Replacement of the deoxycytidine residues in *Rhizobium* bacteriophage RL38JI DNA

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Rhizobium phage RL38JI DNA is resistant to cleavage by a variety of restriction endonucleases, and is only partially sensitive to digestion by pancreatic DNase I or by micrococcal nuclease. We have found that a mixture of DNase I, P1 nuclease, and bacterial alkaline phosphatase will quantitatively digest RL38JI DNA to deoxyribonucleosides. HPLC analysis revealed that dCyd is nearly totally absent among these digestion products, while dGuo, dAdo, and Thd are readily detected. Three additional peaks are always present; their retention properties correspond to no known modified deoxyribonucleosides. Thus it appears that dCyd is replaced in phage RL38JI DNA by as many as 3 different modified residues.

Rhizobium *phage* *DNA modificaton* *Deoxycytidine*

1. INTRODUCTION

The transducing phage RL38JI has a relatively broad host range, infecting strains of *Rhizobium leguminosarum, R. phaseoli*, and *R. trifolii* [1]. Although the plating efficiency of the virus differs by no more than 10-fold on these *Rhizobium* species, transduction of *R. leguminosarum* using phage grown on *R. trifolii* cannot be detected, while the reverse gene transfer proceeds efficiently [1]. This puzzling observation led to a closer examination of RL38JI phage DNA. Based on the observed T_m value, RL38JI DNA has a 41% G + C content; however, a 77% G + C content was calculated from buoyant density measurements [2]. Furthermore, RL38JI DNA was observed to be resistant to in vitro cleavage by 7 different site-specific endonucleases (*Eco*RI, *Hin*dIII, *Eco*RV, *Bam*HI, *Sal*G1, *Hae*III, *Sma*I) ([2]; and unpublished). Host *R. leguminosarum* strain 300 DNA [(60% G + C)] is susceptible to cleavage by all these enzymes. These results suggested that RL38JI DNA may contain an unusual base; similar anomalous behavior of modified DNA has been observed for a variety of bacteriophages.

This work demonstrates that RL38JI contains a new modification that replaces all the DNA deoxycytidine residues.

2. MATERIALS AND METHODS

2.1. *Bacterial and phage strains*

R. leguminosarum 300 and *R. trifolii* were from the collection of John Beringer. *R. phaseoli* 14483 and *R. leguminosarum* 10314 were obtained from the American Type Culture Collection. RL38JI was the same phage used in the previous study by V. Buchanan-Wollaston [2].

Growth medium for phage lysates was TY (5 g Bacto Tryptone, 3 g yeast extract per l) plus 10 mM $CaCl_2$ [3]. Phage assays were performed by the agar overlay method; small, clear plaques developed after 24 h at 30°C. The standard host for phage lysates was *R. leguminosarum* 10314

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because phage stocks gave a 10-fold higher titer on this host than on any strain or species tested.

2.2. *Preparation of phage lysates and phage purification*

R. leguminosarum cultures in TY broth at 30°C were infected with an average multiplicity of 0.01 phage per cell when the culture had reached an absorbance of 0.4 at 550 nm (about 2×10^8 colony forming units/ml). Vigorous aeration was continued until lysis occurred (4–6 h after infection). A few drops of chloroform were added to each liter of lysate and debris was removed by two cycles of low-speed centrifugation for 10 min at $5000 \times g$. The clear supernatant (titer usually 10^{10} pfu/ml) was brought to 0.4 M NaCl and 10% polyethylene glycol 6000 [4] and the suspension was left overnight at 4°C. The polyethylene glycol precipitate was isolated by low-speed centrifugation for 10 min at $5000 \times g$. It was washed 3 times with a small volume of 20 mM Tris buffer, pH 7.4, containing 10 mM $CaCl_2$. These repeated washes extracted the concentrated, bluish phage from the pellet, which still contained bacterial debris. All 3 washes were essential to obtain good recovery of the phage. The phage suspension (2×10^{11} pfu/ml or greater) was layered onto a preparative CsCl step gradient [2] in 20 mM Tris buffer, pH 7.4 (containing 10 mM $CaCl_2$) and centrifuged for 12 h at $50000 \times g$. The blue phage band (buoyant density 1.55) was removed by aspiration and dialyzed against 20 mM Tris buffer containing 10 mM $CaCl_2$. Recovery of original crude lysate plaque forming units was 30%.

2.3. *Hydrolysis of RL38JI DNA*

Enzymic hydrolysis: Between 400 and 500 μg purified DNA isolated from CsCl-purified phage was ethanol precipitated and dissolved in 300 μl water using a vortex mixer. Thirty μmol of Tes, pH 7.4 (Sigma), 3 μmol $MgCl_2$, 15 μmol $CaCl_2$, 1500 units DNase I (Worthington), 3.75 μg bacterial alkaline phosphatase (BAP; Boehringer-Mannheim), and 45 units nuclease P1 (Sigma) were added and the volume adjusted to 675 μl. This mixture was incubated at 37°C for 18 h, and a 2 μl aliquot was removed and tested for the presence of oligonucleotides by ion-exchange HPLC. If greater than 10% of the total absorbance was found to be due to oligonucleotides, fresh DNase I, P1 nuclease, and BAP (750 units, 1.87 μg and 22 units, respectively) were added and the incubation continued overnight. When digestion was complete an equal volume of buffer-saturated phenol:chloroform (1:1) was mixed with the sample; after extraction and centrifugation, the combined aqueous phases were twice re-extracted with chloroform:isoamyl alcohol (24:1) and lyophilized. The dehydrated sample was dissolved in 50 μl or less of water.

Acid hydrolysis: Acid hydrolysis of DNA was carried out in redistilled 88% formic acid at 180°C for 30–45 min in a sealed glass ampoule. If the presence of significant amounts of RNA was suspended, a 1:1 (v/v) mixture of formic and trifluoroacetic acids was used and the incubation time increased to 1.5 h [5]. The hydrolyzed samples were lyophilized to dryness and dissolved in water.

2.4. *Separation of RL38 deoxyribonucleosides and bases by HPLC*

Ion-exchange chromatography was as described by Singhal [6] with only minor modifications [7]; reverse-phase HPLC was similar to that described by Kuo et al. [8], except that elutions were performed under isocratic conditions at a flow rate of about 10 ml/h in 50 mM KH_2PO_4/5% methanol, pH 4.13.

3. RESULTS

3.1. *Resistance of RL38JI DNA to nucleolytic cleavage*

As described earlier, attempts to digest RL38JI DNA with various restriction enzymes were unsuccessful. Mixing RL38JI DNA with phage λ DNA followed by incubation with restriction enzymes yielded the expected λ DNA bands but no detectable RL38JI fragments. This result ruled out the trivial possibility that the RL38JI DNA preparation contained an inhibitor of the nucleases tested. Because *Eco*RV has been reported to restrict hypermodified DNA from T-even phage [9], we tested RL38JI for sensitivity to this nuclease; unexpectedly, RL38JI DNA was resistant to cleavage under conditions where wild-type T4 DNA was digested, albeit incompletely (not shown).

Attempts to digest RL38JI DNA with nuclease mixtures (DNase I, snake venom phosphodi-

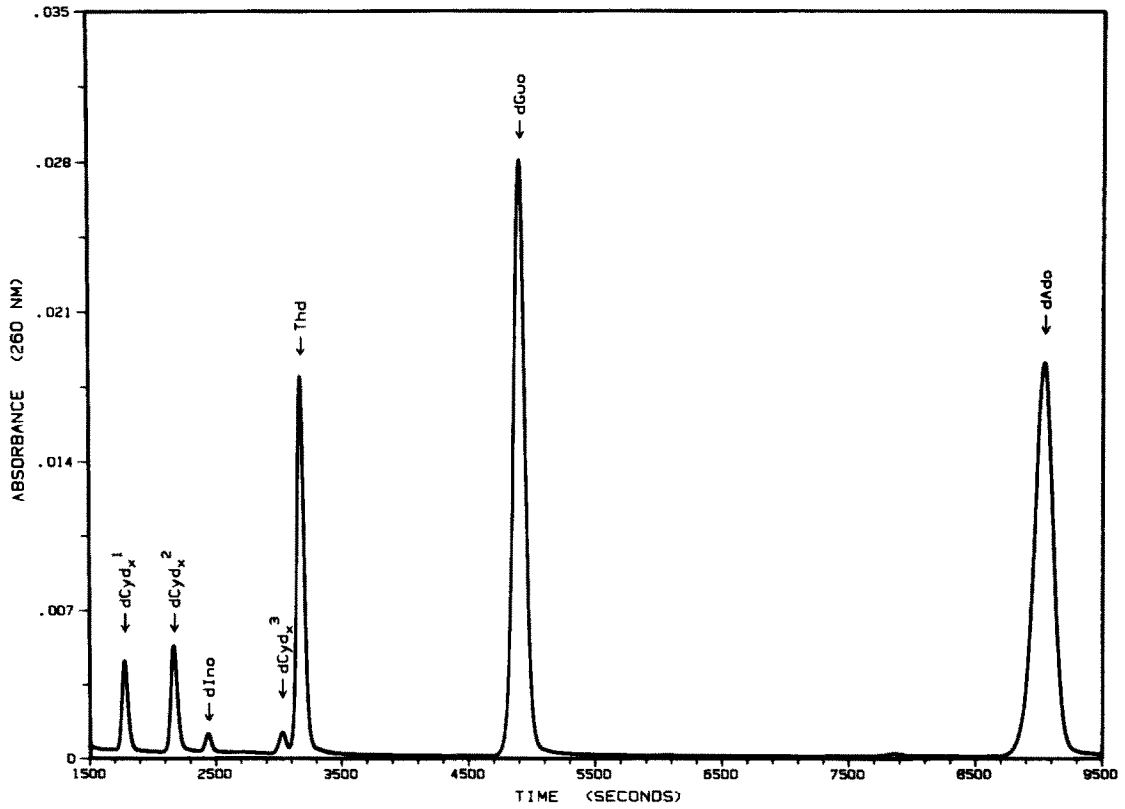

Fig.1. Ion-exchange chromatographic separation of deoxyribonucleosides produced by enzymatic digestion of phage RL38JI DNA. Enzymic digestion of purified RL38JI DNA to deoxynucleosides was carried out by incubation with pancreatic DNase I, P1 nuclease, and bacterial alkaline phosphatase (see section 2). Digestion was incomplete in this sample, so the relative peak areas of dC_x^1, dC_x^2 and dC_x^3 are under-represented.

esterase and BAP, or micrococcal nuclease, calf spleen phosphodiesterase and BAP) led to conversion of less than 25% of the DNA to deoxymononucleosides, as determined by HPLC. Moreover, dCyd was almost totally lacking among the mononucleoside digestion products.

We were further surprised to find that formic acid hydrolysis yielded only the 3 major bases, Ade, Gua and Thy, but not Cyt. Thus, it appeared that Cyt was completely replaced in RL38JI DNA by a base which interferes with enzymic digestion and whose chromophore is sensitive to acid hydrolysis.

In view of the acid lability of this base, we sought to find conditions which would allow complete enzymic digestion. We observed that only treatment with P1 nuclease, DNase I and BAP (see section 2) yielded a complete digest of the sample. Fig.1 shows the chromatographic profile (254 nm) of such a digest fractionated on a strongly acidic ion-exchange resin. In addition to the peaks corresponding to Thd, dGuo and dAdo (and a minute peak corresponding to dCyd), there are 3 additional peaks (labeled dC_x^1, dC_x^2 and dC_x^3) which elute prior to Thd. Fractions containing these 3 peaks were pooled, concentrated and repurified by the same ion-exchange chromatographic method, and the purity of each confirmed by reverse-phase HPLC (not shown). The alkaline absorption spectra of dC_x^1, dC_x^2 and dC_x^3 were identical (fig.2). Acid hydrolysis of each of these samples led to the loss of UV absorption, a fact consistent with our observation that acid hydrolysis of RL38JI DNA yields only Ade, Gua, and Thy.

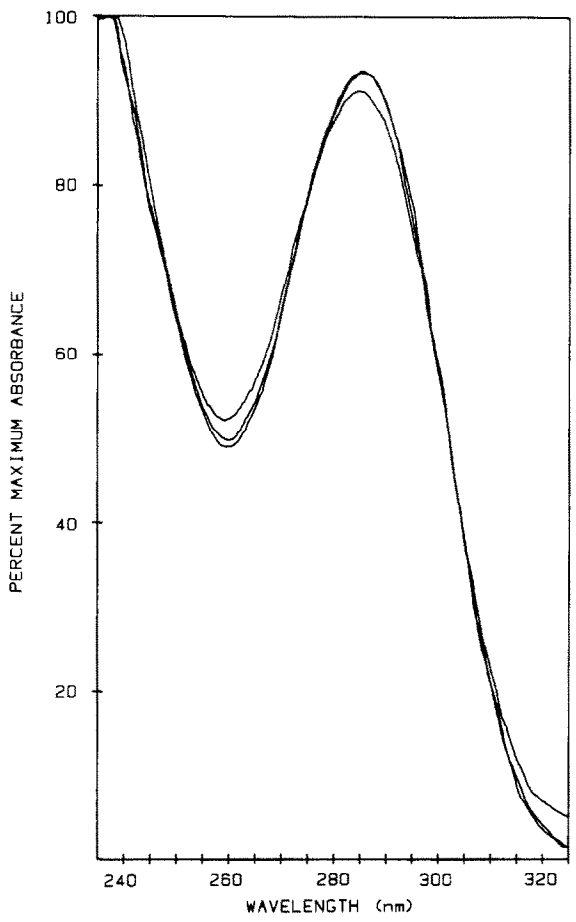

Fig.2. UV-absorption spectra of the 3 modified deoxyribonucleosides from RL38JI DNA. Spectra were measured at pH 11 in a Perkin-Elmer Model 330 spectrophotometer. The full-scale values were 0.8, 0.8 and 0.45 absorbance units for dC_x^1, dC_x^2 and dC_x^3, respectively.

4. DISCUSSION

Two lines of indirect evidence suggest that RL38JI DNA may contain some unusual DNA modification. First, the G + C content calculated from T_m and buoyant density measurements gave significantly different values [2]. Second, RL38JI DNA was refractory to in vitro cleavage by the 7 restriction endonucleases we tested. Furthermore, the DNA was only partially digested by various mixtures of endo- and exonucleases, such as pancreatic DNase I and snake venom phosphodiesterase. Despite this, we were able to find appropriate conditions in which to obtain complete enzymatic digestion. This, in turn, permitted us to measure directly the composition of the DNA. The chromatographic separation (fig.1) revealed that almost no dCyd was present. In place of dCyd we observed 3 new species, dC_x^1, dC_x^2 and dC_x^3, which exhibit identical UV-absorption spectra at pH 11 (fig.2). This suggests that the chromophore is the same in all 3 cases. Based on their HPLC elution positions, we can immediately rule out 5-methyl dCyd and 5-hydroxymethyl dCyd as being one of the dC_x species. Moreover, the dC_x modification causes a greater spectral shift at pH 11 (λ_{max} at 284 nm) from dCyd (λ_{max} at 271.5 nm) than that observed for 5-methyl dCyd (λ_{max} at 279 nm). The differences in HPLC retention times for the 3 modified species may be due to successive derivatizations of a single parent molecule.

From the areas measured for the 3 major deoxynucleosides, we calculate that the G + C content of RL38JI DNA is 54% (a low level of N^6-methyl dAdo was also observed); this value is different from that calculated from either the T_m or buoyant density data. The DNA from host *R. leguminosarum* has a G + C content of 60% based on buoyant density [2]. The host DNA apparently lacks or has very little of this unusual modification, inasmuch as it is readily cleaved by the enzymes that do not digest RL38JI DNA.

The recently described *R. meliloti* transducing phages ϕM12 [10] and N3 [11] contain DNA which is resistant to a number of restriction enzymes; however, *Eco*RI and *Hin*dIII cleaved both DNAs. Since RL38JI DNA is resistant to all restriction enzymes tested, its DNA modification is likely to be different from those of the two *R. meliloti* transducing phages.

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