Phages I α and I $_2$ -2: IncI Plasmid-dependent Bacteriophages

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Phage I α was isolated from sewage from Windhoek, South West Africa. It formed relatively clear plaques about 2 mm in diameter, on sensitive strains of *Escherichia coli* K12 and *Salmonella typhimurium* LT2. The phage had an hexagonal outline with a diameter of about 24 nm, contained RNA and was resistant to chloroform. Phage I α formed plaques or propagated only on organisms carrying I $_1$ plasmids or the I γ plasmid R621a. The efficiency of plating was higher on *E. coli* than on *S. typhimurium* hosts. The phage adsorbed along the length of shafts of I $_1$ pili.

Phage I $_2$ -2 was isolated from Pretoria sewage. It was a filamentous virus and individual virions varied considerably in length. Phage I $_2$ -2 formed turbid plaques which varied from pin point to about 1 mm in diameter on all hosts. It was resistant to RNAase and sensitive to chloroform. Phage I $_2$ -2 had a spectrum of activity limited to strains harbouring I $_2$ plasmids but the adsorption site could not be demonstrated. The phage was not related serologically to phages If1 or PR64FS.

INTRODUCTION

Plasmids belonging to incompatibility groups I $_1$, I $_2$, I γ , I ζ , B, and K determine thin (6 nm) flexible pili (morphological class 1; Bradley, 1980*b*). These pili are usually produced under an efficient repressor system (Lawn *et al.*, 1967; Meynell *et al.*, 1968), but mutants showing constitutive pilus production have been isolated (Meynell & Datta, 1967). Using as hosts, strains carrying pilus constitutive (*pil^c*) I $_1$ plasmids, Meynell & Lawn (1968) isolated a filamentous DNA phage, If1, which adsorbed to pilus tips.

Plasmids determining pili able to adsorb this phage were considered to constitute an 'I complex'. When it was shown that certain other plasmids, which encoded pili that did not adsorb phage If1, had significant DNA homology with some of these plasmids, they too were considered as members of the complex (Grindley *et al.*, 1972, 1973; Hedges & Datta, 1973; Falkow *et al.*, 1974). Thus defined, the I complex was found to be identical with the set of plasmids determining pili of morphological class 1 (Bradley, 1980*b*).

Within this plasmid set, two groups seem almost completely distinct. Plasmids of group I $_2$ show no DNA homology with other plasmids (hereafter described as the 'I $_1$ complex'), and with the sole exception of the anomalous plasmid, R805a (Bradley 1980*b*; D. E. Bradley & J. N. Coetzee, unpublished), the pili encoded by members of the I $_2$ complex are serologically unrelated to those determined by I $_2$ plasmids. Plasmids of the I $_1$ complex show a variety of compatibility interactions with one another, but all are completely compatible with all plasmids not assignable to this complex. They all show significant DNA homologies (Grindley *et al.*, 1973; Falkow *et al.*, 1974; H. Richards, personal communication) and all determine serologically related pili (Bradley, 1980*b*). The I $_1$ complex thus seems to be strictly comparable with the F complex.

The first identified plasmids of this complex were those that constitute group I₁ (=I α) (Meynell *et al.*, 1968; Grindley *et al.*, 1972, 1973; Datta & Barth, 1976; Datta, 1979). Pili determined by all these plasmids adsorb phage If1. Plasmids of group B (=O) (Grindley *et al.*, 1972, 1973; Datta & Olarte, 1974) are compatible with I₁ plasmids, but have significant DNA homology with them and code for pili that are serologically related to I₁ pili (although they do not bind phage If1).

Hedges & Datta (1973) identified a plasmid, R621a, compatible with both I₁ and I₂ plasmids, but determining pili capable of adsorbing phage If1 and showing serological relationships with those encoded by I₁ plasmids (Falkow *et al.*, 1974). R621a has been shown to be incompatible with B plasmids (N. Datta, personal communication) and in a classification based exclusively upon incompatibility, I₇ plasmids cannot be separated from the B plasmids. By DNA reassociation, however, R621a is much more closely related to I₁ plasmids than to those of group B (Falkow *et al.*, 1974).

R805a, designated an I ζ plasmid, (Falkow *et al.*, 1974) is unable to coexist with plasmids of either I₁ or B groups (Datta & Olarte, 1974). By DNA homology it is similar to I₁ plasmids (Falkow *et al.*, 1974). Pili of R805a-carrying cells bind both anti-I₁ and anti-I₂ sera (Bradley 1980*b*).

Plasmids of group K determine pili that are serologically related to I₁ and B pili (Bradley, 1980*b*), but failed to adsorb phage If1. Preliminary evidence suggests that they have significant DNA homology with plasmids of these two groups (H. Richards, personal communication).

I₁ plasmids are unable to transfer to the tribe *Proteae* (Coetzee, 1964; Datta & Hedges, 1972). B plasmids, however, have been transferred to *Proteus mirabilis* (H. Richards, personal communication) and transfer of B plasmids from wild isolates of *Proteus* sp. has been reported (Towner, 1979).

Here are described properties of two phages: phage I α , which was isolated as forming plaques on strains carrying plasmid R144*drd*3 but not on chromosomally isogenic R⁻ strains, and phage I₂-2 which, similarly, was dependent on the I₂ plasmid R721 *pi*^h for plaque formation.

METHODS

Bacteria and plasmids. *Escherichia coli* strains were J53, J53-2 (Coetzee *et al.*, 1979) and JE2571 (Bradley, 1980*a*). *Salmonella typhimurium* strains were LT2*trpA*8 (Coetzee *et al.*, 1979) and M827-2, a spontaneous *rif* mutant of LT2 strain M827 (Spratt *et al.*, 1973). Plasmids were from the collection of Naomi Datta (Datta, 1977) and were transferred by conjugation to the appropriate organisms when necessary. Plasmid-carrying strains are listed in Table 1.

Bacteriophages. The host ranges of phage I α and I₂-2 (see Results) were compared with that of phage PR64FS (Coetzee *et al.*, 1980). The latter phage was also used as a negative control in RNAase sensitivity tests. Phage If1 (Meynell & Lawn, 1968), which is closely related to phage PR64FS, was used in serological tests (see below).

Media, antibiotics, transfer of plasmids by conjugation, general phage techniques, production of phage lysates and organic solvent sensitivity of phage. These were done as described and referred to by Coetzee *et al.* (1979) and Bradley & Rutherford (1975). Incubation temperature was 37 °C.

Phage isolation. Sewage specimens were obtained from various sewage works in the Pretoria area as well as six samples from Windhoek SWA. Attempts were made to isolate an R721 plasmid-specific phage from sewage according to the method of Bradley *et al.* (1981*a*). The partner organisms used for enrichment of sewage were J53-2(R721 *pi*^h) and M827-2(R721 *pi*^h). The method of Bradley *et al.* (1981*c*) was used to isolate a chloroform-resistant phage specific for bacteria harbouring plasmids of the I complex. After the chloroform had been evaporated, these samples of sewage were enriched with partner strains J53-2(R64*drd*11) or LT2*trpA*8(R64*drd*11), J53-2(R144*drd*3) or LT2*trpA*8(R144*drd*3), J53-2(R483*drd*) or LT2*trpA*8(R483*drd*) and J53-2(R821*adrd*) or LT2*trpA*8(R821*adrd*). All enriched samples were then treated as before.

Concentration of phages. Phage I α lysates were concentrated using polyethylene glycol (mol. wt 6000) and NaCl at final concentrations of 11% (w/v) and 0.5 M, respectively (Yamamoto *et al.*, 1970). For phage I₂-2 the concentration of polyethylene glycol was 8% (w/v). Pellets were suspended in small volumes of distilled water.

Ribonuclease sensitivity. This was determined according to Bradley *et al.* (1981*a*), with M827-2(R721 *pi*^h) as the indicator organism for phage I₂-2 and J53-2(R64*drd*11) as host for phage I α and the negative control phage PR64FS.

Serology. Phage PR64FS antiserum (inactivation constant, *K*, 602 mm⁻¹) (Coetzee *et al.*, 1980) was used in neutralization experiments (Coetzee *et al.*, 1979) with phages PR64FS, If1 and I₂-2.

Bacteriophage propagation. Propagation of phages I α , I $_2$ -2 (see Results) and PR64FS (Coetzee *et al.*, 1980) was as described by Bradley *et al.* (1981*a*). In order to exploit the physiological derepression of pilus synthesis in newly infected cells a phage was added to a mixed population of chromosomally isogenic R $^+$ and R $^-$ lines (Datta *et al.*, 1971). Phage I α had a greater efficiency of infection on *E. coli* K12 than on *S. typhimurium* LT2, so strains of the former (J53-2 and R $^+$ derivatives) were used in most phage propagation tests. Positive controls were performed using R144*drd3* in the appropriate host, and negative controls used R $^-$ strains. The final phage concentrations were assayed using an R144*drd3* carrying variant of a strain chromosomally isogenic with the host upon which multiplication had been tested (usually J53-2). Phage I $_2$ -2 was tested for propagation by analogous techniques using either *E. coli* K12 (strain J53-2) or *S. typhimurium* LT2 (strain M827-2). The positive controls carried either TP114*drd1* or R721 *pil* c and these strains were also used to assay final phage concentrations.

A phage titre increase on the test organisms, at least 10 times that of the negative control, on three separate occasions, was taken as a positive result.

Phage titration on bacteria temporarily derepressed for pilus synthesis. This test was used where phage propagation experiments had yielded negative or inconclusive results. It consisted in mixing, on non-selective nutrient agar, 0.05 ml broth cultures of donor and recipient bacteria in the exponential phase of growth. Bacteria in the former culture carried the plasmid, repressed for pilus synthesis, under investigation. After 2 h incubation the mating mixture, now possibly containing recipient bacteria harbouring the plasmid temporarily derepressed for pilus synthesis (see Bradley, 1980*a*), was gently suspended in 2 ml pre-warmed broth. This suspension was immediately used to prepare a soft agar overlay on nutrient agar containing antibiotics for the plasmid selection and rifampicin for donor counterselection. After the agar had set, dilutions of the phage suspension were dropped on the surface.

Electron microscopy. I $_1$ pili were conveniently obtained in large numbers from nutrient agar cultures of J53-2(R64*drd11*). A loopful of bacteria was suspended in a little high-titre phage I α suspension in a small Petri dish to form a thin paste. A little of this was mixed with a few drops of phage suspension. After incubation (tightly covered) for 45 min, a carbon-coated electron microscope specimen support grid was touched on to the surface of the paste, then washed free of excess bacteria by floating on successive baths of 0.1 M-ammonium acetate solution. Negative staining was with a mixture of equal volumes of 2% (w/v) sodium phosphotungstate and 0.1 M-ammonium acetate solutions. Similar procedures were used for phage I $_2$ -2, employing strain JE2571(TP114*drd1*) as a source of I $_2$ pili. Magnification calibration was done with catalase.

RESULTS

The third specimen of Pretoria sewage produced a type of plaque on M827-2(R721 *pil* c) not present on the M827-2 control plates. Phage from one of these plaques was named I $_2$ -2 and was propagated on the above plasmid-containing organism. After examining a further 73 weekly sewage samples (68 from Pretoria and environs and 5 from Windhoek) a chloroform-treated specimen from Windhoek yielded a phage that plated on J53-2(R144*drd3*) and LT2*trpA8*(R144*drd3*) but not on isogenic strains lacking the plasmid. The phage was named I α and was propagated on J53-2(R144*drd3*) or J53-2(R64*drd11*).

Properties of the phages

Plaque morphology. Phage I α formed relatively clear plaques about 2 mm in diameter on all its hosts (Table 1). Plaque size was constant and probably indicated the ready availability of adsorption sites (Meynell, 1978).

The plaques of phage I $_2$ -2 were turbid and varied from pin point to about 1 mm in diameter on all hosts. On *E. coli* the plaques were particularly turbid and vague (Table 1).

Phage morphology and adsorption sites. Phage I α was hexagonal in outline, had the semi-transparent appearance of a typical RNA bacteriophage in the electron microscope and was about 24 nm in diameter (Fig. 1). I $_1$ plasmids determine pili that are thin (6 nm), flexible, and aggregate strongly (Bradley, 1980*b*). Aggregates detached from cells were obtained by the method of preparation described and were easily distinguishable from Type I pili and flagella. Phage I α virions were attached to the aggregates in fairly large numbers (Fig. 1). There were a few detached virions in the preparation, but most were in the immediate vicinity of the aggregates; they probably separated from pili during drying. Because of aggregation of the pili, it was difficult to be sure whether the phage virions adsorbed uniformly along the pilus shaft or preferentially near the tip, but occasionally an I $_1$ pilus protruded from an aggregate and I α phages could be seen adsorbed uniformly along the shaft (Fig. 1, arrowed).

Table 1. *Host ranges of phages I α , I $_2$ -2 and PR64FS*

Drops of phage suspensions (titre $3-9 \times 10^9$ p.f.u. ml⁻¹) were spotted on lawns of organisms contained in top-layer agar. The preparation of bacteria temporarily derepressed for pilus synthesis and phage propagation experiments were done as described in Methods.

Host	Plasmid group	Reference(s)*	Phage action†					
			I α		I $_2$ -2		PR64FS	
			Plaques	Phage propagation	Plaques	Phage propagation	Plaques	Phage propagation
J53-2(R724)	B	1	—	—	—	—	—	—
LT2trpA8(R724)	B	1	—	—	—	—	—	—
M827-2(R ¹⁶)	B	2	—	—	—	—	—	—
J53-2(R ¹⁶)	B	2	—	—	—	—	—	—
J53-2(R621a <i>pil</i> ^c)	B (I γ)	3, 4, 5	+	ND	—	—	+	ND
M827-2(R621a <i>pil</i> ^c)	B (I γ)	3, 4, 5	+	ND	—	—	+	ND
J53-2(R64drd11)	I $_1$	6	+	ND	—	—	+	ND
LT2trpA8(R64drd11)	I $_1$	6	+	ND	—	—	+	ND
J53-2(R144drd3)	I $_1$	3	+	ND	—	—	+	ND
LT2trpA8(R144drd3)	I $_1$	3	+	ND	—	—	+	ND
J53-2(R163drd7)	I $_1$	7	+	ND	—	—	+	ND
M827-2(R163drd7)	I $_1$	7	+	ND	—	—	+	ND
J53-2(Collb-P9)	I $_1$	8	—	+	—	—	—	+
M827-2(Collb-P9)	I $_1$	8	—	+	—	—	—	+
J53-2(R483drd)	I $_1$	9, 10	+	ND	—	—	+	ND
M827-2(R483drd)	I $_1$	9, 10	+	ND	—	—	+	ND
J53-2(JR66a)	I $_1$	3, 9, 10, 15	+ ^a	ND	—	—	—	+
M827-2(JR66a)	I $_1$	3, 9, 10, 15	+ ^a	ND	—	—	—	+
J53-2(R805a)	I ζ	1	—	—	—	—	—	+
M827-2(R805a)	I ζ	1	—	—	—	—	—	+
J53-2(TP114drd1)	I $_2$	2, 11	—	—	+ ^b	ND	+	ND
JE2571(TP114drd1)	I $_2$	2, 11	—	—	+ ^b	ND	+	ND
M827-2(TP114drd1)	I $_2$	2, 11	—	—	+	ND	+	ND
J53-2(RM413)	I $_2$ ‡	12	—	—	—	+	—	+
M827-2(RM413)	I $_2$ ‡	12	—	—	—	+	—	+
J53-2(R821a)	I $_2$ §	10	—	—	—	+	—	+
M827-2(R821a)	I $_2$ §	10	—	—	—	+	—	+
J53-2(R721 <i>pil</i> ^c)	I $_2$ §	4, 13, 14	—	—	+ ^b	ND	+	ND
M827-2(R721 <i>pil</i> ^c)	I $_2$ §	4, 13, 14	—	—	+	ND	+	ND
J53-2(R387)	K	2	—	—	—	—	—	—
LT2trpA8(R387)	K	2	—	—	—	—	—	—

ND, Not done.

* References: 1, Falkow *et al.* (1974); 2, Bradley (1980b); 3, Hedges & Datta (1973); 4, Hedges, unpublished; 5, N. Datta (personal communication); 6, Datta *et al.* (1971); 7, Lawn *et al.* (1967); 8, Meynell *et al.* (1968); 9, Datta & Barth (1976); 10, Jacob *et al.* (1977); 11, Grindley *et al.* (1973); 12, Hedges *et al.* (1974); 13, Jobanputra & Datta (1974); 14, H. Richards (personal communication); 15, Datta & Hedges (1973).

† Phage action upon the specified hosts was classified according to the following scheme.

Plaques. The ability of the phages to form visible plaques is recorded: +, formation of plaques to titre of the phage suspension (stocks of phage I α regularly plate on *S. typhimurium* strains with one-tenth of the titre observed on *E. coli* hosts); +^a, formation of plaques observed on bacteria carrying plasmids producing pili constitutively due to physiological derepression (D. E. Bradley, unpublished); +^b, plaques were observed but these were very turbid and vague; —, no visible lysis.

Phage propagation. The ability of phages to undergo increase on the indicated hosts is recorded: +, a minimum 10-fold increase above the negative control on three separate occasions; —, no increase in titre above the negative control.

‡ Formerly assigned to group I α .

§ Formerly assigned to group I δ .

Phage I $_2$ -2 was an unusually thin filamentous virus (diameter about 7 nm), which did not exhibit the usual uniformity of length in the electron microscope (not illustrated), probably because of incomplete assembly and/or breakage. Electron micrographs of random fields were taken, and the lengths of all 138 virions within them were measured. Some Type I pili may have

Fig. 1. Phage I α virions adsorbed to pili synthesized by *E. coli* strain JE2571(R64drd11). The arrow marks a virion adsorbed to a pilus shaft away from the tip. The bar represents 100 nm.

Fig. 2. Length distribution diagram for filamentous phage I₂-2. The length class measurements providing the three modal length peaks are 100–300 nm for the 240 nm peak, 400–500 nm for the 450 nm peak and 800–1000 nm for the 910 nm peak. Three mean values are obtained by averaging each set of measurements (see text).

been included inadvertently, although they were distinguishable by their straight appearance. Immuno-electron microscopy (see Bradley, 1980*b*) showed that the numbers of I₂ pili from the propagating organism were insignificant. The length range of phage I₂-2 was 28–2606 nm. A length distribution diagram showed three modal lengths (Fig. 2), the mean values within their peaks being ($\pm$ s.e.) 212 ± 36 nm, 440 ± 32 nm, and 912 ± 70 nm. While the shortest length class had the most virions, 212 ± 36 nm seemed an unlikely unit length because insufficient genetic information would be available in the virion to code for replication and assembly. Since the 800–1000 nm length class was the second largest (23 measurements), and since most filamen-

tous phages are about 1000 nm long, we believe that phage I₂-2 has a unit length of 912 ± 70 nm (modal length 910 nm).

We were unable to demonstrate the expected adsorption of I₂-2 virions to I₂ pilus aggregates by electron microscopy and it is presumed that adsorption is inefficient. Neither was it possible to resolve any terminal structure on the virion as was found with phage C-2 (Bradley, *et al.*, 1982) and phage fd (Gray *et al.*, 1981).

Sensitivity to organic solvents or RNAase. The titre of phage I α (1×10^9 p.f.u. ml⁻¹) was unaffected by prior exposure to chloroform, whereas similar treatment of phage I₂-2 (titre 5×10^9 p.f.u. ml⁻¹) reduced the titre by >99% (not shown). Titres of both phages were not reduced by prior exposure to diethyl ether. Plaque formation by phage I α on LT2*trpA8*(R144*drd3*) was totally inhibited by RNAase in the medium whereas the phage plated to titre (1×10^9 p.f.u. ml⁻¹) on media lacking RNAase. The plaque-forming titre of phage I₂-2 and the negative control, PR64FS, were not affected by the presence of RNAase in the medium.

Serology. The plaque-forming titre of phage I₂-2 was not reduced by the phage PR64FS antiserum used. This serum neutralized the plaque-forming titres of phages PR64FS and If1 at *K* values of 580 and 248 min⁻¹, respectively. It was concluded that phage I₂-2 is not serologically related to the latter two filamentous phages (see Coetzee *et al.*, 1980).

Host range. In contrast to phage PR64FS, which formed plaques or propagated on host strains harbouring I₁, I₂, I γ or I ζ plasmids, phage I α only infected organisms carrying I₁ plasmids or the I γ plasmid R621a *pi*^h (Table 1). The efficiency of plating of phage I α on the *E. coli* strain was 10-fold higher than on the corresponding *S. typhimurium* hosts. Phage I₂-2 only formed plaques or propagated on bacteria carrying plasmids of the I₂ group (Table 1). The host range of phage I α did not overlap the spectrum of the I₂ phage.

DISCUSSION

The striking feature of the host range of phage I α is that it distinguished pili encoded by the I₁ plasmids and the I γ plasmid R621a from those determined by other plasmids, whilst phage I₂-2 distinguished between I₂ plasmids and members of the I₁ complex (Table 1). Some of the conclusions on the host range of the phages were based on phage propagation rather than upon plaque formation (Table 1), but results were conclusive and precedents for this procedure exist (Datta *et al.*, 1971; Falkow *et al.*, 1974; Bradley, 1977; Grant *et al.*, 1978).

R64, R144 and ColIb-P9 are typical I₁ plasmids (Meynell *et al.*, 1968; Hedges & Datta, 1973) and specify I₁ pili that are closely related, though R64 pili can be distinguished serologically from those of the other plasmids (Lawn & Meynell, 1970). R144 and ColIb-P9 share extensive DNA homology (Falkow *et al.*, 1974) with the I γ plasmid R621a, and pili of R621a are serologically related to I₁ pili (Bradley, 1980*b*). Presumably, much of the DNA homology is in the region determining pilus synthesis. The presence of I α phage receptors on the shafts of pili of all four plasmids is thus explicable. We suppose that R621a is a recombinant between I₁ and B plasmids, determining the pili of the former and incompatibility reactions of the latter. This suggestion is supported by the fact that, like B plasmids (Towner, 1979; H. Richards, personal communication), but unlike those of group I₁ (Coetzee, 1964; Datta & Hedges, 1972), the derivative R621a-1a (Hedges, 1974, 1975, and unpublished) can transfer to *P. mirabilis* strain PM5006. The replication properties of R621a may resemble those of B rather than I₁ plasmids.

The host ranges of phages I₂-2 and I α taken together are equivalent to that of phage PR64FS except for the failure of either phage to lyse bacteria carrying the I ζ plasmid R805a (Table 1). R805a (incompatible with both I₁ and B plasmids) shares >50% polynucleotide sequences with I₁ plasmids and like I₁ plasmids has sequences (about 20%) in common with B plasmids (Falkow *et al.*, 1974). Its pili are serologically related to both I₁ and I₂ pili (Bradley, 1980*b*) and it constitutes a major anomaly in the plasmid classification system. Although B and K plasmids determine pili serologically related to those of I₁ plasmids (Bradley, 1980*b*), these pili are incapable of adsorbing phages If1, PR64FS or I α . Thus, these pili, though similar are not identical.

Shaft-adsorbing phages tend to be more specific than those adsorbing to pilus tips (Coetzee *et al.*, 1979; Coetzee *et al.*, 1980; Sirgel *et al.*, 1981; Bradley *et al.*, 1981*a*; D. E. Bradley, unpublished). In this respect the host range of phage I α resembles that of phage PRR1, which only adsorbs to the shafts of P-1 pili (Olsen & Thomas, 1973), phage C-1, which adsorbs only to shafts of C pili (Sirgel *et al.*, 1981), and phages t and F $_0$ lac (Bradley *et al.*, 1981*b, c*), which adsorb to the terminal shafts of pili produced by T plasmids and the unique plasmid F $_0$ lac, respectively. The adsorption site of phage I $_2$ -2 could not be determined (see Results) and the limited host range of the phage could not be used to predict this site: the filamentous phage Pf3 has a host range limited to bacteria carrying P-1 plasmids and the adsorption site of the phage has not been determined, although this could be somatic (Bradley & Rutherford, 1975; Stanisich, 1974; Bradley, 1978), while the filamentous phage C-2 (D. E. Bradley, unpublished) adsorbs to the sides of shafts of the pili determined by some but not all C plasmids.

The paucity of phage isolates adsorbing to I pili (Meynell, 1973; Coetzee *et al.*, 1980) suggests that such phages are rare. This conclusion is plausible since no plasmid determining constitutive I pilus production has been observed in nature and the maladaptive properties of such synthesis have been demonstrated (Dowman & Meynell, 1970; Romero *et al.*, 1971; Salisbury *et al.*, 1972). Hence, I pili are probably only rarely available and the range of phages adapted to adsorption to such receptors is likely to be limited, and far narrower than, for example, the F complex (Miyake *et al.*, 1969; Dennison & Hedges, 1972).

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