renaturation kinetics and electron microscopy to determine the 
abundance and size distribution of inverted repeat sequence DNA in the genome of 
Rhodomicrobium vannielli (Potts et al., 1980). In this work a more direct method was used to 
Isolate and assay the abundance of inverted repeat sequences in R. vannielli DNA by TCA 
precipitation of nuclease S1-resistant 0C3r DNA produced by denaturation and rapid
Inverted repeat DNA in R. vannielii

Fig. 1. Autoradiography of a 5% polyacrylamide gel of samples from a nuclease S1 digestion of R. vannielii DNA. IR DNA: ethanol precipitated material from a limit S1 digestion (360 min); arrowheads mark appropriate size limits of IR DNA. Size standards are plasmid pAT153 digested with HindIII or HpaII, and end-labelled with [32P]ATP. Fragment sizes as indicated.

renaturation. S1 digestion of 0Cot renatured 32P-labelled E. coli and R. vannielii DNA yielded limit values of 7% and 9% S1-resistant DNA respectively. The resulting S1-resistant fraction was isolated and again denatured, renatured and digested with nuclease S1. This redigestion should remove all of the genuine inverted repeat sequences from the S1-resistant fraction as they would have been reduced to linear duplex molecules in the first S1 digestion. Thus, only crosslinked DNA, DNA with multiple repeat structure and contaminating acid-precipitable material would survive the second S1 digestion. The S1 redigestion showed that 20% of the R. vannielii S1-resistant fraction and 50% of the E. coli S1-resistant fraction did not consist of IR DNA. This allowed revised estimates of the IR DNA content of DNA extracted from E. coli and R. vannielii as 3-5% and 7% respectively.

The size distribution of IR DNA from R. vannielii was estimated by electrophoresis in 5% polyacrylamide slab gels. Fig. 1 shows an autoradiograph of such a gel from an S1-digestion time course. It is clear that the average fragment size decreased throughout digestion, even at digestion times when the acid precipitable count remained constant. Two classes of fragment
were observed: a heterogeneous smear of fragments from 100–700 bp and a pair of discrete bands of approximately 17 and 27 bp. The low $M_r$ bands made up a significant proportion of the total IR DNA and appeared to be stable at longer digestion times, in fact increasing in intensity.

The high and low $M_r$ IR DNA fractions could be separated by gel filtration through a 3 ml bed volume column of Sephadex G-50 (Pharmacia) and so were used independently as hybridization probes to examine the distribution of IR DNA sequences within the *R. vannielii* genome. Fig. 2 shows one such hybridization experiment. Both probes hybridized to many bands in the EcoRI digests and appeared to have a number of bands in common. There were no distinct differences in hybridization pattern with the different stage-specific DNA preparations used. This suggested that IR DNA sequences were probably not involved in major cell-cycle associated DNA rearrangements.

To determine whether there was any homology between the high and low $M_r$ IR DNA fractions, labelled low $M_r$ IR DNA was hybridized to a 3% agarose gel containing high $M_r$ IR DNA (Fig. 3). It is apparent that the low $M_r$ IR DNA had homology throughout the high $M_r$ smear. More interesting, however, is the hybridization to HaeIII digested chromosomal DNA, which shows four faint hybridized bands.

**DISCUSSION**

The inverted repeat sequence DNA of *R. vannielii* comprised approximately 7% of the total chromosomal DNA and could be divided into two size classes: a heterogeneous high $M_r$ class ranging from 100–700 bp and two low $M_r$ bands of 17 and 27 bp. Both classes hybridized to many bands in chromosomal restriction digests, suggesting that the IR DNA sequences are dispersed throughout the genome. The hybridization of low $M_r$ IR DNA to high $M_r$ IR DNA implied that the 17 and 27 bp fragments are derived from the high $M_r$ fragments, and this view
Inverted repeat DNA in *R. vannielii*

Fig. 3. (a) Ethidium bromide stained 3% agarose gel of *HaeIII* digested *R. vannielii* DNA (RM5: *HaeIII*, 2 μg), heat denatured *R. vannielii* DNA (RM5 denatured, 0.5 μg) and *R. vannielii* 100–700 bp IR DNA (IR DNA, 1 μg) with digested pBR322 size standards; (b) autoradiograph of the corresponding hybridization with end-labelled 17–27 bp IR DNA probe (>10^5 c.p.m.).

was supported by the timing of the appearance of the low *M*, fragments (Fig. 1). Hybridization of low *M*, IR DNA to *HaeIII*-digested chromosomal DNA was considerably different from that with other restriction digests. The four bands observed here (Fig. 3) suggested that the low *M*, IR DNA sequences have a clustered distribution in the genome, in contrast with the hybridization in Fig. 2. It is possible, however, to incorporate all of these observations into a single model which describes the distribution of IR DNA sequences within the *R. vannielii* genome. We propose that the 17 and 27 bp IR DNA fragments are organized in clusters which make up the 100–700 bp IR DNA fraction. These high *M*, fragments are arranged in groups which produce the four hybridizing bands on digestion with *HaeIII*. These groups of IR DNA fragments are dispersed throughout the genome, as demonstrated by the hybridization of both IR DNA fractions to many *EcoRI* restriction fragments whose average size is much larger than the *HaeIII* fragments.

A family of palindromic sequences, similar in size to the low *M*, IR DNA sequences described here, has been found by computer searches of the available sequence data for *E. coli* (Gilson et al., 1984; Stern et al., 1984). These elements comprise 0.5–1.0% of the *E. coli* genome, generally occur between genes, and may have some regulatory function. Indeed, one member of this family has been suggested to act in attenuation of transcription within the *deoC–deoA* operon (Valentin-Hansen et al., 1984). In S1 digestion experiments similar to those described here, low *M*, IR DNA fragments of this size have been observed in *E. coli* (Russell, 1984). In contrast with the IR DNA sequences of *R. vannielii*, however, these fragments appeared early in digestion and were not visibly stable to prolonged S1 digestion. This may reflect differences in the sequence homology between the repeats and in their organization within the genome.
It will be of interest to investigate whether any homology exists between the IR DNA sequences of *R. vannielii* and the genome of *E. coli*, and also to look at the sequence structure and heterogeneity within the IR DNA sequences of *R. vannielii*, particularly in the low *M*, fraction.

**REFERENCES**


