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Identification of an endogenous plasmid in *Dictyostelium discoideum*

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A plasmid has been discovered in a strain of the eukaryote, *Dictyostelium discoideum*, which has an unstable, non-chromosomal, cobalt resistance phenotype. The plasmid, termed Ddp1, is ~13.5 kbp in size and is found in the nucleus. It has an A-T content typical of *Dictyostelium* DNA as judged by its restriction enzyme digestion pattern, and it is not related to either mitochondrial or ribosomal DNA. Similar or identical plasmids have been found in two original, cobalt-sensitive, isolates, NC4 and V12, but no plasmid was detected in three other isolates (WS472, WS526, WS584). The plasmid codes for non-essential functions since it is absent from the latter isolates, and it is lost from mutant strains which are capable of axenic growth.

Key words: cellular slime mold/*Dictyostelium discoideum*/ eukaryotic plasmid/heavy metal resistance/plasmid

Introduction

Plasmids occur widely in prokaryotes. The same has not been shown in eukaryotes although there is one well studied eukaryotic plasmid, the 2 μm circle in *Saccharomyces cerevisiae* (Broach, 1981). Eukaryotic plasmids have also recently been reported in *Podospora anserina* (Kück *et al.*, 1981), and *Fusarium oxysporum* (Guardiola *et al.*, 1982). Furthermore, closed circular DNA of uncertain origin has been reported in man (associated with *Alu* repeats, Calabretta *et al.*, 1982) and *Drosophila melanogaster* (Flavell and Ish-Horowicz, 1981). Here we report the discovery of a plasmid in the cellular slime mold *Dictyostelium discoideum*.

This plasmid was discovered as a result of an apparent association with cobalt resistance in *D. discoideum*. Studies of resistance to heavy metals have produced a series of surprising new phenomena. Arsenate and antimony resistance in *Escherichia coli* is plasmid mediated (Silver and Keach, 1982; Mobley and Rosen, 1982). Cadmium resistance results from amplification of the metallothionein-I gene in mouse cells (Beach and Palmiter, 1981), while copper resistance in *S. cerevisiae* appears to result from a tandem duplication of a chromosomal gene (Fogel and Welch, 1982).

In previous studies cobalt resistance mutants of *D. discoideum* were isolated (Williams and Newell, 1976; Williams, 1978); most of the mutations mapped to the *cobA* locus on linkage group VII (Welker and Williams, 1980). The properties of one particular mutant – instability, high level of resistance and dominance to wild-type (Williams, 1978) led us to suspect that the resistance was plasmid mediated. Here we confirm that this cobalt resistance is not chromosomal and report the existence of a plasmid in the mutant strain, HU32, although we have not yet rigorously established that the cobalt resistance is plasmid mediated.

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Results

Extrachromosomal unstable cobalt resistance

In studies extending over several years a series of cobalt resistance mutations in *D. discoideum* have been isolated and characterised (Welker and Williams, 1980). With one exception, *cob*-354, all cobalt resistance mutations studied map to the *cobA* locus on linkage group VII (Williams, 1978; Welker and Williams, 1980). The *cob*-354 mutation is unstable, being lost when strains carrying it are passaged in the absence of cobaltous chloride [see Williams (1978) for growth on bacteria, Figure 1 for growth in axenic medium]. Genetic studies have failed to locate the *cob*-354 mutation to any known linkage group, and it certainly does not map to linkage group VII (where *cobA* is located). This is clear from the cobalt resistance phenotype of the strains shown in Table I in which all known linkage groups have been exchanged, while *cob*-354 was retained. The unstable nature of the resistance was confirmed by isolating cobalt-sensitive derivatives of strains HU1315 (strain HU1656) and HU1491 (HU1626) after passage on SM agar without cobalt. All other phenotypes of HU1656 and HU1626 were identical with those of HU1315 and HU1491, respectively, (Table I) except for the coumarin sensitivity phenotype (see below).

Discovery of a plasmid in strains carrying the cob-354 mutation

The extrachromosomal location (Table I), instability (Figure 1), high level of resistance and dominance to wild-type (Williams, 1978) indicated that the *cob*-354 phenotype

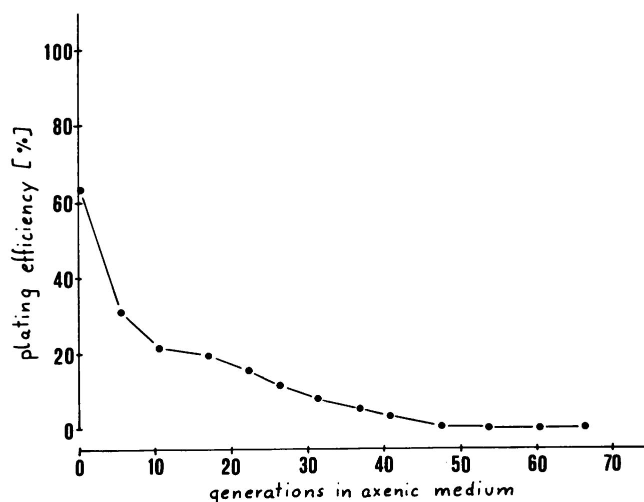

Fig. 1. Loss of cobalt resistance from strain HU32 during growth in axenic medium. Cells from a culture of HU32 growing in axenic medium containing 150 μg/ml $CoCl_2$ were serially passaged in axenic medium (without cobalt) and the plating efficiency (percentage colony formation on SM-agar plates containing 300 μg/ml $CoCl_2$ compared with SM-agar alone) was determined at intervals of ~5 generations. Plating efficiency on SM agar was always close to 100% of the numbers determined by haemacytometer counts.

Table I. Exchange of chromosomes in strains carrying the *cob*-354 mutation

Haploid strain	Parents	Chromosomes exchanged	Linkage group I	II	III	IV	VI	VII
HU32	NP81		+	axeA1 axeC1 oaaA1	axeB1 tsgA1	bwnA1 ebrA1	+	+
HU1159	DU1569 (HU32/ HU892)	I,II,III IV,VII	cycA1	acrA1 tsgD12 whiA1	bsgA5	bwnA1	+	couA351 tsgK21 frtB353
HU1184	DU1716 (HU1159/ AX3)	I,II	+	axeA1 axeC1 oaaA1	bsgA5	bwnA1	+	couA351 tsgK21 frtB353
HU1315	DU1865 (HU1184/ HU485)	III,IV	+	axeA1 axeC1 oaaA1	axeB1	whiC351	+	couA351 tsgK21 frtB353
HU1491	DU2143 (HU1315/ HU526)	I,III VI,VII	cycA1	axeA1 axeC1 oaaA1	bsgA5	whiC351	manA2	+

The *cob*-354 cobalt resistance was transferred from HU32 to HU1491 using parasexual genetic techniques *via* three haploid intermediates: HU1159, HU1184 and HU1315. Strains designated HU, AX or NP are haploid and DU are diploid.

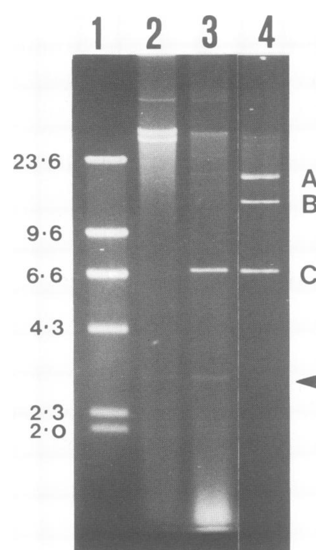

Fig. 2. Undigested plasmid Ddp1 from axenically and bacterially grown cells. *D. discoideum* plasmid from bacterially grown NP161 (no plasmid detected from ~10^8 cells, **lane 2)**, HU32 (~6 x 10^7 cells, **lane 3)** and axenically grown HU32 (~6 x 10^7 cells, **lane 4)**. All three samples were spermine precipitated (Hoopes and McClure, 1981). A, B, C refer to open circular, linear and supercoiled forms, respectively, of plasmid Ddp1. The arrow indicates the 5.5-kbp plasmid from *K. aerogenes* which is present as a faint band in **lanes 2** and **3. Lane 1** is phage λ DNA digested with *Hin*dIII; the size (in kbp) of fragments is given.

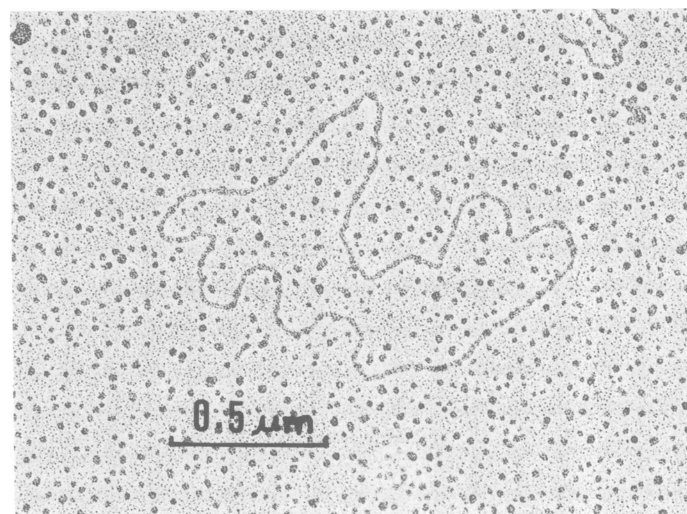

Fig. 3. Electron micrograph of plasmid Ddp1.

might be due to the presence of a plasmid. Using techniques developed for isolating closed circular DNA from bacteria (see Materials and methods), DNA was isolated from HU32 grown with *Klebsiella aerogenes* on medium containing cobaltous chloride. This DNA migrated as three major bands on 0.5% w/v agarose gels.

To exclude the possibility that these bands were due to contaminating *K. aerogenes* plasmid, we decided to grow HU32 in axenic medium. This was not simple as, although HU32 has genes for axenic growth, there was a narrow range of cobalt concentrations which allowed growth and still maintained cobalt resistance. Nevertheless, when HU32 was grown in axenic medium containing 150 µg/ml of cobaltous chloride, the same three major bands were observed when closed circular DNA was isolated (Figure 2, lane 4). These bands correspond to open circle, linear and supercoiled forms of a single plasmid (see below), named Ddp1. Ddp1 DNA extracted from bacterially grown cells was usually enriched in the supercoiled form (Figure 2, lane 3) while the open circle and linear forms were prominent in preparations from axenically grown cells (Figure 2, lane 4). When DNA was isolated from an axenic culture of cobalt-sensitive strain NP161, no plasmid bands were observed (Figure 2, lane 2).

Electron microscopic characterisation of plasmid Ddp1

Plasmid DNA from strain HU32 was prepared for electron microscopy (see Materials and methods) and circles ~4 µm in size were observed (Figure 3). This corresponds to ~13 kbp.

Restriction enzyme digestion of plasmid Ddp1

Since *D. discoideum* DNA has a very high A-T content (Kimmel and Firtel, 1982), it was expected that the plasmid would be cut by restriction enzymes having A-T-rich recognition sequences. In fact there is a strong correlation between

Table II. Restriction enzyme sites in Ddp1

Enzyme	Number of sites in Ddp1	Recognition site %GC
*Hind*III	6	33
*Cla*I[a]; *Nde*I	4	33
*Eco*RI; *Eco*RV	3	33
*Bcl*I[a]; *Bgl*II	2	33
*Hpa*I	1	33
*Kpn*I	3	67
*Pst*I	2	67
*Nco*I; *Pvu*II, *Sph*I	1	67
*Bam*HI[c]; *Pvu*I[c]; *Sal*I[c]	0	67
*Ava*I[c]	0	67 – 100[b]
*Mlu*I; *Sst*I; *Xho*I	0	67
*Bgl*I; *Sma*I; *Xma*III	0	100

[a]Minimum number, as some sites may be protected from digestion by the restriction enzyme due to normal bacterial methylation.
[b]Redundant recognition sequence.
[c]Positive controls were included in these incubations which proves that each enzyme was active. *Mlu*I, *Sst*I, *Xho*I, *Bgl*I, *Sma*I, *Xma*III failed to cut the plasmid but no positive controls were conducted.
Ddp1 was cloned into pBR322 at the SphI site using standard techniques (Ward, unpublished data). The chimeric plasmid, pBMW3, was digested with various restriction enzymes as recommended by their suppliers. The Table is based on examination of 1.5% w/v agarose gels in which all fragments bigger than ~300 bp should be visible.

the A-T content of the restriction site and the degree of cutting (Table II). Twenty three restriction enzymes were tested, 13 of which cut the plasmid at one or more sites (Table II). Figure 4 shows the results of treating Ddp1 with eight restriction enzymes. The data in Table II are, however, largely derived from studies which will be reported elsewhere on Ddp1 cloned in pBR322.

The size of plasmid Ddp1 estimated by summing the restriction fragments is 13 – 14 kbp, in good agreement with that estimated by electron microscopy.

Relationship of the three bands present in plasmid preparations

The relationship of the three major bands seen in plasmid preparations was investigated by isolating each from a preparative gel followed by digestion with three restriction enzymes. All three bands gave the same products. Thus the three bands do not represent three different plasmids. Secondly, band C (Figure 2) was partially converted to band A (Figure 2) in a control incubation containing no restriction enzyme. Thirdly, band B (Figure 2) has the same mobility as the band produced by treating Ddp1 with an enzyme that cuts it once. Finally band C (Figure 2) was enriched in the more dense 'supercoil' region, of CsCl-EtBr equilibrium density gradients. Bands A and B (Figure 2) were enriched in the less dense 'linear' region. From all this evidence we conclude that bands A, B and C represent the open circle, linear and supercoil form, respectively, of a single plasmid.

Subcellular localisation of plasmid Ddp1

Microscopically intact nuclei can be readily isolated from *D. discoideum*. When parallel preparations were made from whole cells and nuclei, the yield of plasmid from the nuclei was similar to that obtained from whole cells. DNA from a crude mitochondrial preparation which was free of nuclei was found not to contain plasmid, although the mitochondrial DNA itself was somewhat degraded. From these experiments we conclude that plasmid Ddp1 is located in the nucleus.

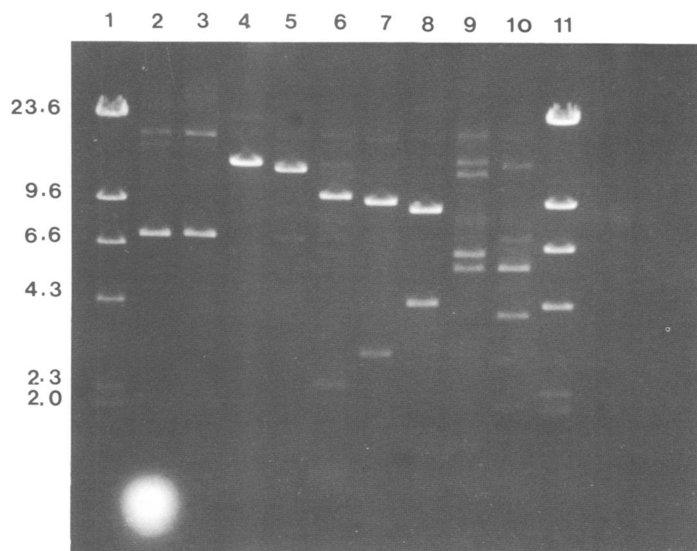

Fig. 4. Restriction enzyme digestion of CsCl-EtBr purified plasmid Ddp1. Ddp1 was treated with RNase (~50 μg/ml), digested with a series of restriction enzymes in buffers recommended by their suppliers, and electrophoresed in a 0.5% w/v agarose gel. Partial digestion products are present as faint bands in most lanes but are most prominent in **lanes 9** and **10**. **Lanes 1** and **11**, *Hind*III digest of λ DNA, fragment sizes in kbp; **lane 2**, untreated Ddp1; **lane 3**, *Sal*I. In other experiments *Sal*I digested pBR322 DNA, which was included in the reaction mixture, without cleaving Ddp1; **lane 4** *Sph*I; **lane 5**, *Bgl*II; **lane 6**, *Eco*RI; **lane 7**, *Eco*RV; **lane 8**, *Pst*I; **lane 9**, *Bcl*I; **lane 10**, *Hind*III.

The plasmid is clearly maintained throughout the asexual life cycle, including spores, as strains are stored as spores lyophilised or desiccated on silica gel.

The origin of plasmid Ddp1 in strain HU32

The isolation of Ddp1 from axenically grown cells made it clear that this is an authentic *D. discoideum* plasmid. We then proceeded to investigate its origin.

Bacterial origin? *D. discoideum* genetics is like bacteriophage genetics – the amoebae form plaques on a bacterial lawn. When the cobalt studies were commenced, it was necessary to isolate a cobalt-resistant strain of the bacterial associate *K. aerogenes* (Williams and Newell, 1976). It was later discovered that the cobalt-resistant strain of *K. aerogenes* contained a plasmid (Li *et al.*, in preparation). At first it was thought that this plasmid carried a gene specifying bacterial cobalt resistance which might have been transferred to *D. discoideum*. This possibility was excluded for the following reasons. (i) The bacterial plasmid [5.5 kbp seen as a faint band (arrow) in Figure 2] has been cloned in both orientations in pBR322 and tested for its ability to confer cobalt resistance to *E. coli*. No increased resistance was found (Li *et al.*, in preparation). (ii) The restriction enzyme digestion pattern of the *K. aerogenes* plasmid DNA is completely different from that of Ddp1 (Li *et al.* in preparation); for example, the *K. aerogenes* plasmid has *Sma*I, *Bgl*I and *Bam*HI sites, but no *Eco*RI, *Pst*I or *Hind*III sites, whereas the reverse is true for Ddp1. (iii) Southern analysis using nick-translated Ddp1 showed no hybridisation to the *K. aerogenes* plasmid or other *Klebsiella* sequences. Control experiments with nick-translated pBR322 sequences gave hybridisation with the *Klebsiella* plasmid.

Relationship to other D.discoideum repeated DNA. Plasmid Ddp1 yielded restriction fragments differing in length from those of mitochondrial DNA and ribosomal DNA of *D. discoideum* when the three were digested with a

series of restriction enzymes. In addition, no hybridisation was observed on Southern analysis between plasmid Ddp1 and mitochondrial or ribosomal DNA bands obtained by *Eco*RI digestion of whole cell DNA of strain AX3. Hence Ddp1 is not related to the other repeated extrachromosomal DNA sequences in *D. discoideum.*

The plasmid occurs in other strains. In preliminary experiments we failed to detect a chromosomal copy of plasmid Ddp1, so its origin was unclear until we discovered that other (cobalt-sensitive) strains carry plasmid. In further experiments, a plasmid was isolated from cobalt-sensitive strain X22. This plasmid had the same restriction pattern as Ddp1 using eight restriction enzymes and so we conclude that the two plasmids are very similar if not identical. Both strains HU32 and X22 are derived from the type strain NC4 isolated by Raper in 1933 (Raper, 1935). We investigated an early stock of NC4 (see Materials and methods) and also found a similar plasmid in this strain. A separate wild isolate, strain V12, which is of opposite mating type, was also found to carry a plasmid which is the same as Ddp1 on the basis of limited restriction analysis. Three other wild isolates WS472, WS526, WS584 (Erdos *et al.*, 1973) did not carry plasmid detectable by our standard isolation techniques.

Relationship between axenic strains and plasmid curing

Although it is possible to grow *D. discoideum* in a fully defined medium (Franke and Kessin, 1977), the selection of strains capable of axenic growth (without bacteria) is difficult. Only a small number of independently isolated axenic strains exist (Sussman and Sussman, 1967; Watts and Ashworth, 1970; Loomis, 1971; Williams, 1976), because at least three chromosomal mutations are required for rapid axenic growth (Williams *et al.*, 1974; North and Williams, 1978). We have attempted to isolate plasmid from two axenic mutants – strains AX3 (Loomis, 1971), which is derived from the NC4 isolate, and HU1231 (a derivative of strain NP84; North and Williams, 1978) which is derived from the V12 isolate. In neither strain have we detected plasmid. This may explain why others have not observed plasmid Ddp1, as axenically grown cells are used extensively in biochemical and molecular studies.

Our hypothesis is that the plasmid is in some way deleterious for axenic growth (see below) and that loss of the plasmid was one step in the selection of axenic mutants. However, the plasmid can be reinserted into an axenic genetic background using parasexual techniques. In early experiments we were unable to detect plasmid in either the axenic strain X2 (essentially the parent of HU32) or HU978 (a cobalt-sensitive derivative of HU32). However, the cells used in these experiments had been extensively passaged in axenic medium. Recently we have been able to isolate plasmid from newly established axenic cultures of X2 and HU978. We conclude that selective conditions are required for maintenance of the plasmid during extended axenic growth. Strain X2 is derived from a cross between an AX3-derived axenic strain and a non-axenic strain M28, which contains plasmid. This explains the presence of the plasmid in strains X2 and its derivatives HU32 and HU978.

Is the cobalt resistance phenotype associated with plasmid?

The genetic evidence presented in Table I suggests a relationship between cobalt resistance and the presence of the plasmid. Cobalt-resistant strains HU1315 and HU1491 both carry the plasmid, while the plasmid was not detected in cured, cobalt-sensitive, derivatives HU1656 and HU1626, respectively. However, the discovery of a similar plasmid in standard cobalt-sensitive strains makes the issue more complicated. Our initial studies suggested that the copy number of the plasmid in HU32 grown axenically in the presence of cobaltous chloride was ~100/cell. More recent studies suggest the copy number in cobalt-sensitive strains may be considerably less than this. Experiments aimed at determining the precise copy number as well as studies on transcription and translation of plasmid in several strains are in progress. Thus, we hope to elucidate the relationship between copy number, expression of the plasmid and cobalt resistance.

A second phenotype associated with the plasmid is the suppression of *couA*351, a coumarin sensitivity mutation in *D. discoideum* (Welker and Williams, 1982). Strain HU1315 (Table I) which is cobalt resistant, carries Ddp1, and should be coumarin-sensitive (*couA*351), had a 40% plating efficiency on SM agar containing 1.3 mM coumarin; i.e., the *couA*351 phenotype was not expressed. The cured strain HU1656 plated at 1.6% on 1.3 mM coumarin. Similar results were obtained with other pairs of cobalt resistant and cured strains.

Discussion

This paper reports the discovery of a closed circular duplex DNA molecule of ~13–14 kbp (~4 μm), which we call Ddp1, in the cellular slime mold *D. discoideum.* Ddp1 or closely related plasmids are found in different wild isolates. They do not seem to code for essential functions since some wild isolates appear not to have the plasmid and long term growth in axenic medium results in its loss. Ddp1 is located in the nucleus and can be transferred between strains by parasexual genetic techniques. Ddp1 was discovered in a strain, HU32, that is unstably cobalt resistant, and at first it was thought that the plasmid carried a cobalt resistance gene. Our current interpretation is that the resistance arises in a less specific manner, such as a membrane alteration. Firstly, the plasmid also suppresses coumarin sensitivity associated with the *couA*351 mutation. Secondly, the loss of the plasmid is associated with selection for the ability to grow in axenic medium, which undoubtedly requires membrane alterations (North and Williams, 1978). However, Ddp1 can be inserted into an axenic genetic background and maintained indefinitely in a strain growing axenically under selective pressure (cobalt chloride) and for a short time in strains not under selection. All axenic strains appear to lose the plasmid on prolonged passage without selection, particularly in axenic medium.

Autonomously replicating double-stranded DNA molecules are not well characterised in eukaryotes. The best understood example is the 2 μm circle in yeast (Broach, 1981) and Ddp1 shares some of its features, e.g., lack of essential functions, presence in some but not all isolates, nuclear location. However, the two plasmids show no homology on the basis of Southern analysis. The yeast 2 μm circle has been useful in the construction of cloning vectors in *S. cerevisiae* (Struhl *et al.*, 1979) and we hope to use the Ddp1 plasmid for similar studies in *D. discoideum*.

A brief report of some of this work has been published elsewhere (Metz *et al.*, 1983).

Materials and methods

Strains

D. discoideum strains derived from the following five wild isolates were

used: NC4, *matA*1 (Raper, 1935); V12, *mata*2 (Raper, 1951); WS472, *matA*1659; WS526, WS584, asexual strains (Erdos *et al.*, 1973). The NC4 and V12 isolates are very early stocks obtained from K.B.Raper through R.H.Kessin. NC4 was isolated in 1933 (Raper, 1935) and maintained until 1941 when it was lyophilised. This stock was subsequently relyophilised in 1948 and 1954 and our stock came from a 1954 lyophil (Raper, personal communication). Strain V12 was isolated in 1937, passaged until 1941 and then lyophilised. This was subsequently relyophilised in 1954 and the stock used was derived from one of these lyophils (Raper, personal communication). Strains NP161 and HU1231 are capable of growth in axenic medium and are derived from strain NP84 (North and Williams, 1978), a growth temperature-sensitive (*tsgN*350), axenic (*axe*-352) derivative of strain V12 (mating type *mata*2). Strain HU32 (Williams, 1978) (*bwnA*1, *axeA*1, *axeB*1, *axeC*1, *oaaA*1, *tsgA*1, *cob*-354, *ebrA*1), a parent strain X2 (Williams *et al.*, 1974) *bwnA*1, *axeA*1, *axeB*1, *axeC*1, *oaaA*1, *tsgA*1) and a cured (cobalt-sensitive) derivative of HU32, HU978, (*bwnA*1, *axeA*1, *axeB*1, *axeC*1, *oaaA*1, *ebrA*1, *tsgA*1) were all capable of growth in axenic medium. The genotypes of the following strains are already published: M28 (Katz and Sussman, 1972), X22 (Williams and Newell, 1976), AX3 (Loomis, 1971; North and Williams, 1978). The genotypes of the strains used to establish that *cob*-354 was not a chromosomal mutation are listed in Table I.

When *D. discoideum* was grown on bacteria, a cobalt-resistant mutant of *Klebsiella aerogenes* was used (Williams and Newell, 1976).

Media

D. discoideum amoebae were grown either in axenic medium (Watts and Ashworth, 1970), per litre: oxoid bacteriological peptone, 14.3 g; oxoid yeast extract, 7.15 g; D-glucose 15.4 g; $Na_2HPO_4.12H_2O$, 1.28 g; KH_2PO_4, 0.48 g; dihydrostreptomycin sulphate 250 mg, pH 6.5 or in association with *K. aerogenes* on solid SM agar, per litre: D-glucose, 10 g; oxoid bacteriological peptone, 10 g; oxoid yeast extract, 1 g; $MgSO_4.7H_2O$, 1 g; KH_2PO_4, 2.2 g; $K_2HPO_4.3H_2O$, 1.3 g; Bacto-Difco agar 13 g. Cobaltous chloride (May & Baker analytical reagent grade $CoCl_2.6H_2O$) was added from a sterile solution (100 mg/ml) to agar (60°C) or axenic medium after autoclaving. The levels of $CoCl_2.6H_2O$ used were 300 μg/ml in solid medium and 150 μg/ml in liquid axenic medium. Note that toxicity of cobaltous chloride may vary if the source of the nutrients is different from that specified above (Williams, 1978).

Genetic techniques

Standard techniques for parasexual genetic analysis and handling of cobalt mutants were used (Williams, 1978; Welker and Williams, 1980, 1982). When HU32 was grown in axenic medium containing 150 μg/ml $CoCl_2$, amoebae grown on SM agar containing 300 μg/ml $CoCl_2$ were inoculated at $10^5 - 2 \times 10^5$/ml. In this medium the doubling time was usually ~20 h.

DNA preparation from D. discoideum

Plasmid isolation. Plasmid DNA was isolated from bacterially or axenically grown cells by a modification of the alkaline-SDS method (Birnboim and Doly, 1979). The cells were washed free of culture medium or bacteria by three centrifugations (250 g x 3 min) involving washes with salt solution (0.6 g NaCl; 0.75 g KCl, 0.3 $CaCl_2$ per litre water). The pellet of cells, usually ~5×10^9 cells, was resuspended in between one and four pellet volumes of cold TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) plus 50 mM glucose and 1% diethylpyrocarbonate (Fluka). An equal volume of alkaline SDS (0.2 N NaOH, 1% w/v SDS, <1 week old) was added and the lysate stood on ice for 15 min. Then 3 M Na, 6 M acetate (pH 4.8) was added (3/4 volume of alkali) and the mixture stood on ice for 2 h. The mixture was centrifuged (12 000 *g* x 20 min), the supernatant ethanol precipitated (2 volumes of ethanol) and redissolved in a small volume of TE buffer. These preparations, especially from axenic cells, contain large amounts of white flocculant material that precipitates at 4°C. We believe this to be carbohydrate. It was removed either by spermine precipitation (Hoopes and McClure, 1981) or CsCl-EtBr centrifugation.

Whole cell DNA. The method of Jacobson (1976) was used.

Nuclear and mitochondrial DNA. Nuclei were prepared by detergent lysis and differential centrifugation (Jacobson, 1976) and mitochondria were obtained from the post-nuclear supernatant. DNA was then prepared as for whole cells.

DNA preparation from K. aerogenes. Standard methods were used to prepare plasmid and total DNA from *K. aerogenes* (Clewell and Helinski, 1969).

Electron microscopy of plasmid

The plasmid was spread and analysed in the electron microscope as described by Ferguson and Davis (1978).

Electrophoresis and hybridisation

DNA samples were electrophoresed through 0.5% w/v agarose gels (Loening, 1969) and stained with ethidium bromide. For Southern (1975) hybridisation, gels were transferred to nitrocellulose filters (Schleicher & Schüll, BA85), DNA probes (pBR322, Ddp1 or pBMW3) labelled by nick-translation (Rigby *et al.*, 1977) with [α-^{32}P]dATP (NEN) and hybridised to immobilised DNA by the methods of Wahl *et al.* (1979).

Enzymes

Restriction enzymes were obtained from Boehringer, New England Biolabs or Bethesda Research Laboratories, and used as described by the manufacturers. Phage λ *Hind*III DNA mol. wt. standards were from Boehringer.

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