Studies on the Deoxyribonucleic Acid of *Serratia marcescens*

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SUMMARY

A method for the quantitative isolation of pure highly polymerized DNA from *Serratia marcescens*, based on Kirby's phenol extraction procedure (Kirby, 1956, 1957), has been developed. No statistically significant differences were detected in the base composition or sequence between the DNA of a wild and a mutant strain of the organism. 6-Methylaminopurine was found in the DNA of *Serratia marcescens*. The properties of this base and its N-nitroso derivative were compared with those of a synthetic sample.

INTRODUCTION

Deoxyribonucleic acids (DNA) are usually extracted from bacteria after they have been mechanically broken or lysed with lysozyme or sodium deoxycholate (e.g. McCarty & Avery, 1946; Zamenhof, Alexander & Leidy, 1953; Jones, 1953). The DNA thus obtained has usually been found to contain a much lower phosphorus content than the theoretical value, indicating the presence of non-phosphorus containing impurities (Overend, Stacey, Webb & Ungar, 1951; Dutta, Jones & Stacey, 1953; Burton, 1960). In the present work Kirby's method for the isolation of DNA from animal tissues (Kirby, 1956, 1957) was used to extract DNA from *Serratia marcescens* without the necessity for mechanical breakage of the cells, and the DNA so obtained was purified and analysed for purines and pyrimidines.

METHODS

Organism. Two strains of *Serratia marcescens* were used: (i) a normal wild pigmented strain (NCTC 1377); (ii) a colourless mutant produced in this laboratory as follows. A culture of the pigmented strain was grown for 18 hr. at 25° on a medium containing NaCl (0.5 %, w/v), Oxoid Lab Lemco (0.5 %, w/v) and Oxoid peptone (1.0 %, w/v) (10 ml.). The culture was centrifuged and the sediment of organisms washed three times with 0.85 % (w/v) NaCl solution. The organisms were finally suspended in 0.85 % NaCl (10 ml.) and a sample, in a 1 cm. quartz spectrophotometer cell, irradiated with ultraviolet light from a Hanovia Dectectolite unit (Hanovia Lamps Ltd. Slough, Bucks.) for 5 min. at a distance of 15 cm. The suspension was then plated on the medium described below and incubated at 25° for 24 hr. A colourless colony was picked off and propagated as usual. This strain gave colonies which were colourless when grown for 24 hr. but gave a very faint pink on prolonged incubation. When grown on a medium containing only inorganic salts and glucose this faint pink colour did not develop. The organism retained these properties after regular subculture for 6 years. The following characteristics were found to be

identical with those of the wild pigmented strain: motility +; indole reaction -; acid only produced from glucose, sucrose, inositol, mannose and maltose; no fermentation of lactose, dulcitol, arabinose, rhamnose and xylose.

Medium. The organism was grown on a nutrient agar containing NaCl (0.5%, w/v), Oxoid Lab Lemco (0.5%, w/v), Oxoid peptone (1.0%, w/v) and shred agar (0.2%, w/v): Astell Lab. Service Co., Ltd., London, S.E. 6).

Conditions of growth. The nutrient agar was poured into drying trays (Jencons Ltd., Hemel Hempstead, Herts.) 12 in. $\times$ 12 in., which were then covered with glass and allowed to cool. The inoculum was prepared from a 24 hr. nutrient broth culture, and was added (2 ml.) to each tray, which was then incubated at 26° for 48 hr.

Harvesting. The organisms were scraped off the surface of the agar, which was then washed with a little physiological saline. Organisms and washings were centrifuged at 4000 g for 20 min. and the deposit washed three times with saline solution.

Nitrogen determination. DNA samples were digested with the reagent described by Ma & Zuazaga (1942) containing selenium (0.05%, w/v) and the nitrogen determined by developing the indophenol blue colour by using the method of Holbrook & Jones (unpublished results). After digestion the solution was allowed to cool and saturated sodium bicarbonate solution (16 ml.) added, followed by sodium hypochlorite solution (1 ml., 1.0–1.4% (w/v) available chlorine, containing sodium nitroprusside 0.025% (w/v)). After 5 min., sodium phenate solution was added (1 ml., phenol 5.6% (w/v), sodium hydroxide 16.6% (w/v)) and the absorption of the solution read at 625 m μ after 20 min.

Total phosphorus was estimated by the method of Jones, Lee & Peacocke (1951).

Inorganic phosphorus was estimated by the method of Berenblum & Chain (1938).

Pentose was determined by the method of Euler & Hahn (1946) with a purified yeast nucleic acid as a standard.

Base analysis. Samples of DNA (5 mg. dried *in vacuo* at 110° for 3 hr.) were hydrolysed in formic acid (98–100% (w/v), 0.6 ml.) at 175° for 1 hr. Duplicate samples were hydrolysed and for each hydrolysate four chromatograms were run in propan-2-ol + 10 N-hydrochloric acid + water; (68 + 20.5 + 11.5, by vol.) on Whatman No. 1 paper.

RESULTS

Estimation of the DNA content of Serratia marcescens. The method was a modification of Schmidt & Thannhauser's (1945) method introduced by Jones, Rizvi & Stacey (1958). The cells were harvested, weighed while wet, washed, freeze dried and reweighed, thus enabling a wet/dry weight ratio of the cells to be obtained so that the amount of DNA obtained in future isolations could be expressed as a percentage of the total amount present once the wet weight of cells harvested was known. Freeze-dried cells (70 mg.) were extracted with ether after refluxing with 95% methanol to remove the lipids, the acid soluble material was extracted with cold trichloroacetic acid (2 ml., 5%, w/v) and the residue dispersed in N-KOH (4 ml.) and incubated at 37° for 15 hr. The phosphorus content of the solution was measured before and after the precipitation of the DNA with perchloric acid (0.4 ml., 60%, w/v). It was found necessary to centrifuge the suspension at

173,000 g (Spinco ultracentrifuge model L). The amount of DNA obtained was 2.18 mg. and the DNA content of *Serratia marcescens* was calculated to be 3.1 %.

Isolation of the DNA

All operations carried out at 0–4° unless otherwise stated. The organisms (7 g. wet weight, 1 g. dry weight from 6 trays) were added to a mixture of 6 % (w/v) sodium *p*-aminosalicylate (200 ml.) and phenol (Analar, 200 ml.) which had been saturated with salicylate solution, and the mixture shaken for 1 hr. The mixture was then centrifuged at 1000 g for 10 min. the water layer removed, and the phenol layer re-extracted with a salicylate solution (200 ml.) saturated with phenol until no more material precipitable with 3 volumes of ethanol was extracted from the cell debris; usually five extractions were necessary. Ethanol (3 volumes) was added to the combined aqueous layers and the crude DNA removed on a glass rod. The DNA was dissolved in M-NaCl (20 ml.) and left overnight. The solution was centrifuged at 25,000 g for 20 min. to remove cell debris and microsomal RNA, and brought to 20°. Cetyltrimethylammonium bromide (CTAB, British Drug Houses, Ltd.) was added to the supernatant liquid so that its final concentration was 1 % (w/v), and the concentration of the sodium chloride was decreased to 0.6M by the addition of water (13.3 ml.). The precipitate of the cetyltrimethylammonium salt of DNA was removed on a glass rod, dissolved in M-NaCl, and the sodium deoxyribonucleate precipitated by adding ethanol (3 volumes).

The DNA so obtained was found by analysis to have a very low phosphorus content (about 7 % of the dry weight), which indicated the presence of impurity. The DNA was dissolved in water and shaken with chloroform to remove any traces of CTAB and protein, and the aqueous layer was then centrifuged at 80,000 g. The supernatant liquid was dialysed against frequent changes of distilled water and freeze dried (yield 30 mg., 97 % recovery). The precipitate, which in the case of DNA from the wild strain was coloured red, was very stable to acid-hydrolysis, but eventually gave a mixture of amino acids and sugars, indicating that the impurity probably contained cell-wall material.

Analysis of the DNA

The DNA was isolated in three separate experiments from both the strains of *Serratia marcescens*. The analyses are shown in Table 1. The RNA and protein contents of all samples were less than 1 %. The base contents of the DNA samples were also determined. Adenine, guanine, cytosine and thymine were present in the amounts shown in Table 2.

Identification and properties of 6-methylaminopurine

6-Methylaminopurine was identified chromatographically in the DNA of *Serratia marcescens* by using the method described by Dunn & Smith (1958), and by comparing its spectra in neutral, alkaline and acid solution with that given by a solution of the base which has been prepared from 6-methylmercaptapurine (Nutritional and Biochemical Corporation, Cleveland, Ohio) by the method of Elion, Burgi & Hitchings (1952). The R_F values of the two compounds corresponded in the solvent systems butanol + ethanol + water (4 + 1 + 5, by vol.; R_F 0.61), and propan-2-ol + 10 N-hydrochloric acid + water (68 + 20.5 + 11.5 by vol.; R_F 0.50).

Fig. 1. Spectrum of N-nitroso derivative of 6-methylaminopurine (13.6 $\mu\text{g./ml.}$) in aqueous solution at pH 7.0.

Table 1. *Analysis of DNA samples isolated from Serratia marcescens*

Values are given for dry DNA. Nitrogen was determined by the method of Holbrook & Jones (unpublished results) and phosphorus by the method of Jones, Lee & Peacocke (1951). % DNA isolated from the cells calculated from the yield of wet cells obtained.

	Wild strain			Colourless mutant		
	S1	S2	S3	S4	S5	S6
Nitrogen (%)	14.6	15.1	15.1	14.7	15.3	14.9
Phosphorus (%)	8.7	9.0	9.0	8.7	9.1	8.9
ϵ_p	7900	7800	8000	7800	8200	8200
% isolated from cells	97	100	97	100	100	100

S1–3, Separate samples of DNA isolated from the wild strain; S4–6, separate samples of DNA isolated from the colourless mutant; ϵ_p , molar absorptivity, based on one gram-atom of phosphorus/litre.

The N-nitroso derivative of the synthetic 6-methylaminopurine was made by dissolving the base (100 mg.) in glacial acetic acid (2 ml.), adding a saturated solution of sodium nitrite (2 ml.) and allowing the solution to stand at room temperature overnight. Water (2 ml.) was added, the precipitate (80 mg.) filtered off and recrystallized from water to give orange needles (65 mg.; m.p. 242–6° (*d*). Found: C, 40.36; H, 3.76; N, 46.00%; calculated for $\text{C}_6\text{H}_6\text{N}_6\text{O}$: C, 40.44; H, 3.37; N, 46.12%). The spectrum given by this sample in neutral aqueous solution is given in Fig. 1; it does not agree with the spectrum given previously for this compound by Dunn & Smith (1958). This can be explained by the fact that they took their

Table 2. *Base composition of the DNA of Serratia marcescens*

The amounts were determined from the ultraviolet absorption of the eluate from paper chromatograms after hydrolysis of the DNA in formic acid. The number of samples hydrolysed is shown in parentheses and the mean amounts $\pm$ standard deviation are given in moles of base/100 g. atoms of P.

Source	Adenine	6-MAP*	Thymine	Guanine	Cytosine	Nitrogenous material recovered (%)
Wild strain	19.61 $\pm$ 0.21 (6)	0.69 (2)	20.16 $\pm$ 0.14 (6)	29.70 $\pm$ 0.50 (6)	29.84 $\pm$ 0.27 (6)	97.7
Colourless mutant	19.59 $\pm$ 0.41 (6)	0.73 (2)	19.76 $\pm$ 0.14 (6)	29.98 $\pm$ 0.59 (6)	29.94 $\pm$ 0.39 (6)	97.9

* 6-Methylaminopurine.

Table 3. *Ratio of the inorganic phosphorus liberated during a Burton (1960) degradation to the total phosphorus content of Serratia marcescens DNA*

DNA (5 mg.) was taken in each case and allowed to stand for 16 hr. at 30° C. in diphenylamine (2%, w/v) in formic acid (30 ml., 68% w/v). Inorganic phosphate was measured by Berenblum & Chain's (1938) method and total phosphorus by Jones, Lee & Peacocke's (1951) method.

Phosphorus	Wild strain						Colourless mutant					
	S1		S2		S3		S4		S5		S6	
Inorganic (I) μ g.P	3.45	4.15	4.38	4.15	4.14	4.91	3.81	5.05	4.17	4.50	5.00	5.80
Total (T) μ g.P	14.2	19.0	19.0	18.0	17.5	22.0	14.9	22.2	17.7	19.2	19.7	21.0
I/T	24.2	21.8	23.0	23.0	23.6	22.3	25.5	22.7	23.5	23.4	25.3	25.2
Average I/T			23.0 $\pm$ 0.7						24.2 $\pm$ 1.1			

S1-3, Separate samples of DNA isolated from the wild strain; S4-6, separate samples of DNA isolated from the colourless mutant.

spectra of the compound after they had eluted it from a chromatogram either at pH 1 or at pH 13. Under these conditions we found that the N-nitroso derivative breaks down to yield the parent base.

The base (17 μ g. eluted from paper chromatograms in 0.1 N-HCl, 3.4 ml.) isolated from the DNA of *Serratia marcescens* gave when treated with nitrous acid a compound with the same R_f values as N-nitrosomethylaminopurine, but when an attempt was made to obtain the spectrum of this compound by eluting it from chromatography paper, the blank reading was so high, that it was impossible to obtain an accurate spectrum, but the peak was in the region of 300 m μ .

From the value obtained for the molar extinction coefficient of the pure synthetic 6-methylaminopurine (15.1×10^{-3} at 267 m μ ; pH 1.0), it was found to be present to the extent of 3.4 % of the adenine content of the wild strain DNA, and 3.6 % of the adenine in the colourless strain DNA.

No 5-methylcytosine was found in the DNA preparations of *Serratia marcescens* examined.

Table 4. *Amount of thymidine liberated from the Serratia marcescens DNA during the Burton (1960) degradation*

The amounts were determined from the ultraviolet absorption of the eluates from paper chromatograms (Burton & Petersen, 1960). The mean amounts $\pm$ standard deviation are given in moles of thymidine/100 g. atoms of P on the chromatograms.

	Wild strain			Colourless mutant		
Thymidine liberated	S1	S2	S3	S4	S5	S6
	5.62	5.44	5.37	5.38	5.47	5.58
Average	5.48 $\pm$ 0.11			5.48 $\pm$ 0.08		

S1-3, Separate samples of DNA isolated from the wild strain; S4-6, separate samples of DNA isolated from the colourless mutant.

Base sequence of the DNA

To detect any difference in the base sequences of the DNA of the two strains of *Serratia marcescens* especially in the thymine distribution (since only this base occurred in what might be significantly different amounts in the DNA of the two strains; Table 2), the DNA was subjected to the degradation procedure described by Burton & Petersen (1957, 1960). The same preparations of the DNA were used as were taken for the base analysis (S1-6); the results are given in Table 3. The products of the degradation were separated by removal of the terminal phosphate groups, followed by a chromatographic separation of the oligonucleotides (Burton, 1960). The amount of thymidine thus liberated from the DNA of the two strains was then determined and is shown in Table 4.

DISCUSSION

When the phenol extraction method (Kirby, 1956) for isolating DNA is applied to micro-organisms, it is usually necessary to break the cells mechanically. In the present case with *Serratia marcescens* on the addition of phenol and water, the DNA at once passed into the aqueous layer. By using this method and with a suitable

salt concentration for the aqueous layer, the ribosomal RNA was left with the cell debris, enzyme activity was completely inhibited and the protein was completely removed from the DNA. When this method was combined with the method of Dutta *et al.* (1953) for separating DNA from RNA by the use of cetyltrimethylammonium bromide, it was possible to isolate quantitatively the DNA from *S. marcescens*, and to obtain it in an undegraded form, free from protein (microsomal and transfer), RNA and polysaccharide. The DNA as initially isolated contained an impurity, probably cell-wall material, which was removed from the solution by centrifugation at 80,000 *g* for 1 hr. Samples of DNA which have been isolated from bacterial sources have often had low phosphorus contents (Burton, 1960); perhaps these too were contaminated by cell-wall material, although in one case the contaminant was shown to be a polysaccharide (Overend *et al.* 1951).

For comparison, DNA was isolated from the two strains of *Serratia marcescens*, and care was taken to ensure that identical growth, harvesting, isolation and purification procedures were used in each case; even so no statistically significant difference was found in the base contents of the DNA preparations from the two strains. No difference in the distribution of purines and pyrimidines in the DNA's from the two strains could be detected by analysing the results of the percentage inorganic phosphorus liberated during a Burton degradation. Nor was any difference detected in the amounts of thymidine liberated during the degradation. However, because no difference was found in the base composition or sequence between the DNA preparations from the two strains, this does not mean that they are identical, but rather that any difference was too small to be detected by the methods used.

The amount of 6-methylaminopurine found in the DNA is the highest so far known to occur naturally and is only exceeded by the amount found in the DNA from *Escherichia coli* 15T⁻ grown under conditions of thymine deficiency (Dunn & Smith, 1958). If it be assumed that 6-methylaminopurine has similar base-pairing properties to adenine, then it can be seen that in this *Serratia* DNA there are typical Watson & Crick (1953) base pairs, i.e. adenine + 6-methylaminopurine = thymine; guanine = cytosine.

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