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Characterisation of a flocculating strain of *Pseudomonas putida*

by

Marian Corkill

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of the requirements for the degree of
Master of Science**

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Abstract

Autoflocculation is a feature of some strains of *Pseudomonas putida* , a bacterium which is of interest commercially because it synthesises dehalogenases. Using shake flask cultures, it was shown that flocculation resulted from growth in magnesium-depleted conditions and the suggestion is made that flocculating strains of *P. putida* could have higher magnesium requirements than non-flocculating strains. Electron microscope evidence points towards some form of bridging mechanism for flocculation, possibly involving secreted polymers, rather than neutralisation of surface charge. Changes in cell shape were also observed in both flocculating and non-flocculating cultures grown in magnesium-depleted conditions. Measurements of floc strength and re-formation of flocs after break-up tend to support the idea that flocculation could be of use in commercial downstream separation of cells.

Contents

Abbreviations

L-broth	Luria broth
S2	Seed 2 minimal medium
SDS	sodium dodecyl sulphate
EDTA	ethylenediaminetetra-acetic acid
L-CPA	L-chloropropionic acid
TEMED	tetramethylethylene diamine
A_{600}	absorbance at 600 nm

Introduction

1.1 Methods of separating liquids and solids: why flocculate?

Many processes require the separation of a suspension into solid and liquid fractions, in order to recover one (or sometimes both) of the fractions with minimal contamination by the other. Principal separation techniques include electrical, mechanical and gravitational methods and those involving surface actions. On a large scale, some of these processes are impractical or not economically sound because they involve too high an energy input, the commonest methods in use being centrifugation and filtration.

Centrifugation is generally the preferred technique where the solid fraction of the suspension is the one to be retained. The rate of sedimentation of particles in a centrifuge depends on the square of the particle diameter, the viscosity of the suspending fluid and the difference in density between particles and liquid, such that V= d2(sp-sl)g /18μ, where V =sedimentation velocity, d = diameter of spherical particles, μ = viscosity of liquid, sp = density of particles, sl = density of liquid and g = gravitational force.

Where the particles are very small and have a similar density to that of the liquid, for example *Pseudomonas* sp. cells grown in broth, centrifugation is often not a very efficient method of separation. One way to improve the sedimentation rate would be to increase the particle size and this can be done by flocculation - making the particles come together and then adhere to form aggregates.

If, on the other hand, filtration is used as the separation technique, very small particles may quickly block the filter. Flocs are larger and also pack on to the filter

in a different way from single particles, so flocculation may improve the flow rate of the filtrate by making the filter more porous (Cumming, 1987). Flocculation could therefore be a useful step in recovery of solids or liquids by either of these two main techniques.

1.2 Causes of flocculation

1.2.1 Stable suspensions.

Particles in suspension, such as microbial cells grown in liquid culture, are maintained in a stable state by the surface charges on the particles themselves. One type of particle will have a certain net electrical charge, positive or negative, resulting from the ions present on its surface. (Microbial cells, for example, when suspended in a medium of neutral pH, carry an overall negative charge.) This attracts a layer of oppositely-charged ions from the suspending liquid which stay very close to the particle surface and, in turn, attract a more diffuse layer of counterions extending out into the liquid and forming together the so-called ionic "double layer". The particles also experience attractive Van der Waals forces but, while the suspension is stable, the diffuse layer keeps the particles sufficiently far apart for these forces not to be significant.

Flocculation will only be possible if the particle surface charge can be lowered sufficiently for the particles to come close enough together to adhere or if some bridging mechanism operates between particles, in which case the repulsive forces can still be present as the particles can remain further apart. The attractive Van der Waals forces are fixed and cannot be manipulated.

1.2.2 Addition of flocculating agents.

Flocculation can be induced in both non-living particles and micro-organisms by the addition of flocculating agents such as lime, aluminium sulphate or organic cations. These agents act in one of three ways: by compressing the ionic double

layer around the particles as a result of charge neutralisation, by specific ion adsorption, where particular counterions become complexed with certain ions on the surface and reduce the charge sufficiently for the particles to come close enough together to adhere, or by sweep flocculation, in which hydrolysing metal salts are added and an insoluble hydroxide precipitates, trapping the particles as it settles. In many instances, it seems that more than one mechanism may be operating simultaneously (Halverson and Panzer, 1980). Addition of acid to a bacterial culture, for instance, will reduce the number of charged carboxyl and other anionic groups in the cell walls and thus lower the negative surface charge, leading eventually to flocculation. The iso-electric point for Gram-negative bacteria is generally between 2.0 and 3.8 (Harden and Harris, 1953).

1.2.3 Presence of polymers.

The water supply industry uses synthetic organic chemicals for clarification of water; some of these polymers, eg. sodium polyacrylate, poly(sodium styrene sulphonate), also flocculate bacteria, this time by the "patch"method or by cell bridging. Water-soluble polymers having some sub-units of the molecule charged are known as polyelectrolytes, overall positive charges producing cationic polymers and negative charges anionic ones. Some polymers, such as pure polyacrylamide, are uncharged (nonionic) but can still induce flocculation. The patch method of flocculation involves adsorption of a cationic or anionic polymer having a high charge density along its chain to localised areas of the particle surface. Positive and negative regions of the surface can therefore attract each other and flocculation occurs. If the optimum polymer concentration is exceeded, the suspension restabilises. Nonionic polymers usually function by bridging mechanisms. The molecules are large and linear and tend to form loops and free ends as they adsorb to particles. These stretches can attach to other particles and hold them together without the need for reduction in surface charge. In this case, no reversal

of flocculation occurs with high polymer concentration.

Adding a chemical to induce flocculation in a fermenter, however, would then require further downstream processing to remove it. It would be preferable to employ natural flocculation, if the conditions inducing it could be sufficiently accurately identified.

1.3 Methods of measuring flocculation.

Measurement of flocculation can be based on reduction in particle numbers, increase in particle size or settling of the flocs (Cumming, 1987). Direct measurement of particle size may be made using light scattering techniques, microscopic measurements with an automatic image analyser or by enlarging photographs, or a Coulter counter. The last measures the change in electrical resistance of a suspension as it flows between two electrodes, the difference in resistance between solid particles and liquid being a function of particle size. Sedimentation measurements are only an indirect way of assessing flocculation but the most popular method used, nevertheless, has been measuring the clarity of the supernatant after a period of settling of the suspension, using optical density. It is also possible to use sediment volume as a measure of flocculation, this being larger if flocs have formed because of the spaces within the flocs which themselves are relatively incompressible.

Another physical parameter of interest if flocculation is to be used as a major separation process is floc strength. Non-compressible flocs are best for filtration and strong flocs are needed for centrifugation and other handling stages such as pumping. Centrifugation will produce shear forces on the flocs as the particles accelerate through the liquid and while the liquid itself flows through the entry ports into the centrifuge, causing turbulence effects. If the flocs break up under shear, less solid material will be recoverable from the centrifuge. A measure of floc strength can be obtained by comparing flocculation before and after treatment of

the suspension in a blender or homogenizer.

1.4 Industrial applications of microbial flocculation.

Two industries already make use of microbial flocculation on a large scale, brewing and waste water treatment. In beer production, ale yeasts flocculate spontaneously (autoflocculation) towards the end of fermentation, provided calcium ions are present. Some yeasts sink, other types trap gas bubbles as they flocculate and rise to the surface. In either case, it is relatively easy to remove the yeast from the liquid. In the activated sludge method of sewage treatment, bacteria of various kinds flocculate in the aerated waste water and, having removed the waste materials, can be easily separated from the purified liquid by sedimentation.

Autoflocculation has also been observed in micro-algae grown in shallow waste oxidation ponds in high light intensity, usually in association with a high pH (Oswald and Golueke, 1968; Eisenberg *et al*, 1981). Flocculation is thus a characteristic of a wide variety of micro-organisms.

1.5 Genetic control of microbial flocculation.

Flocculation is known to be under polygenic control in yeast, environmental factors further affecting gene expression (see below). In this context, it is interesting to note the ability of cycloheximide (an inhibitor of protein synthesis) to prevent floc formation in a flocculating strain of yeast, confirming that acquisition of floc-forming ability requires cytoplasmic protein synthesis (Beavan *et al*, 1979).

Cell aggregation of *Escherichia coli* in liquid culture is also genetically controlled. A gene known as *mor* controls variations in surface properties of the cells, such that mutants with the *mor* gene deleted grow into colonies with rough crenated edges and in liquid culture will form clumps and sediment. The gene has been cloned and transferred via a plasmid to *mor* -deficient *E. coli* where it fixes the colony phenotype as smooth and the suspension as non-flocculating (Warne,

1988).

Unless the ability to flocculate confers some selective advantage, however, it is likely to be lost from a culture over successive generations. The two industrial processes currently employing autoflocculation, activated sludge treatment and brewing, both exert selective pressures in this way by recycling flocculated cells.

1.6 The bacterial envelope

1.6.1 Chemical structure

The nature of the microbial surface is clearly a major factor in flocculation. Gram-negative bacteria have a complex cell envelope with four layers, an inner cytoplasmic membrane composed of phospholipids and proteins, a layer of peptidoglycan, the periplasmic space containing soluble proteins and oligosaccharides and an outer membrane, sometimes referred to as the cell wall. The outer membrane contains a high proportion of lipoproteins and lipopolysaccharides with the polysaccharide chains, usually uncharged, extending outwards from the cell surface. The lipopolysaccharides, characteristic of Gram-negative bacteria, are amphipathic, having a hydrophobic fraction, lipid A, and a hydrophilic sugar chain, usually substituted with O-antigen. In some species, colonies with O-antigen exhibit smooth morphology while those without O-antigen appear rough (Lugtenberg and van Alphen, 1983). Divalent cations have a high affinity for lipopolysaccharides which contain binding sites for both magnesium and calcium, probably phosphate groups in the lipid A and polysaccharide backbone regions. Cationic bridges between such groups are thought to be required for maintenance of the normal structural organisation of the outer membrane.

Detailed structure of the bacterial envelope, especially the outer membrane, has been shown to vary considerably according to the environment. For example, a marine pseudomonad secreted a polysaccharide under conditions of starvation (ie.

absence of carbon and nitrogen sources) which made the cells more hydrophilic and less liable to adhere (Wrangstadh *et al*, 1986). Many experiments have been performed on nutrient deprivation, including work on high salt concentration and depletion of cations such as iron and magnesium. Various effects are described, ranging from changes in the phospholipid and lipopolysaccharide content of the cell envelope to production of exopolysaccharides and induction of outer membrane protein synthesis (Brown and Williams, 1985). This type of study may have particular relevance to fermenter growth of micro-organisms where the nutrient supply can be accurately controlled.

Also present in many bacteria are numerous fimbriae or pili, rigid cylindrical rods of protein typically 0.2 - 20μm long and 5 - 25nm in diameter, extending outwards from the cell wall. These have roles in the transfer of nucleic acids between bacteria (the so-called "sex pili") and in adhesion of the cells to other surfaces. Studies of the attachment of fimbriated *E. coli* to mammalian epithelial cells showed that adhesion was influenced by D-mannose, methyl α -D-mannopyranoside and mannan and also the lectin, concanavalin A, suggesting that binding is mediated by a mannose-specific, lectin-like substance in the bacterial envelope (Ofek *et al*, 1977). Cousland's work on flocculating and non-flocculating strains of *Pseudomonas putida* failed to find any differences between the strains in respect of lipopolysaccharide composition, nor were fimbriae discernible in either strain (Cousland, unpublished). Lack of fimbriae in this species of *Pseudomonas* had earlier been reported by Fuerst and Hayward (1969).

1.6.2 Cell surface charge

The overall negative surface charge carried by microbial cells results mainly from the high proportion of phosphate and carboxyl groups in the cell walls. This surface charge density can be calculated via the zeta potential, the electric potential in the liquid very close to the cell surface where there is no movement

of liquid in an electric field. The diffuse layer of ions is assumed to be stripped away, leaving the inner or Stern layer of ions, and the zeta potential is measured at this point, the shear boundary. Measurements made on bacteria and yeasts show them to have a zeta potential of between -10 and -40mV at pH 7 (George, 1986). The actual value varies with pH, the ionic strength of the suspending fluid and the type of electrolyte ions present and can be used to draw conclusions about the biophysical nature of the cell surface. Zeta potential (or more usually, in practice, electrophoretic mobility) has been correlated with flocculation in a number of studies (Eriksson and Axberg, 1981; Cumming,1987) but the relationship has not always been conclusive.

1.6.3 Hydrophobicity

As well as ionic effects, nonionic forces (Van der Waals forces) affect the behaviour of cells in suspension. Collectively, these forces may be termed "surface energy"and they provide the cells with hydrophobic characteristics, leading to a tendency for the particles to aggregate. Quantitative measurements of cell surface energy can be given via (i) contact angles of drops of liquid on the surface of a layer of cells or (ii) partition coefficients of cells between immiscible liquid phases. A direct relationship has been demonstrated between these two parameters (Gerson, 1980; Gerson and Akit, 1980).

Gram-negative bacteria may have a more hydrophobic cell surface than Gram-positive bacteria owing to the high proportions of lipopolysaccharide and lipoprotein in their outer membrane. Differences in hydrophobicity between species could be linked to differences in lipopolysaccharide content and this could lead to changes in the surface interactions of the cells, such as flocculating behaviour.

1.7 Environmental control of microbial flocculation

Chemicals which can induce flocculation in a variety of suspensions have been

previously described (Sections 1.2.2 and 1.2.3) but the dynamic nature of microbial cultures means that many more variables may have a role in cell aggregation than with a non-living system. The culture medium itself may be complex, the stage of growth of the organisms may be significant and, in batch culture, the composition of the medium is constantly changing.

Flocculation is most likely to occur during late logarithmic phase or stationary phase of batch culture (Cumming, 1987). The cell concentration is high, so cells will collide more frequently through Brownian motion, even if the culture is not stirred. In certain yeast strains, which would flocculate in stationary phase but not in mid-logarithmic phase, the carboxyl group density in the cell walls apparently increased with growth to a threshold point above which flocculation could occur (Beavan *et al*, 1979). Cells at this stage in growth are also more likely to leak proteins and nucleic acids into the medium, both of which can cause flocculation in some bacteria (Sakka and Takahasi, 1981; Sakka *et al*, 1984). Activated sludge flocs have been shown to contain proteins, nucleic acids and considerable quantities of extra-cellular polymeric material (Harris and Mitchell, 1973). Polymers have been implicated in a number of studies, acting either by bridging or patch adsorption (Cumming, 1987). In lager yeasts, for example, bridges form between calcium ions and anionic groupings, probably carboxyl fractions, in cell surface polymers (Beavan *et al*, 1979; Bowen and Cooke, 1989). Flocculent bacteria isolated from activated sludge have been shown to secrete cellulose, forming fibrils in which the cells can become entrapped (Deinema and Zevenhuizen, 1971). The mere presence of macro-molecules in a culture medium is not evidence of their involvement in flocculation but some workers have been able to show that removal of the polymers, for instance by washing or by enzymic breakdown, prevents flocculation. Thus, high levels of cellulase or proteinase K broke up flocs of *Pseudomonas putida* (Cousland, unpublished).

It has been suggested that the presence of inducers in the growth medium might be essential for flocculation in some strains of yeast. A study of ale yeasts showed that some strains would not flocculate in a chemically defined medium, indicating the need for an extracellular flocculating agent. A specific peptide fraction, rich in acidic amino acid residues, has been isolated from wort which was able to bring about flocculation, possibly through bridging with divalent cations (Stewart *et al*, 1973; Bowen and Cooke, 1989). The nature of the culture medium has been shown to affect flocculation in *Pseudomonas putida* also; strains which would flocculate in Luria broth did not do so in Seed 2 medium (Cousland, unpublished). Cousland also found that the tryptone component of the Luria broth from different suppliers affected the response; non-flocculating strains flocculated in tryptone supplied by Difco but not in tryptone from Oxoid.

1.8 *Pseudomonas putida* : a case for flocculation?

Pseudomonas putida is a Gram-negative rod-shaped bacterium with polar flagella. It occurs naturally in water and in putrefying materials but is non-pathogenic to humans, being killed at temperatures above 34^0C. Its respiration is aerobic and its nutritional requirements fairly simple, nitrates sufficing as a nitrogen source and a single organic compound as a source of carbon and energy.

Some strains of *P. putida* can grow on halogenated alkanoic acids as their carbon source by producing dehalogenases which convert the substrates to non-halogenated forms. Thus:-

$$R.CH.X.COOH + OH = R.CHOH.COOH + X$$

where X = halogen and R = H, CH_3 or $CH_3.CH_2$

The rate of the reaction can be determined by measuring the free halogen (usually chloride) ion concentration in a cell-free extract sample (Weightman and Slater, 1980). Along with hydrolysis of the 2-halo-carboxylic acid to produce a

2-hydroxy acid goes inversion of the configuration, eg. L-2-chloropropionic acid (L-CPA) is converted to D-lactic acid (Motosugi *et al*, 1982). L-CPA is an intermediate in the production of a number of herbicides including the ICI product, "Fusilade". Isolation of L-CPA from a racemic mixture by enzymic transformation is a potential alternative to currently available manufacturing processes.

Dehalogenase activity has been identified in a number of *P. putida* strains; some, such as AJ-1, produce two dehalogenases and can metabolise both optical isomers of chloropropionic acid but a chemical mutant of this strain, AJ-1/23, has been isolated which can hydrolyse only D-CPA. Production of L-CPA from a mixture of D and L forms is therefore possible using this specific dehalogenase. As the dehalogenase is retained in the cells, rather than being secreted into the medium, recovery of the enzyme would involve initial recovery of the cells. An efficient method of solid/liquid separation must therefore be employed downstream of the fermenter. *Pseudomonas* cells are very small and can be difficult to separate in a centrifuge but the discovery of autoflocculating strains of *P. putida* has opened a way for flocculation to lead to more effective centrifugation.

1.9 Aims of the project.

Before it can be determined whether or not flocculation has any potential as a means of separation for *Pseudomonas putida* , the conditions governing flocculation must be thoroughly understood. The project was designed to investigate the environmental factors affecting flocculation and to discover, as far as possible, the mechanism behind the process. This involved attempting to identify significant components of the culture medium, comparison of the physiology and cell wall chemistry of flocculating and non-flocculating strains and physical studies of the flocs themselves. Some electron microscope work was also undertaken to look at surface structures.

Materials and methods

2.1 Organisms and culture methods

Pseudomonas putida non-flocculating strains AJ-1 and AJ-1/23 and flocculating strains AJ-1 C-4 and AJ-1/23 C-2 were provided by ICI. Storage was in glycerol in the deep freeze. A freeze-dried sample of flocculating strain AJ-1/23 C-2 F-1 was obtained from B. Cousland. Stock plates were maintained on nutrient agar at 26^0C. Broth cultures were grown at 26^0C in 250ml baffled conical flasks on an orbital shaker at 150 rpm, each flask containing 50ml of culture medium.

To ensure that the ability of *P. putida* strains to autoflocculate was not lost by repeated plate-to-plate culturing, the stock plates were tested at intervals by growing up the bacteria in L-broth and fresh stock plates were made from a known flocculating culture.

2.2 Bacteriological media

2.2.1 Luria broth (L-broth)

Tryptone	$10g.l^{-1}$
Yeast extract	$5g.l^{-1}$
NaCl	$5g.l^{-1}$

Tryptone was Tryptone T from Oxoid unless otherwise stated.

2.2.2 Seed 2 minimal medium (S2 medium)

Phosphate buffer, pH 7.2	20ml
Ammonium sulphate (36% w/v)	5ml
Magnesium sulphate (40% w/v)	0.5ml
Iron (III) chloride (0.972%)	0.5ml
Fisons trace elements	1ml

All above made up to 1 litre with distilled water.

Phosphate buffer: 95g K_2HPO_4 and 78g NaH_2PO_4 in 1 litre distilled water

Fisons trace elements:	calcium	720ppm
	zinc	22ppm
	manganese	25ppm
	copper	5ppm

After autoclaving, this has glucose added to a final concentration of 0.5%.

2.3 Buffers, stains and solutions

2.3.1 Phosphate buffer diluent

1 litre 50mM K_2HPO_4 has added to it 50mM KH_2PO_4 until the pH is 7.4

2.3.2 Gram staining procedure for bacteria

Crystal violet	0.5% w/v in distilled water
Lugol's iodine	5g iodine + 10g potassium iodide in 100ml distilled water. Diluted for use 1 in 5 with distilled water.
Acetone/ethanol	50:50 mixture
Neutral red	1g neutral red + 2ml 1% acetic acid in 1 litre distilled water

1. Fix bacterial smear by heating slide gently. 2. Cover slide in crystal violet for 1 minute. Wash with water. 3. Cover slide with iodine for 1 minute. Wash with water. 4. Rinse with acetone/ethanol until no more violet colour appears in

solvent. Wash with water. 5. Cover with neutral red for one minute. Wash with water and blot dry.

2.3.3 Stains used for lipid analysis

All the reagents were obtained from Sigma Ltd. (i) Dragendorff's reagent: 0.11M potassium iodide and 0.6mM bismuth subnitrate in 3.5M acetic acid. Supplied ready-made. (ii) Molybdenum blue: 1.3% molybdenum oxide in 4.2M sulphuric acid. Supplied ready-made. (iii) Ninhydrin: 0.2% solution in ethanol, freshly-made.

2.3.4 SDS solubilizing buffer

Tris HCl	0.355g
Tris Base	0.212g
EDTA	0.148g
SDS	10g
B-mercaptoethanol	20ml

Made up to 200ml with distilled water. For use, dilute to half stock strength.

2.3.5 Slab gel for electrophoresis: SDS discontinuous system

(i) Stacking gel

Acrylamide - bis-acrylamide (30:0.8)	2.5ml
Stacking gel buffer (0.5M Tris-HCl, pH 6.8)	5.0ml
10% SDS	0.2ml
1.5% ammonium persulphate	1.0ml
Water	11.3ml
TEMED	0.015ml

(ii)Resolving gel

Acrylamide - bisacrylamide (30:0.8)	12.5ml
Resolving gel buffer (3.0M Tris-HCl pH 8.8)	3.75ml
10% SDS	0.3ml
1.5% ammonium persulphate	1.5ml
Water	11.95ml
TEMED	0.015ml

(iii) Reservoir buffer

30.3g Tris, 144.0g glycine, 10.0g SDS made up to 1 litre with distilled water.

Buffers and acrylamide stored at 4^0C.

(iv) *Coomassie blue stain*

45% methanol, 9% glacial acetic acid and 0.1% w/v Brilliant blue R (Coomassie blue) warmed at 37^0C for 30 - 45 mins. De-stain: 5% methanol and 7% glacial acetic acid left at room temperature at least overnight.

2.3.6 Acrylamide-based polymer flocculating agents

Zetag 76 and Magnafloc LT22, from Allied Colloids Ltd, are both acrylamide-based cationic polymers. 1% w/v stock solutions were made up by wetting the dry agent with methanol and adding it slowly to distilled water on a magnetic stirrer. The solution was left to stand for a few hours to condition it. To standardise the method of addition, the flocculant was added to the top of the culture sample in a sedimentation tube and the tube was inverted twice to mix.

2.4 Measurement of flocculation

2.4.1 Sedimentation test

Flocculation was monitored indirectly by measuring the decrease in optical density of a sample allowed to sediment under gravity for 30 minutes. The ab-

sorbance at 600nm (A_{600}) was measured with a Perkin-Elmer 550S UV-VIS spectrophotometer. If necessary, samples were diluted with potassium phosphate buffer pH 7.4, to give a reading of no more than about 1.3 (above which the spectrophotometer is much less reliable) and phosphate buffer was used as the blank. The first sample of 1ml was taken aseptically from the culture flask and its A_{600} measured. 10ml of culture were placed in a sterile Universal bottle and allowed to sediment at room temperature for 30 minutes. 1ml of supernatant was then removed and its A_{600} measured. % decrease in A_{600} was calculated.

2.4.2 Other methods

Two possible methods for measuring flocculation were rejected. A Coulter counter was available but flocculated particles tend to break up under the forces used to make the suspension flow so results were likely to be less accurate, especially where large flocs had formed. An attempt was made to measure sedimentation rate using a strobe centrifuge but the flocs of AJ-1/23 C-2 in L-broth were too large and settled out too rapidly to enable a rate to be determined. Unflocculated cells also remained in suspension at the speeds available with this machine.

2.5 Measurement of particle size

Particle size was measured with a MasterSizer particle size analyser from Malvern Instruments Ltd of Malvern, Worcs. Particles are introduced into a laser beam by direct spraying and the diffraction patterns produced by the particles are analysed by computer to give a size distribution. The fundamental measurement is one of volume. Subsequent calculations, assuming spherical particles, mean that the printout generally gives derived diameters rather than volumes. Particles of size 0.1 - 600μm can be measured.

This machine was only purchased towards the end of the project; much greater use could have been made of it for monitoring flocculation in the early stages.

2.6 Measurement of growth rate

Growth rate of *P. putida* in liquid culture was measured via change in optical density (A_{600}). 400ml of L-broth in a 1-litre baffled conical flask was inoculated with a sample of overnight culture from a 250ml flask, such that the initial A_{600} was approximately 0.2. The culture was maintained at 26^0C on an orbital shaker (150 rpm) and the A_{600} of a 1ml sample measured every 30 minutes.

2.7 Viable counts

Viable counts were performed using the method of Miles and Misra (Harrigan and McCance, 1976). Serial dilutions down to 10^{-7} were prepared and the last 4 dilutions plated out. Three 10μl samples were dropped on to well-dried nutrient agar plates, two dilutions per plate, and the drops allowed to dry before the plates were inverted and incubated at 26^0C for 24 hours. Counts were made of drops showing colonies without confluence and the mean value used to calculate the original bacterial concentration.

2.8 Separation of proteins by gel electrophoresis

2.8.1 Total soluble protein

A 1ml sample from an actively growing culture was centrifuged for five minutes in an Eppendorf centrifuge and the supernatant discarded. SDS solubilizing buffer (Section 2.3.4) was added in the ratio of 10μl per 0.1 O.D. unit of the original culture. The mixture was boiled in a water bath for 10 minutes to dissolve proteins, centrifuged for 2 minutes and the supernatant subjected to polyacrylamide gel electrophoresis (500V, 40-50mA for 3 hours) and Coomassie blue staining.

2.8.2 Soluble wall proteins

A 25ml sample of culture was centrifuged for 5 minutes at 4000 rpm and the pellet resuspended in 25ml phosphate buffer. The cells were lysed by French

pressing and the suspension recentrifuged at 4000 rpm for 10 minutes. SDS solubilizing buffer was added to the pellet, the mixture was boiled for 10 minutes and recentrifuged and the supernatant subjected to SDS-PAGE and Coomassie blue staining.

2.9 Lipid analysis using thin layer chromatography

2.9.1 Lipid extraction

A sample of broth culture containing approximately 40-50mg dry weight of cells was centrifuged at 4000 rpm for 5 minutes (flocculating strain) or 10 minutes (non-flocculating strain) and the pellet resuspended in 5ml of a 2 : 1 chloroform : methanol mixture. After homogenization with a blender, the sample was left at room temperature for four hours, with intermittent shaking. The mixture was then filtered and the cells washed twice with 5ml chloroform-methanol (2:1 v/v). Water was added to the collected filtrate to give a final ratio of 2 : 1 : 0.75 chloroform : methanol : water and the mixture was left to stand overnight. The aqueous layer was then aspirated off and discarded and the lower solvent layer evaporated under nitrogen to prevent oxidation of the lipid as it dried. The dry lipid was resuspended in 0.5ml chloroform, containing butylated hydroxytoluene to a final concentration of $50mg.l^{-1}$, again to prevent oxidation, and stored at -20^0C.

2.9.2 T.L.C. and staining

Silica gel G plates were used with a solvent system consisting of 65 : 25 : 4 v/v chloroform : methanol : water. Two plates were set up; after drying the first one was sprayed with Dragendorff's reagent which produces orange spots after a few minutes if choline is present. The second plate was sprayed with ninhydrin and placed in the oven at 100^0C for 5 minutes; this produces purple-mauve spots with phosphatides or lipids having a free amino group. The same plate was then sprayed

with molybdenum blue, which stains phospholipids blue with no extra heating, and placed in the oven for approximately 2 hours to char. The last treatment will turn all organic material brown and is used to identify neutral lipids by elimination of the previous classes.

2.10 Electron microscope studies of bacterial cell structure

Fresh samples of bacterial cultures were prepared for viewing with the transmission electron microscope by placing 5 μl of culture on a nickel-copper grid, allowing the cells to settle for 1-2 minutes, draining off the liquid and washing with water. Some preparations were stained with uranyl acetate.

Freeze-dried samples of bacterial cultures were also embedded and sectioned.

2.11 Measurement of zeta potential

Zeta potential was measured using the DELSA 440 system of Coulter Electronics Ltd. A sample of cells is exposed to an electric field and the cells move according to their electrophoretic mobility.

$$\text{Mobility} = \frac{velocity\ of\ particle\ (\mu m.s^{-1})}{electric\ field\ (volts.cm^{-1})}$$

Mobility is detected using laser light scattered by the moving cells and subsequently detected at four different angles. Using the principle of the Doppler effect, changes in frequency of these beams compared with a reference beam will determine electrophoretic mobility and from this the zeta potential is derived (Sugrue *et al*, 1989).

2.12 Measurement of floc strength

The main quantitative method of investigating flocs once they have formed, is to try to obtain a measure of the force needed to break them up. 100ml samples of culture were stirred for varying lengths of time, using a Stirrer Timer Type 131

of Triton Electronics Ltd. This cannot give an actual value for shear rate but, as the speed is constant, the extent of shearing can be assumed to be proportional to the stir time. Particle size distribution was measured with the Malvern particle size analyser before and after stirring.

2.13 Trace element analysis

Calcium and magnesium levels in culture media were measured using a Perkin Elmer 5000 atomic absorption spectrophotometer with a nitrous oxide/ acetylene flame. Results are expressed in parts per million.

Results

3.1. Initial experiments on growth and flocculation.

Preliminary tests were made on several strains of *Pseudomonas putida* in different media to compare growth rates and to establish the variation in flocculation. Figs. 1 and 2 show growth of the non-flocculating strain AJ-1 in L-broth made with Difco tryptone and Oxoid tryptone T respectively and Figs. 3 and 4 growth of a flocculating strain, AJ-1/23 C-2 F-1, in the same conditions. During the exponential phase of growth there is very little difference in growth rate with medium for AJ-1, with Difco tryptone giving slightly faster growth for AJ-1/23 C-2 F-1. Oxoid tryptone T was selected as the peptone component of the L-broth for all future work.

Comparison of these two strains in L-broth for a longer period of growth extending into stationary phase is shown in Figs. 5 and 6. Again, the two graphs show very similar patterns.

Four different strains of *P. putida* were tested for flocculation using the sedimentation test (see Methods). During early log phase of growth, the A_{600} usually increased during the sedimentation period owing to multiplication of the cells at a laboratory temperature of around 21^0C , not much lower than the normal incubation temperature. In late log or stationary phase, the amount of flocculation varied considerably, as shown in Table 1. Greater experimental error is introduced if the initial A_{600} is very low, so subsequent measurements were made on cultures of 16 hours old or more, wherever possible, preferably with A_{600} values of at least 0.5.

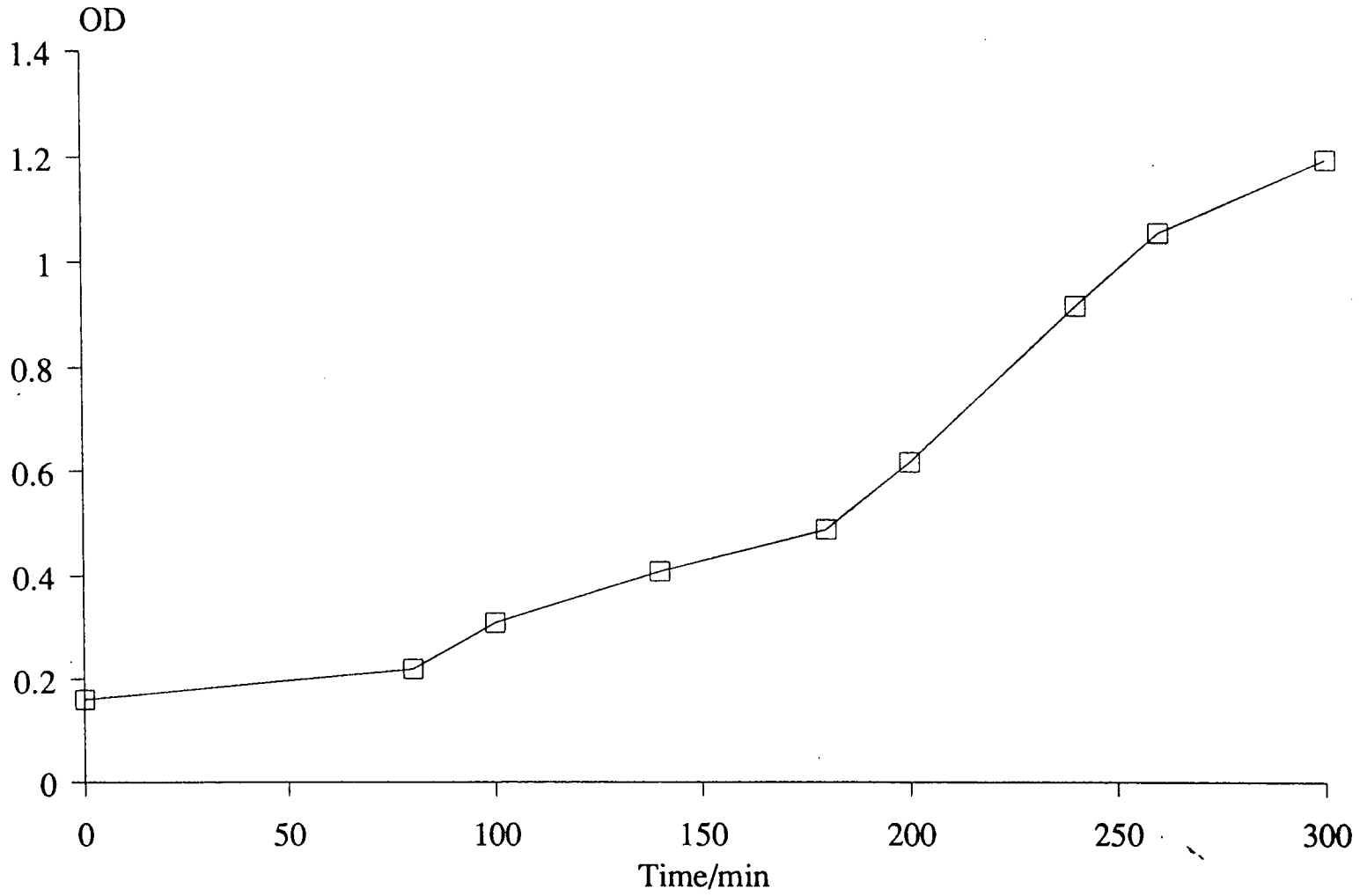

Figure 1. AJ-1 growth in Difco L-broth

Fig 2. AJ-1 growth in Oxoid L-broth.

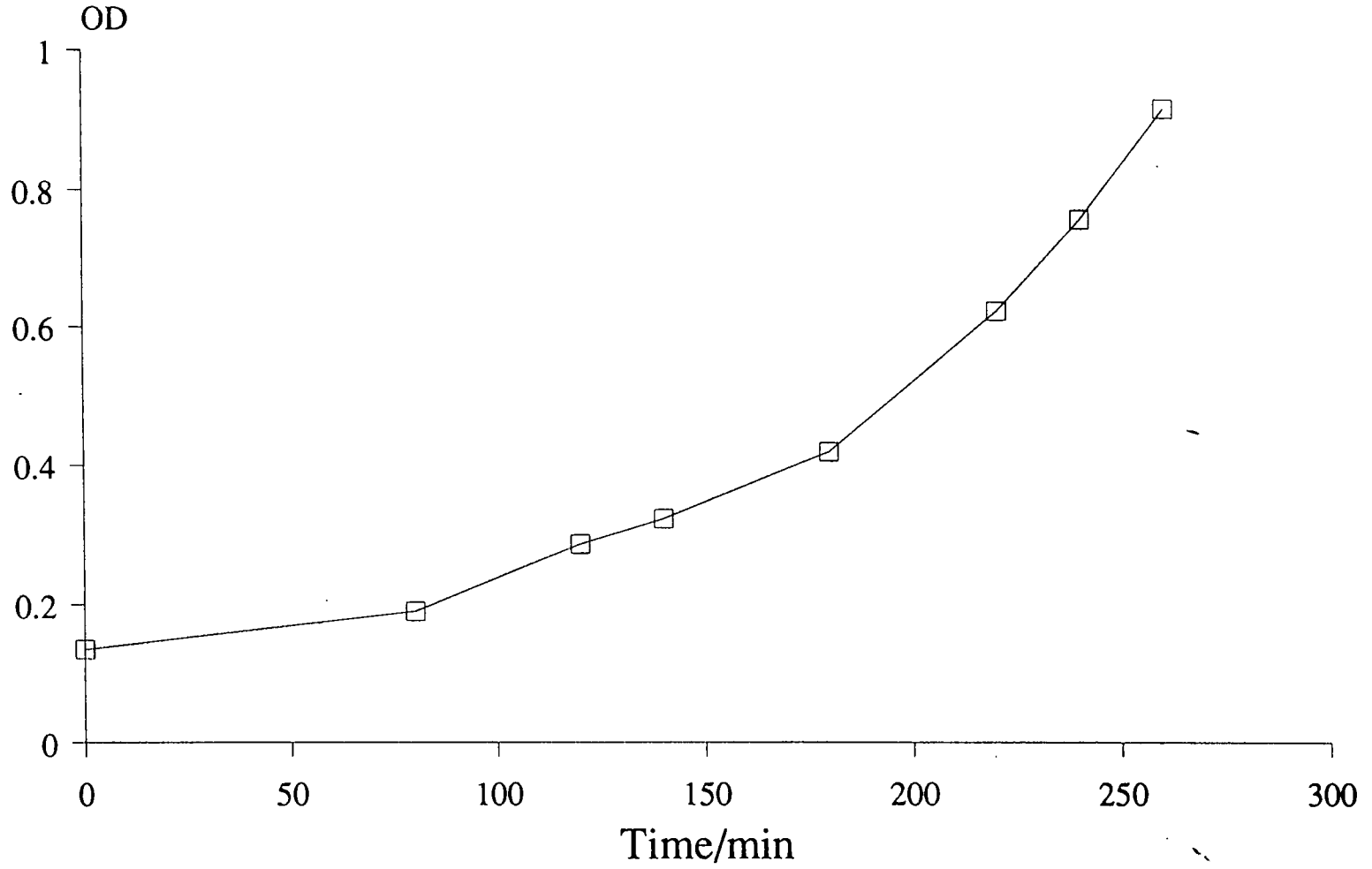

Fig 3. AJ-1/23 C-2 F-1.
Growth in Difco L-broth.

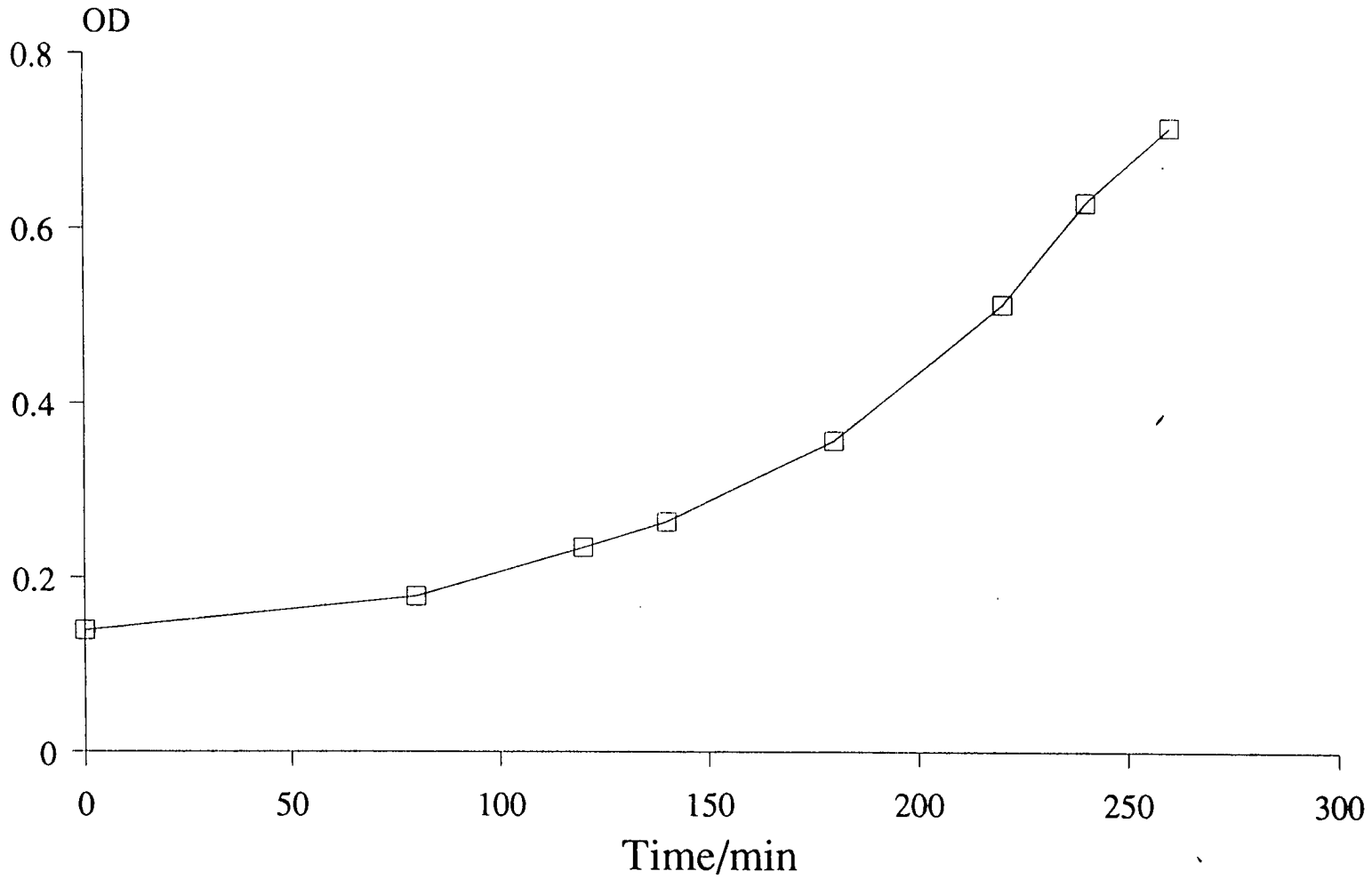

Fig 4. AJ-1/23 C-2 F-1
Growth in Oxoid L-broth

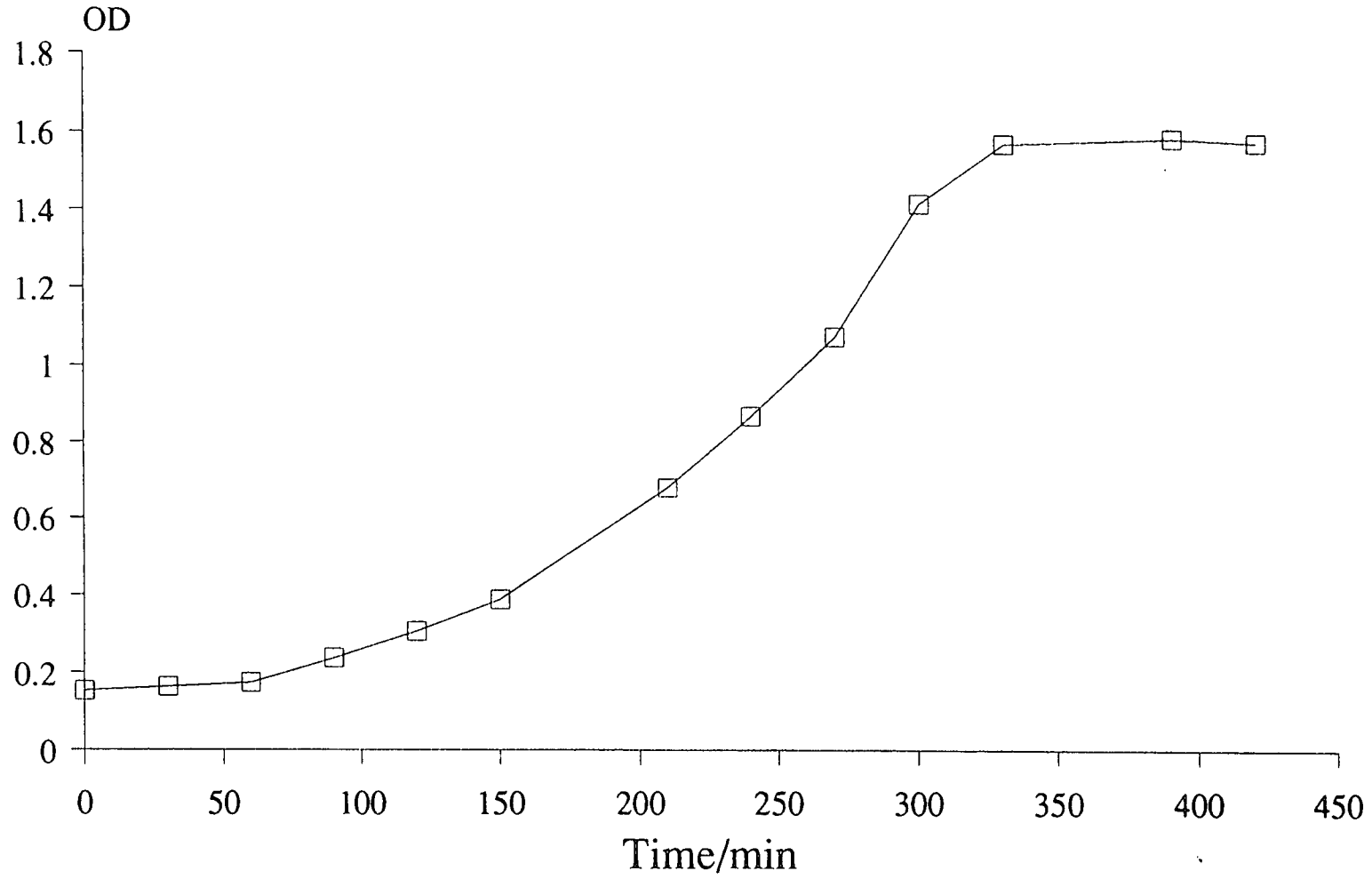

Fig 5. AJ-1 growth in Oxoid L-broth.

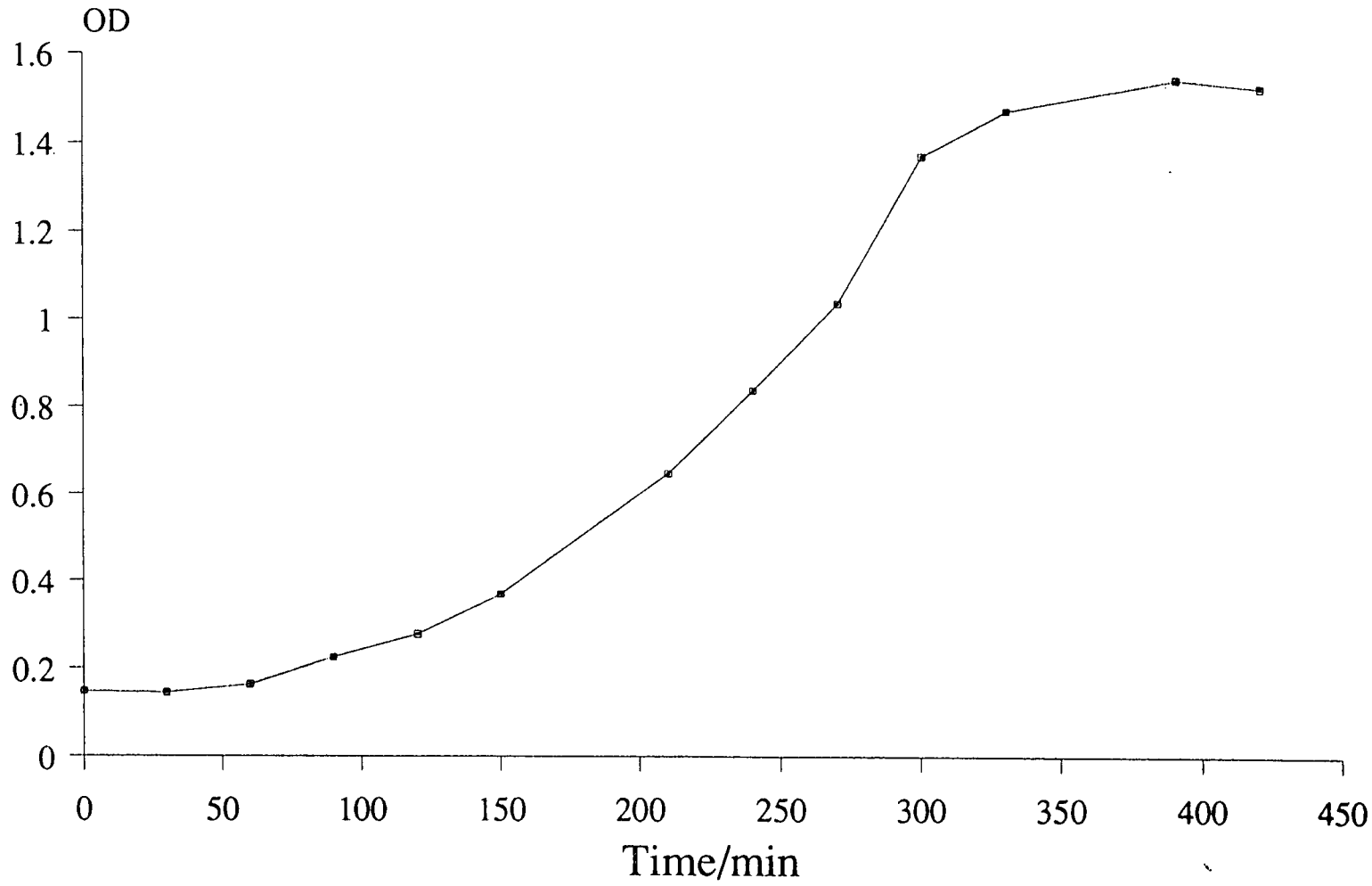

Fig 6. AJ-1/23 C-2 F-1
Grown in Oxoid L-broth

Table 1 Variation in flocculation in strains of *P. putida* grown in L-broth

Strain	Mean % decrease in A_{600}	Standard deviation (S.D.)
Non-flocculating:		
AJ-1	17.3	12.1
AJ-1/23	24.9	7.9
Flocculating:		
AJ-1/23 C-2 F-1	20.0	14.4
AJ-1 C-4	32.7	13.6

range should be given

3.2 Identification of flocculating and non-flocculating strains

Selection of one flocculating and one non-flocculating strain of *Pseudomonas putida* for further study was made by testing fresh ampoules of cultures, stored frozen in glycerol, for sedimentation. The results are shown in Table 2.

AJ-1 was chosen as the non-flocculating strain and AJ-1/23 C-2 as the flocculating strain. These two were used throughout the rest of the investigation.

Flocs are visible with Gram-stained light microscope preparations (compare Figs. 7 and 8) and with whole cell preparations under the electron microscope (Fig. 9). The cells can become joined together at any point on their surface, producing flocs with an irregular but often elongated shape. No surface structures such as pili were visible in these preparations.

Both strains grown in L-broth exhibited considerable variation between cells in terms of length, as can be seen from Figs. 10 and 11. It was not clear whether the flocs in AJ-1/23 C-2 tended to consist more often of cells of one particular length.

Figs. 12 and 13 show the two strains under higher magnification; the layered structure of the cell envelope typical of Gram-negative bacteria can be seen in both preparations.

The particle size analyser was used to investigate the distribution of sizes within each strain. The results are shown in Figs. 14 and 15.

Table 2 Flocculation of fresh samples of *P. putida* to identify working strains

Strain	Initial A_{600}	Final A_{600}	% decrease in A_{600}
AJ-1	1.960	1.656	16
AJ-1/23	2.724	2.208	19
AJ-1 C-4	0.421	0.442	0
AJ-1/23 C-2	2.280	0.700	69

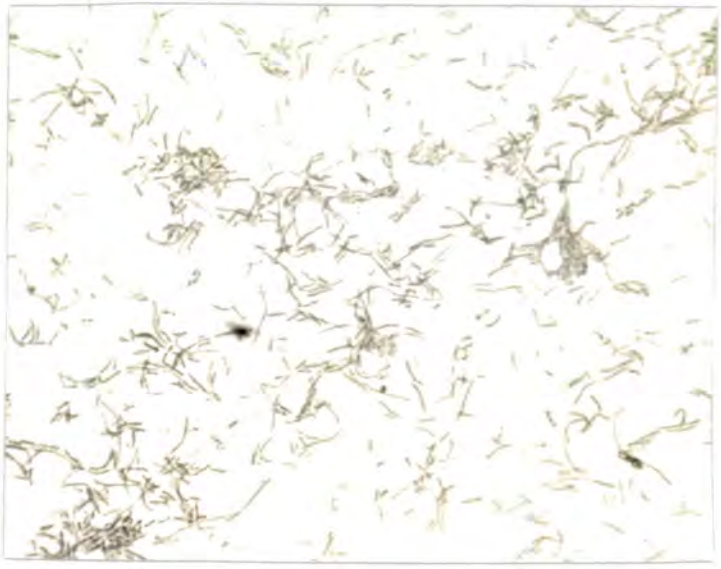

Fig. 7 Gram-stained preparation of AJ-1 grown in L-broth. x100

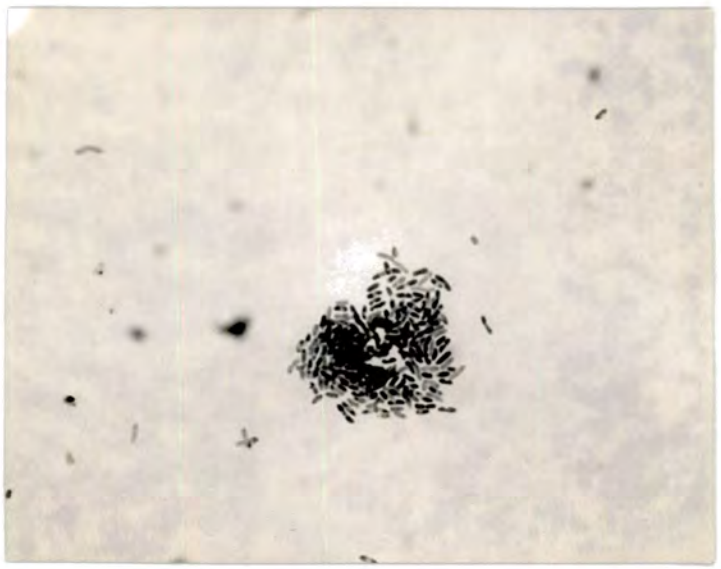

Fig. 8 Gram-stained preparation of AJ-1/23 C-2 in L-broth showing floc. x100

Fig. 9 A floc as seen with the transmission electron microscope. x9000

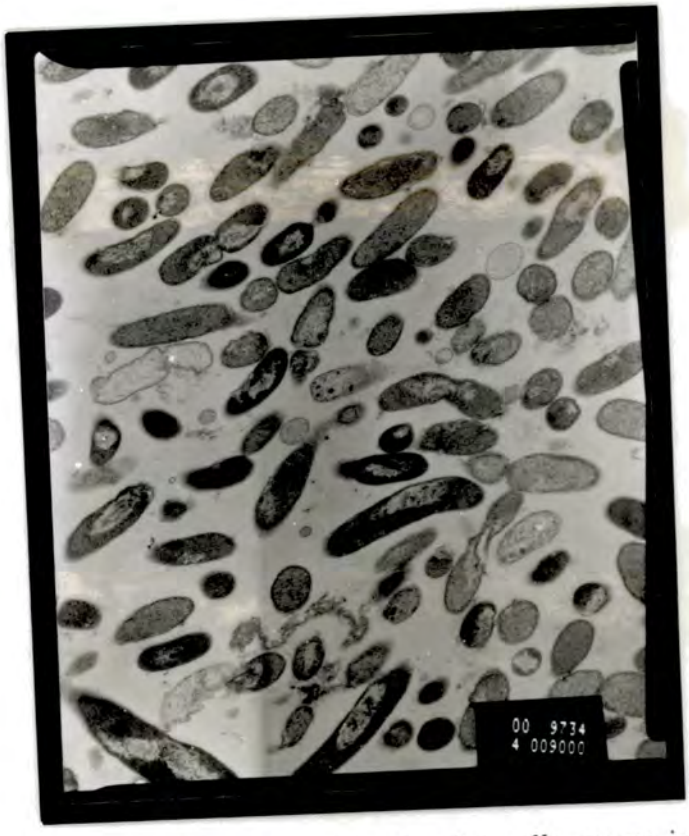

Fig. 10 TEM preparation of sectioned AJ-1 cells grown in L-broth. x9000

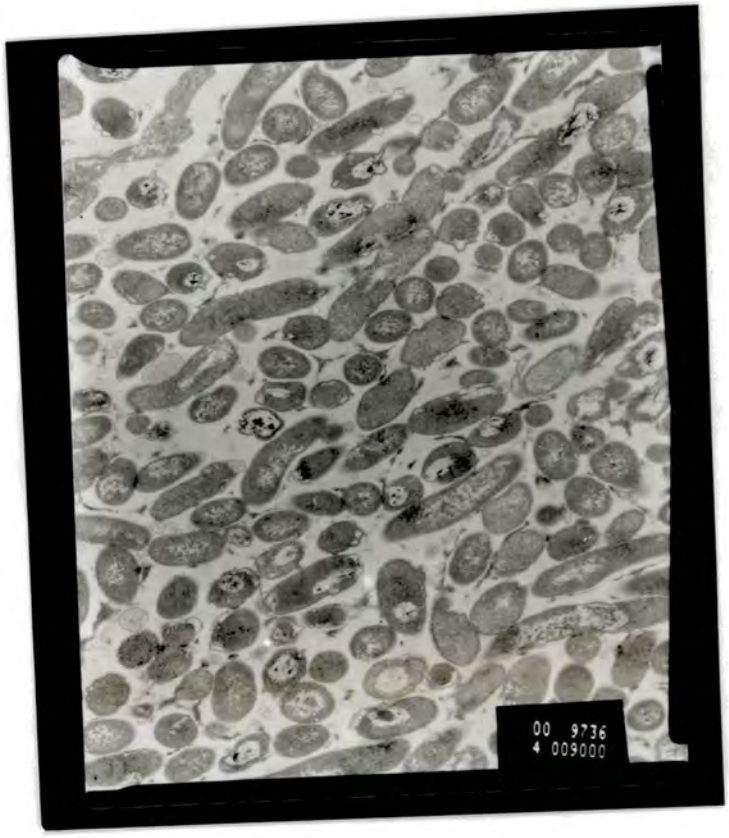

Fig. 11 TEM preparation of sectioned AJ-1/23 C-2 cells grown in L-broth. x9000

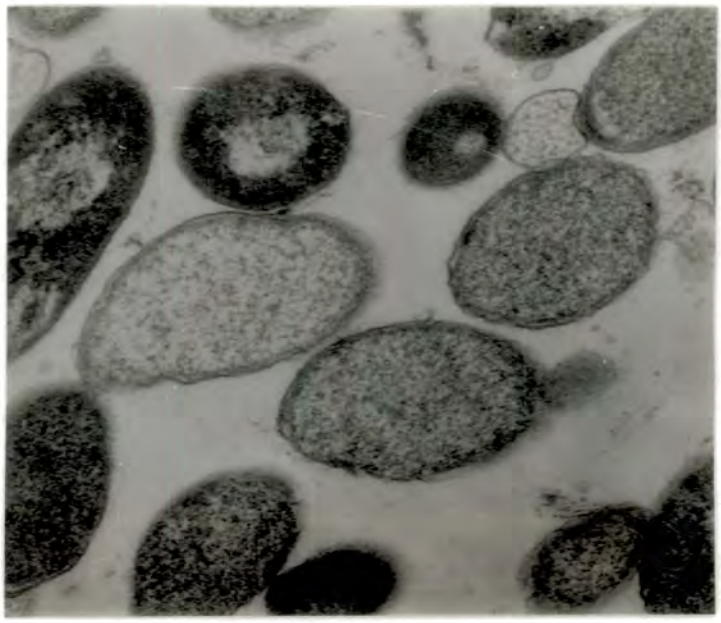

Fig.12 TEM preparation of sectioned AJ-1 cells grown in L-broth. x80 000

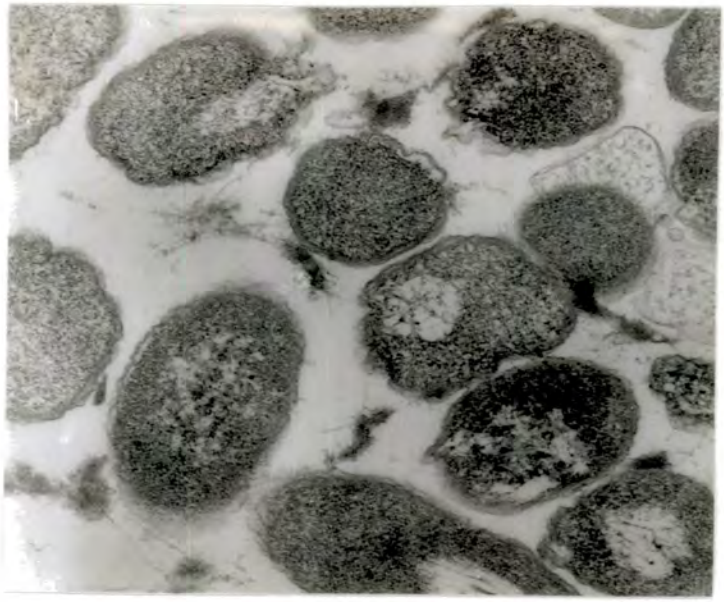

Fig. 13 TEM preparation of sectioned AJ-1/23 C-2 cells grown in L-broth. x80 000

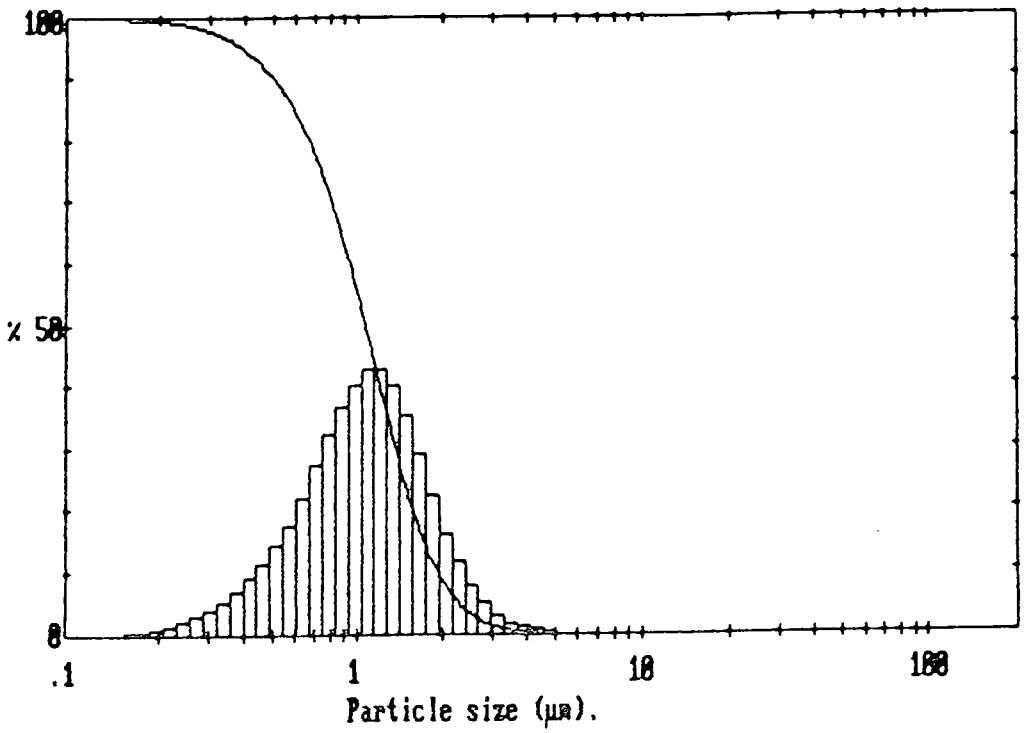

Fig. 14 Particle size distribution of AJ-1 grown in L-broth

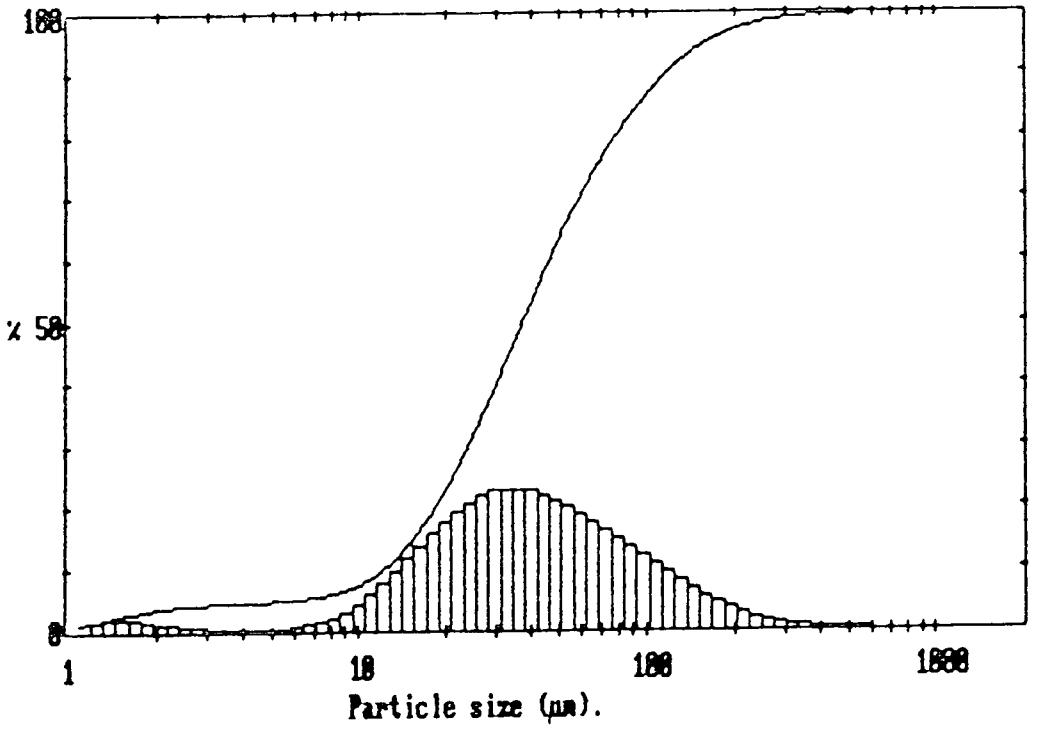

Fig. 15 Particle size distribution of AJ-1/23 C-2 grown in L-broth

3.3 Flocculation and viable counts during stationary phase.

Measurement of flocculation at different times in stationary phase were made for cultures of AJ-1 and AJ-1/23 C-2 and at the same points viable counts of the cultures were established using the surface colony count technique (see Methods). Results are shown in Figs. 16 and 17.

The results indicate that flocculation during stationary phase is not due to cell death; the viable counts do not fall during this stage of culture growth, for either strain.

Concentration effects were also shown to play only a minor role in flocculation. A culture such as AJ-1 in L-broth, which had a more rapid growth rate than AJ-1/23 C-2 in the same medium, would have cells colliding more frequently, but this did not result in flocculation.

3.4 Effect of culture medium on flocculation of AJ-1/23 C-2

3.4.1 Seed 2 minimal medium

It had already been stated that flocculation is not observable in Seed 2 minimal medium (Cousland, unpublished). This experiment was repeated and flocculation of AJ-1/23 C-2 was found to be much lower in Seed 2 medium than in L-broth. Different components of the L-broth were added separately and then together to S2 medium, in the concentrations used for the complex medium, and flocculation of AJ-1/23 C-2 grown in these modified media was measured. The results are shown in Table 3.

In none of the S2 flasks did flocculation approach that of the control cultures in L-broth, even when all the L-broth components were added to S2 medium together. It was concluded that some ingredient in the minimal medium must be inhibiting flocculation and the next series of experiments was designed to test this hypothesis.

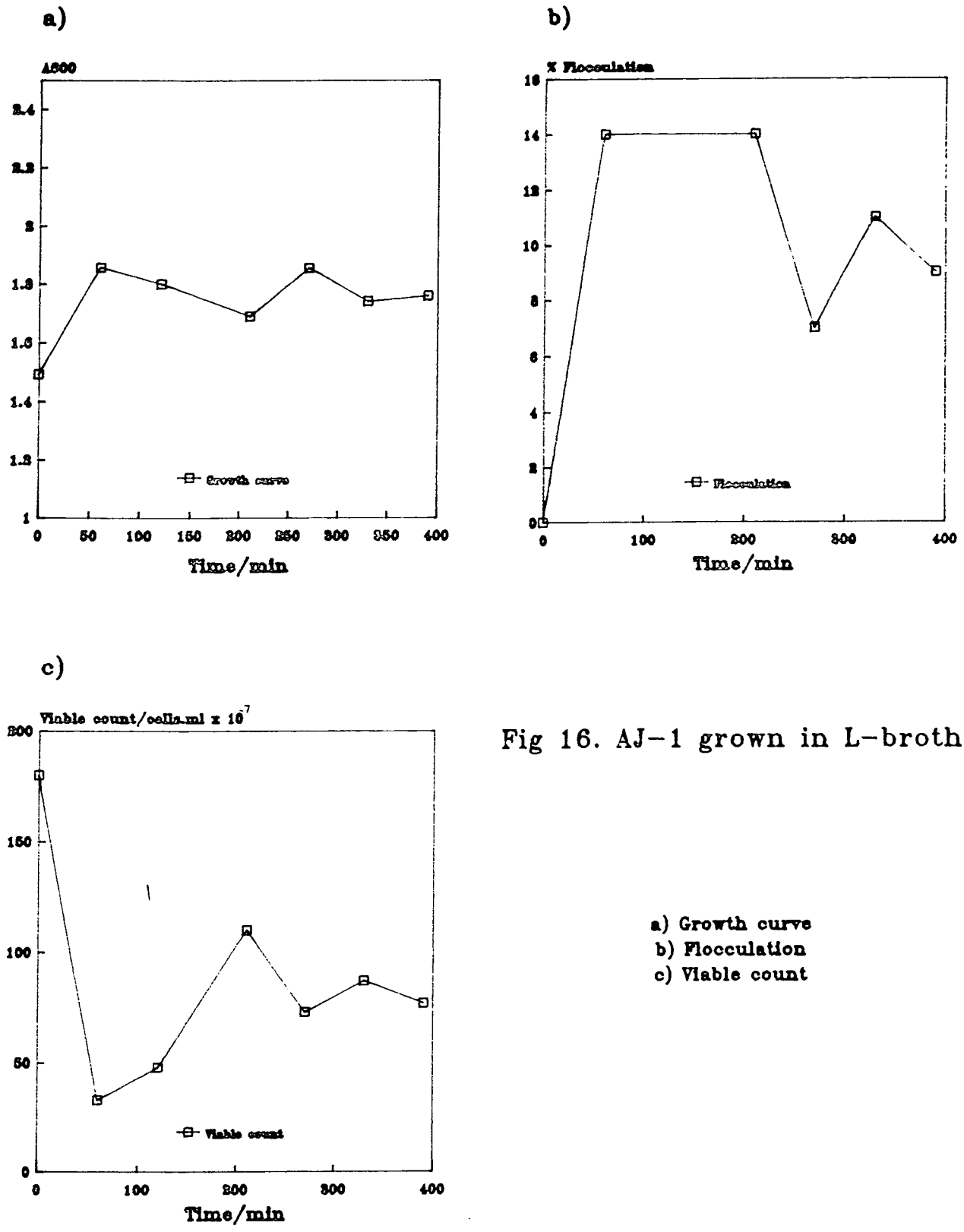

Fig 16. AJ-1 grown in L-broth

a) Growth curve
b) Flocculation
c) Viable count

how may replicates for these points

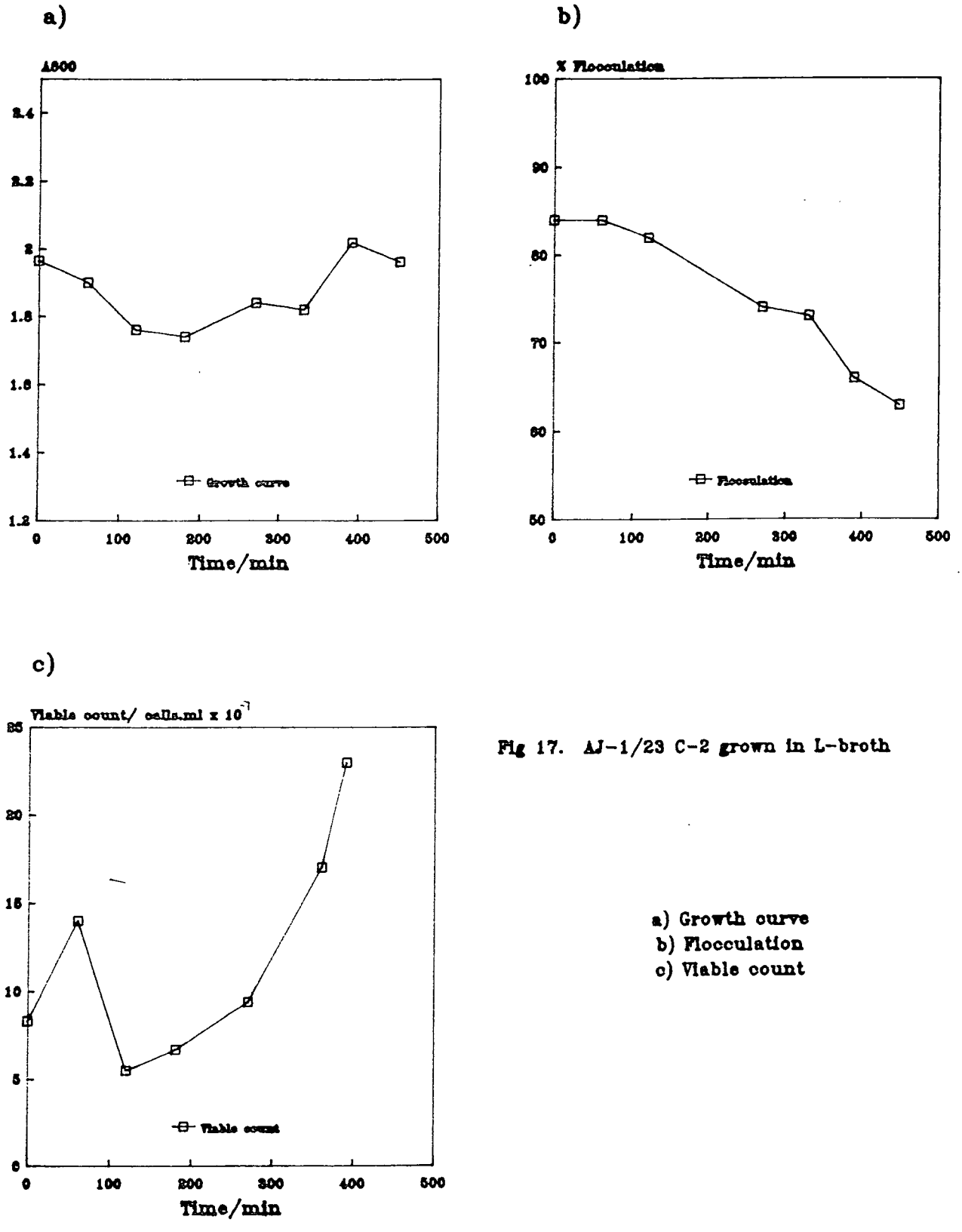

Fig 17. AJ-1/23 C-2 grown in L-broth

a) Growth curve
b) Flocculation
c) Viable count

Table 3 Effect of L-broth components on flocculation in Seed 2 medium

Medium	Mean % decrease in A_{600}	S.D.
L-broth	52.3	12.7
S2	7.6	12.3
S2 + yeast extract	2.3	2.6
S2 + NaCl	3.2	2.6
S2 + tryptone T	6.1	4.6
S2 + Oxoid tryptone	4.5	3.2
S2 + Difco tryptone	4.3	3.3
S2 + yeast extract, NaCl & tryptone T	4.5	1.8

3.4.2 L-broth with added S2 components

Individual components of S2 medium were added separately to L-broth, in appropriate concentrations, and shake flask cultures of AJ-1/23 C-2 maintained overnight. Measurements of sedimentation in the various combined media are shown in Table 4.

Magnesium sulphate consistently reduced flocculation in AJ-1/23 C-2 by a large amount whereas the ammonium sulphate did not, indicating that the magnesium ions rather than the sulphate ions were the active ones. The other components had no significant effect on flocculation, apart from the trace elements, the last suggesting that the stabilising effect could result from the presence of divalent cations generally, rather than magnesium alone.

3.4.3 Effect of magnesium and calcium chlorides on flocculation

AJ-1/23 C-2 was grown in L-broth with either magnesium chloride or calcium chloride added to a concentration equal to that of magnesium sulphate in S2. L-broth and L-broth plus magnesium sulphate were also used as media. Sedimentation tests gave the results shown in Table 5.

This confirms that the magnesium ions produce the stabilising effect rather than their associated anions. The effect of calcium was less marked, although sedimentation was reduced in all the samples compared with controls, in some cases down to less than 10% .

3.4.4 Varying the concentration of magnesium sulphate

The volume of magnesium sulphate solution added to L-broth was reduced to see if a critical point for stabilising AJ-1/23 C-2 suspensions could be established. Sedimentation results are shown in Table 6.

Table 4 Effect of S2 components on flocculation in L-broth

Medium	Mean % decrease in A_{600}	S.D.
L-broth	52.4	19.5
L-broth + glucose	67.3	11.8
L-broth + $FeCl_3$	74.0	4.2
L-broth + phosphate buffer	71.0	17.0
L-broth + $(NH_4)_2SO_4$	65.8	4.3
L-broth + trace elements	30.2	21.2
L-broth + $MgSO_4$	5.7	5.6

Table 5 Effect of magnesium and calcium chlorides on flocculation in AJ-1/23 C-2

Medium	Mean % decrease in A_{600}	S.D.
L-broth	51.8	17.2
L-broth + $MgSO_4$	5.7	5.6
L-broth + $MgCl_2$	3.8	2.6
L-broth + $CaCl_2$	15.5	8.4

Table 6 Sedimentation of AJ-1/23 C-2 in L-broth with varying concentrations of $MgSO_4$

Volume 40% $MgSO_4$ added to 50 ml L-broth (μl)	Mean % decrease in A_{600}	S.D.
25 (as in S2)	6.3	2.1
15	6.3	0.9
5	9.3	0.5
3	10.7	8.1
1	52.7	8.1
0	52.0	7.2

Needs this in conc. terms?

3μl $MgSO_4$ added to 50ml of culture was enough to stabilise the cell suspension. This is equivalent to 2.4 ppm Mg^{2+} in the culture medium. This is in addition to the magnesium contained in the L-broth. Atomic absorption analysis of the L-broth showed it to contain 1.3 ppm Mg^{2+} of which approximately 1.1 ppm came from the yeast extract and the remainder from the tryptone.

3.4.5 Use of different peptone components

If the magnesium concentration is the controlling factor in flocculation, it should be possible to change the flocculating behaviour of AJ-1/23 C-2 by substituting peptones of higher Mg^{2+} content for the normal tryptone T. Sedimentation was compared in L-broth made with tryptone (283 ppm Mg) and soya peptone (2630 ppm) (Oxoid, 1982), which would give values 10% of these in the final broth. Both are above the critical level established in the previous experiment. Results are shown in Table 7.

Again, these results support the hypothesis that high magnesium levels prevent flocculation in this strain of *Pseudomonas putida* . It should be borne in mind, however, that these media differ in other components as well, for example calcium and iron.

3.4.6 Minimal medium with reduced magnesium

Seed 2 medium was made up with decreasing amounts of magnesium sulphate to see if flocculation could be induced in minimal medium without reducing the magnesium to a level insufficient for growth. Sedimentation results are shown in Table 8.

A few large flocs were visible in the flasks with 0.4 and 0.2 ppm Mg^{2+} but these did not contribute to the sedimentation results. Growth, especially with the lowest Mg^{2+} concentration, was very much reduced but it does appear that flocculation can be induced by lowering the magnesium level.

Table 7 Sedimentation of AJ-1/23 C-2 with different types of peptone

Peptone	Mean % decrease in A_{600}	S.D.
Tryptone T	45.3	3.3
Tryptone	13.0	4.5
Soya peptone	6.7	0.5

Table 8 Flocculation of AJ-1/23 C-2 in S2 with different levels of magnesium

Magnesium content (ppm)	Mean % decrease in A_{600}	S.D.
20 (normal level)	2.3	1.2
12	1.0	0.8
4	1.7	1.2
1.6	1.3	0.9
0.6	1.7	0.5
0.4	4.7	0.9
0.2	7.0	5.9

3.4.7 Addition of aluminium sulphate

50μl of 30% w/v aluminium sulphate solution (a standard flocculating agent) was added to a 10ml sample of AJ-1/23 C-2 grown in L-broth plus extra $MgSO_4$, in a sedimentation tube. Flocculation was induced by the aluminium sulphate although the flocs were less dense than those resulting from autoflocculation and failed to sediment comparably in the 30 minutes allowed for the test.

It was possible , later on, to repeat this experiment using the Malvern particle sizer and thus to avoid the problem of slow sedimentation. Particle size distributions are shown in Fig. 18 (immediately after addition of aluminium sulphate) and Fig. 19 (after 1 hour on an orbital shaker). Flocculation is shown to increase with time when the culture flask is exposed to shaking.

Some precipitation, however, does occur if aluminium sulphate is added to L-broth alone, so further tests need to be done to ascertain how much of the total flocculating effect is attributable to broth components, rather than cells.

3.5 The effect of pH on flocculation

Lowering the pH of a suspension where the cells are negatively charged should induce flocculation. Using 2M HCl, the pH of L-broth cultures of both AJ-1 and AJ-1/23 C-2 was lowered in stages and sedimentation tests were carried out. The results are shown in Fig. 20.

Flocculation of cells below pH 4 was immediate and clearly visible in both strains.

The pH of L-broth alone was lowered from 6.8 to 2.7 and no precipitation of any of the broth components occurred.

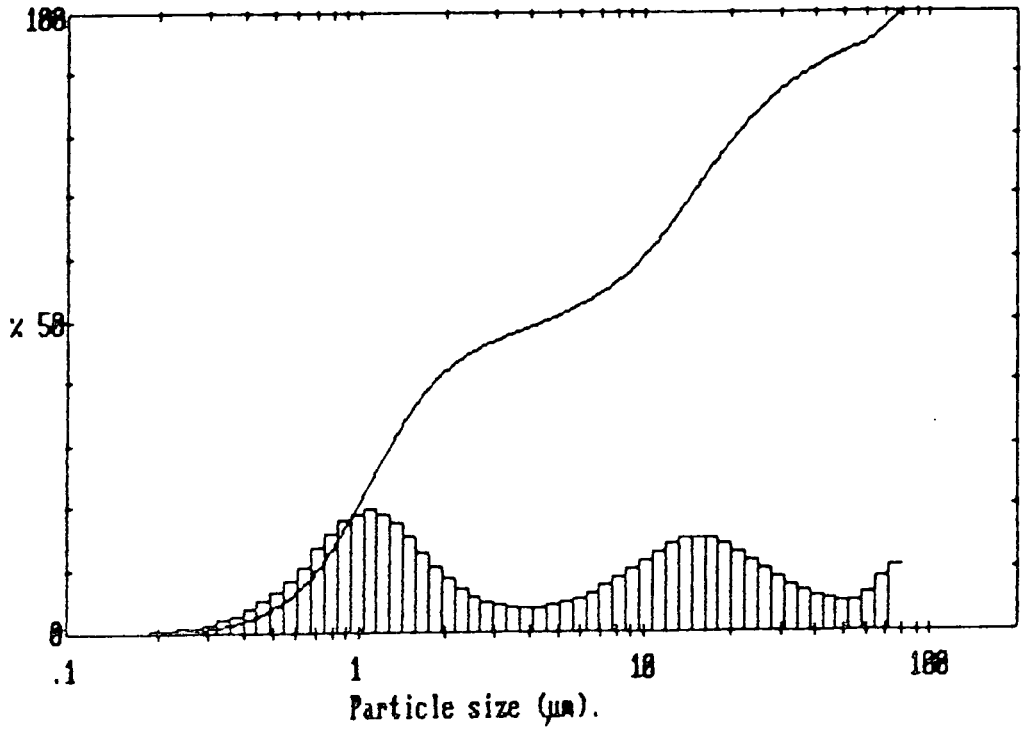

Fig. 18 Particle size distribution of AJ-1/23 C-2 immediately on addition of aluminium sulphate. Median = 4.57 μm

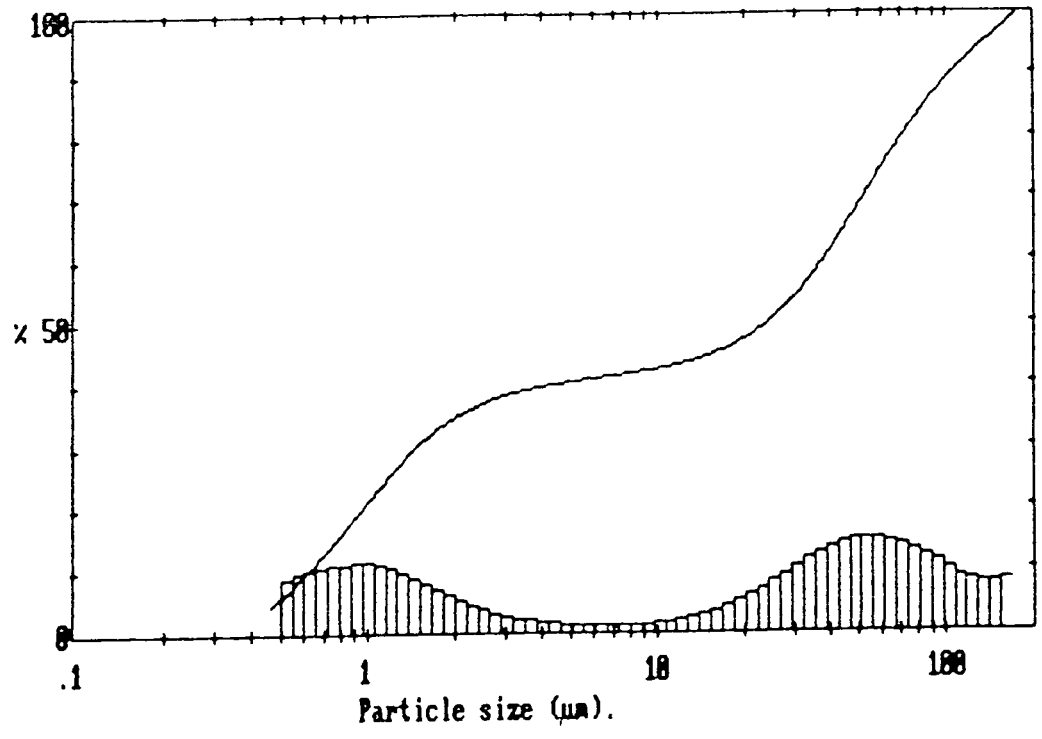

Fig. 19 Particle size distribution of AJ-1/23 C-2 plus aluminium sulphate after one hour on shaker. Median = 24.19 μm

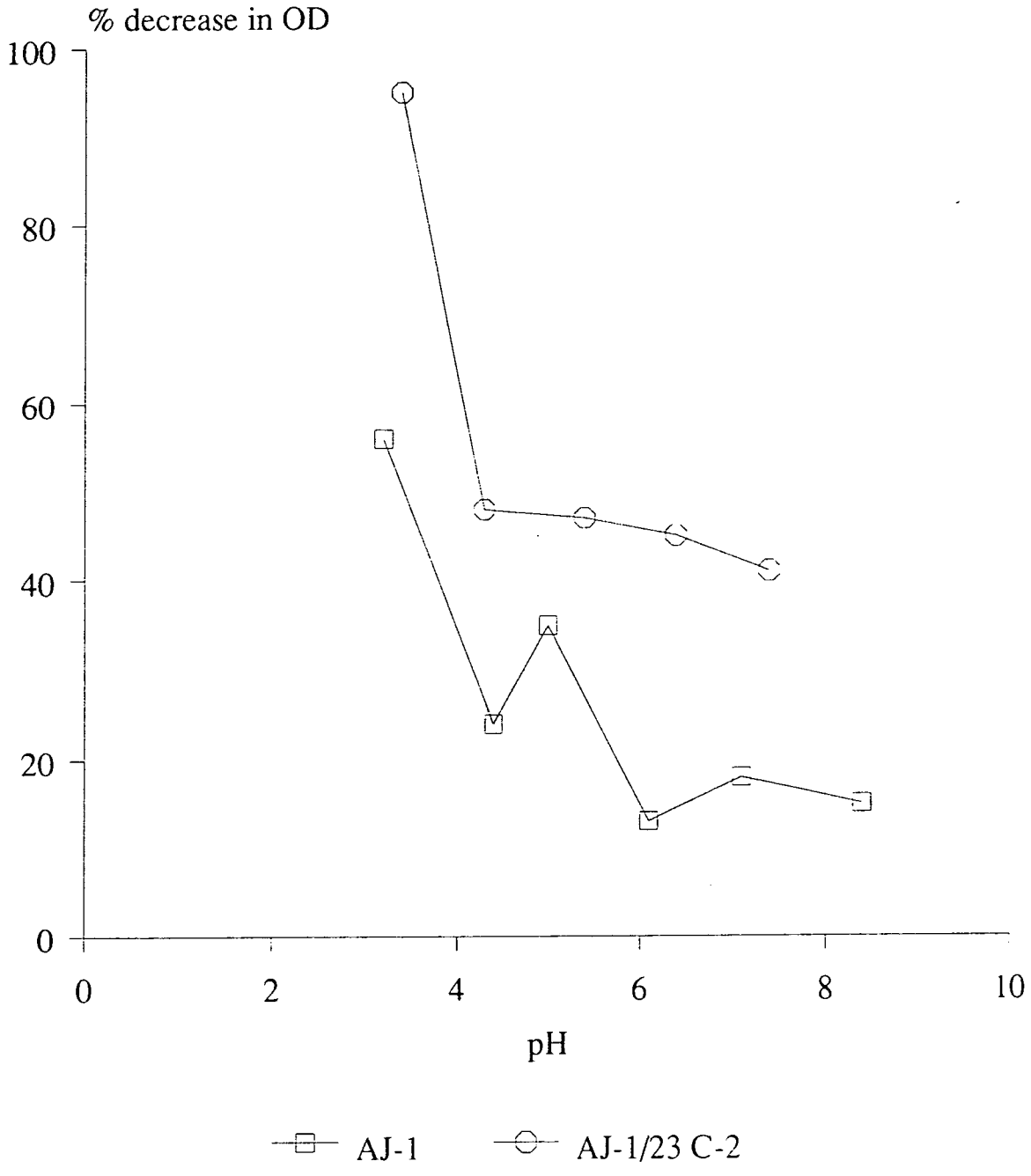

Fig 20. Effect of pH on flocculation

Increasing the pH with 20% NaOH did not increase flocculation in either AJ-1 or AJ-1/23 C-2. Above pH 11 the cells lysed, as evidenced by a considerable decrease in initial optical density and an increase in viscosity of the suspension.

3.6 Inducing flocculation in the non-flocculating strain AJ-1.

3.6.1 Effect of magnesium and calcium

AJ-1 was grown in L-broth with additional $MgSO_4$, $MgCl_2$ and $CaCl_2$, in the concentration equivalent to that of $MgSO_4$ in S2 medium, and flocculation measurements were made. Results are shown in Table 9.

Although calcium in particular is a flocculating agent for a wide variety of suspended particles, neither calcium nor magnesium will induce flocculation in AJ-1.

3.6.2 Addition of mannan

Soluble mannan (known to agglutinate *E. coli*) was added to 10ml of AJ-1 L-broth culture in the sedimentation tube to give a final concentration of either $7\mu g.l^{-1}$ or $14\mu g.l^{-1}$. Flocculation measurements are shown in Table 10.

Addition of mannan made no apparent difference to flocculation.

3.6.3 Action of aluminium sulphate

1ml of a 30% solution of aluminium sulphate was added to a 10ml sample of AJ-1 L-broth culture in a sedimentation tube. Flocculation was immediate but the flocs trapped air bubbles and floated or stayed suspended. It was therefore not possible to measure the optical density of the liquid.

Table 9 Effect of magnesium and calcium on flocculation in AJ-1

Medium	Mean % decrease in A_{600}	S.D.
L-broth	9.4	2.1
L-broth + $MgSO_4$	2.7	3.8
L-broth + $MgCl_2$	7.3	2.6
L-broth + $CaCl_2$	6.0	2.4

Table 10 Effect of mannan on flocculation in AJ-1

Medium	Mean % decrease in A_{600}	S.D.
L-broth	4.0	0.7
L-broth + 7 $\mu g.l^{-1}$ mannan	9.0	0.0
L-broth + 14 $\mu g.l^{-1}$ mannan	5.0	0.8

Lower concentrations of aluminium sulphate were then tested, both 100μl and 50μl additions producing visible flocculation and a measurable decrease in A_{600}. The flocs were not very dense, however, and took a long time to settle, therefore values for % decrease in A_{600} were only small and could not validly be compared with those measured previously. There was some degree of precipitation resulting from mixing aluminium sulphate with L-broth alone, so it is difficult to isolate the cell effect in this instance.

3.6.4 Use of commercially available flocculating agents

Zetag and Magnafloc stock solutions (see Methods) were added to 10ml samples of AJ-1 L-broth culture in sedimentation tubes, to give 5% v/v, 1% v/v and 0.5% v/v concentrations. Both flocculants produced visible flocculation of the cells, especially at 5% concentration, but the flocs were very diffuse and frequently did not sediment so quantitative measurements could not be obtained. Zetag produced some coagulation in the L-broth itself whereas Magnafloc when tested on the broth had no visible effect.

3.7 Effect of magnesium on cell morphology

Extra magnesium in the L-broth had the effect of reducing the average size of the cells in both AJ-1 and AJ-1/23 C-2. Gram-stained preparations are shown in Figs. 21 and 22 – compare with cells from L-broth in Figs. 7 and 8. The addition of calcium chloride to L-broth did not reduce the cell size in the same way, at least for AJ-1 (Fig. 23). Table 11 shows the median cell size resulting from additional magnesium compared with AJ-1 in L-broth, as measured by the Malvern particle size analyser.

Cells of AJ-1/23 C-2 grown in Seed 2 minimal medium (in which they do not flocculate) exhibit a similar morphology to cells grown in L-broth with extra $MgSO_4$.

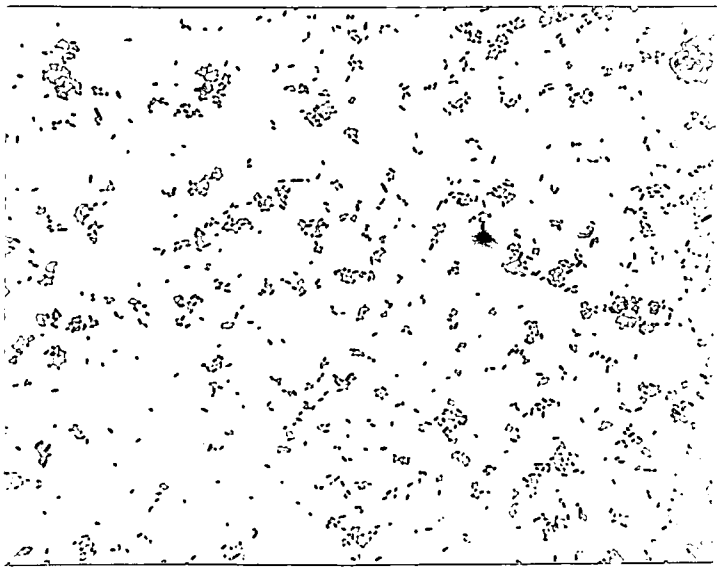

Fig. 21 Gram-stained preparation of AJ-1 grown in L-broth with extra magnesium sulphate. x100

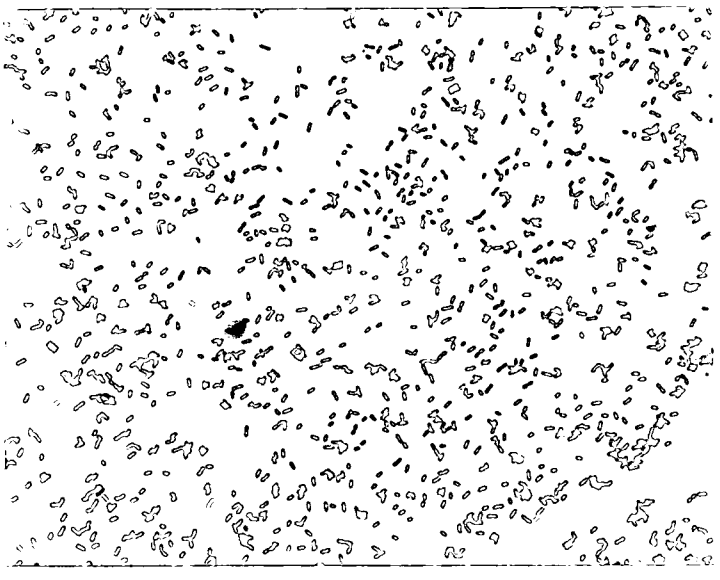

Fig. 22 Gram-stained preparation of AJ-1/23 C-2 grown in L-broth with extra magnesium sulphate. x100

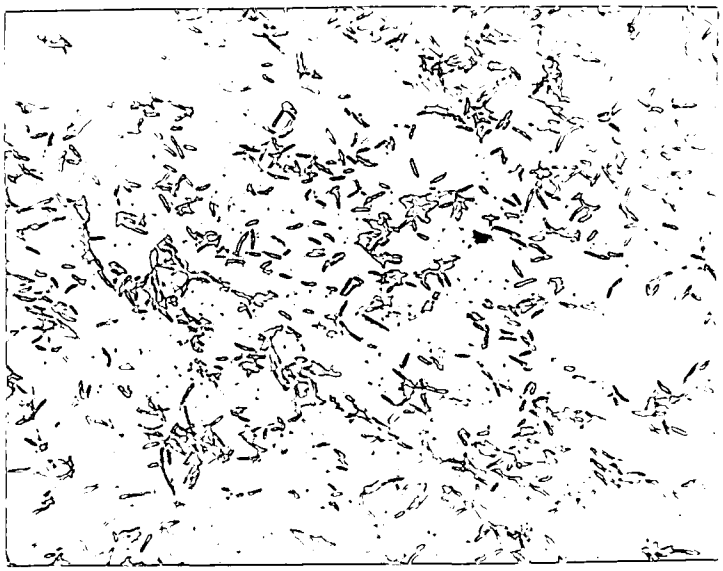

Fig. 23 Gram-stained preparation of AJ-1 grown in L-broth with extra calcium chloride. x100

Table 11 Median cell size of culture samples

Strain / Medium	Median size (μm)
AJ-1 in L-broth	1.06
AJ-1 in L-broth + $MgSO_4$	0.86
AJ-1/23 C-2 in L-broth + $MgSO_4$	0.74

Figs. 24 and 25 show the effect of extra magnesium in the L-broth on AJ-1/23 C-2, using samples prepared for the electron microscope. At higher magnification, it can be seen that the cell envelope is smoother than those of cells grown in normal L-broth (compare Figs. 12 and 13).

3.8 Binding of magnesium sulphate to AJ-1/23 C-2

3.8.1 Addition of magnesium sulphate in sedimentation tube

Magnesium sulphate, in the concentration used in S2 medium, was added to a 10ml sample of L-broth culture of AJ-1/23 C-2 and a sedimentation test was carried out. A control sample had the same volume of NaCl added. Results are shown in Table 12.

Adding magnesium sulphate to the outside of the cells for a short time does not inhibit flocculation, suggesting that the mechanism which stabilises the suspension takes some time to operate and may involve some metabolic activity inside the cells.

3.8.2 Washing the cells

To discover whether the magnesium was easily removable from the flocculated cells, a sample of L-broth culture containing $MgSO_4$ was centrifuged, the cells washed in L-broth, re-centrifuged and resuspended in fresh L-broth. A control sample from an L-broth culture without $MgSO_4$ was tested in the same way. Flocculation results are shown in Table 13.

This indicates that the magnesium is exerting its stabilising effect either by entering the cells or by binding relatively firmly to the cell walls such that simple washing will not remove it. Centrifugation itself was shown to have no effect on flocculation.

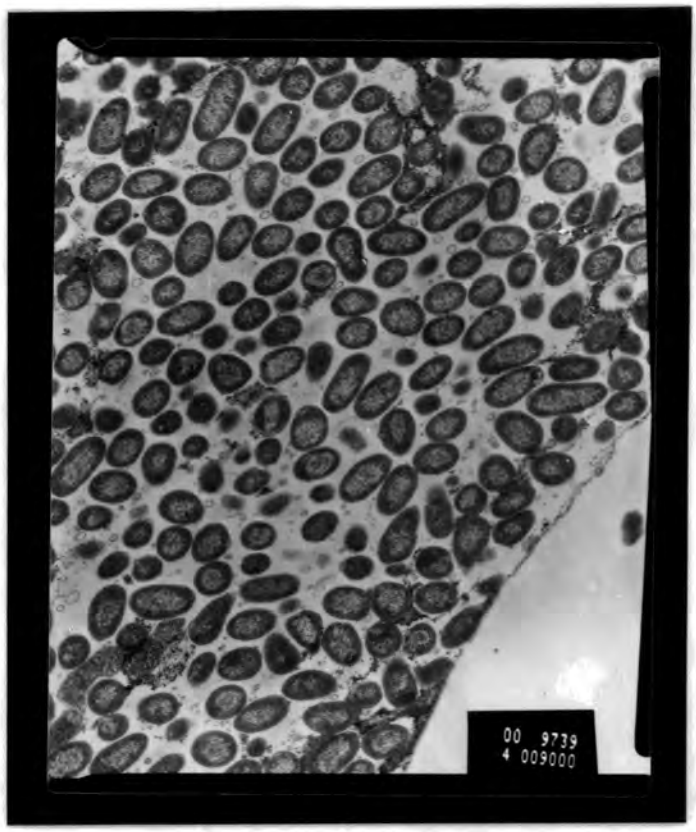

Fig. 24 TEM preparation of sectioned AJ-1/23 C-2 cells grown in L-broth with extra magnesium sulphate. x9000

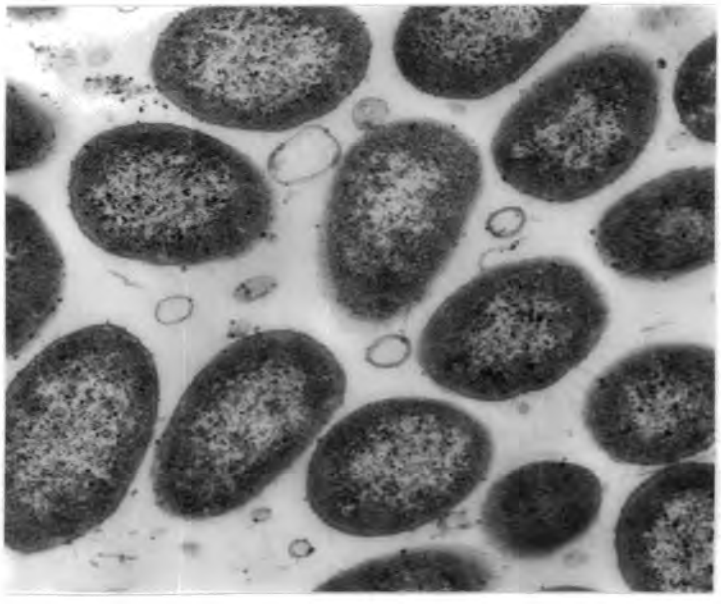

Fig. 25 TEM preparation of sectioned AJ-1/23 C-2 cells grown in L-broth with extra magnesium sulphate. x80 000

Table 12 Addition of salts to sedimentation tube

Medium	% decrease in A_{600}
L-broth	42
L-broth + $MgSO_4$	51
L-broth + NaCl	44

Table 13 Effect of washing on $MgSO_4$ stabilisation of AJ-1/23 C-2 suspension

Medium	% decrease in A_{600}
L-broth	55
L-broth, washed and resuspended	55
L-broth + $MgSO_4$	7
L-broth + $MgSO_4$, washed and resuspended	5

3.8.3 Washing with EDTA

Cells grown in L-broth with extra $MgSO_4$ were centrifuged, washed in phosphate buffer containing 25mM EDTA (ethylenediaminetetra-acetic acid disodium salt), re-centrifuged and suspended in fresh L-broth. Results of sedimentation tests are shown in Table 14.

A sample of cells grown in L-broth alone was also washed with EDTA and re-suspended in L-broth. This treatment did not alter the flocculation of the culture, showing that EDTA itself does not affect flocculation.

The absence of any effect of a chelating agent such as EDTA suggests that the Mg^{2+} is exerting its stabilising effect from within the cells rather than being adsorbed to the cell surface.

3.9. Flocculation in strain AJ-1/23

Overnight cultures of the parent strain to AJ-1/23 C-2, AJ-1/23, a non-flocculating strain, were set up in L-broth, Seed 2 and Seed 2 with minimum magnesium (0.2 ppm). Sedimentation test results were as shown in Table 15.

Flocs were clearly visible in the low Mg^{2+} S2 culture, although growth in this medium was considerably less than in the other two, the flocs being small and therefore sedimenting only slowly.

The morphology of AJ-1/23 in L-broth was similar to that of AJ-1 and AJ-1/23 C-2 in L-broth, ie. the cells varied considerably in length. In S2 with both high and low Mg^{2+}, the cells were less variable in size and generally shorter. Measurements were made with the particle size analyser and the results are shown in Figs. 26 to 28. Conclusions ?

Table 14 Effect of washing with EDTA on $MgSO_4$ stabilisation of AJ-1/23 C-2 suspension

Medium	Mean % decrease in A_{600}
L-broth + $MgSO_4$	7
L-broth + $MgSO_4$, washed with EDTA	0
L-broth	26
L-broth, washed with EDTA	23

Table 15 Flocculation of AJ-1/23 in different media

Medium	Mean % decrease in A_{600}	S.D.
L-broth	8.7	2.6
S2	0.0	0.0
S2 with 0.2 ppm Mg	17.3	2.5

Figs. 26 – 28: Particle size distributions showing effect of medium on growth of AJ-1/23.

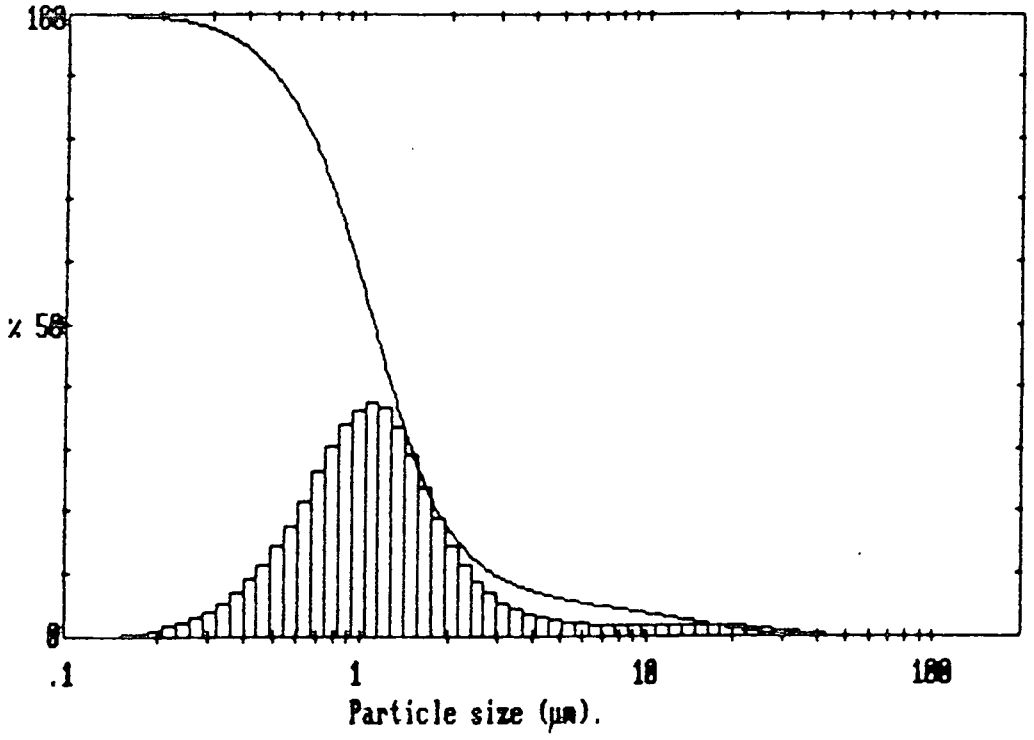

Fig. 26 AJ-1/23 grown in L-broth

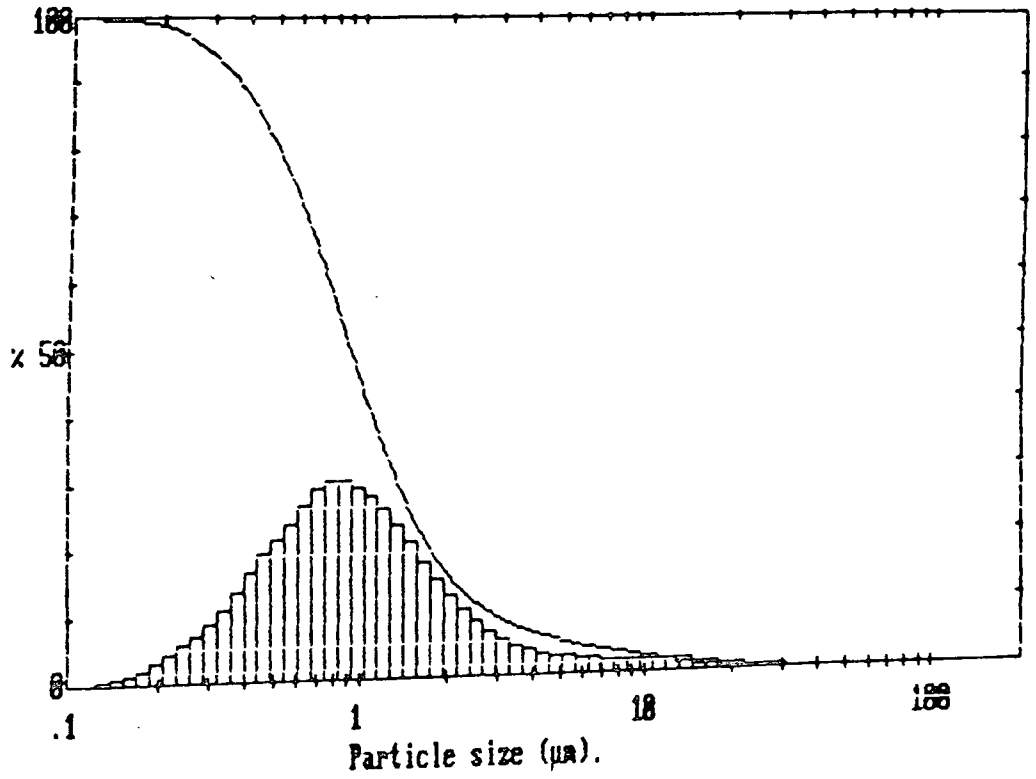

Fig. 27 AJ-1/23 grown in Seed 2

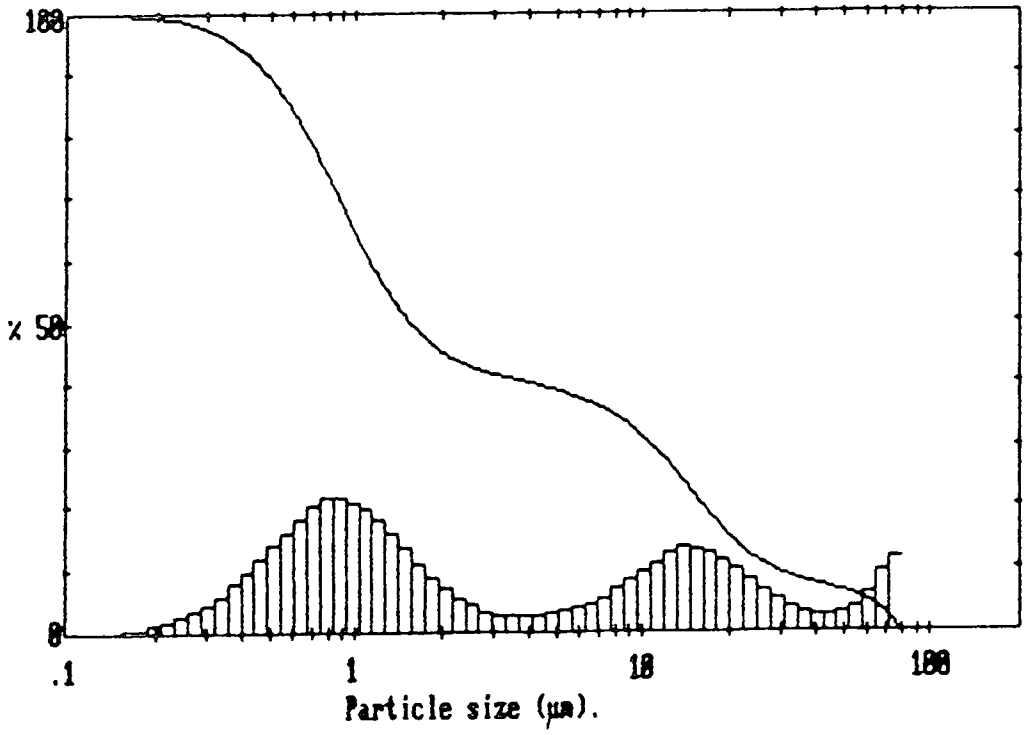

Fig. 28 AJ-1/23 grown in Seed 2 with minimal magnesium

3.10. SDS - PAGE analysis of cell proteins

Electrophoresis was carried out using vertical slab gels in a discontinuous system (Tris-glycine reservoir buffer and Tris-HCl gel buffer). Strains AJ-1, AJ-1/23 and AJ-1/23 C-2 were compared (see Methods). Fig. 29 shows the results for total soluble protein and Fig. 30 those for cell wall proteins. In both cases, there are no apparent differences in the band patterns between the flocculating and non-flocculating strains, implying that flocculation is not a direct result of differences in cell wall proteins between the strains.

3.11. Lipid analysis using thin layer chromatography

No qualitative differences were found in the lipid content of AJ-1 and AJ-1/23 C-2. Three separate spots appeared after charring, one identifiable as phospholipids (blue with Molybdenum blue), one as choline (orange with Dragendorff's reagent) and a third which stained with all three dyes and contained lipids having both phosphorus and amine/choline groups, eg. phosphatidylethanolamine. The pattern was identical for both strains. The mass of cell material used was not determined, although equal volumes of culture were employed, so it is possible that the strains could differ in relative amounts of the types of lipid present.

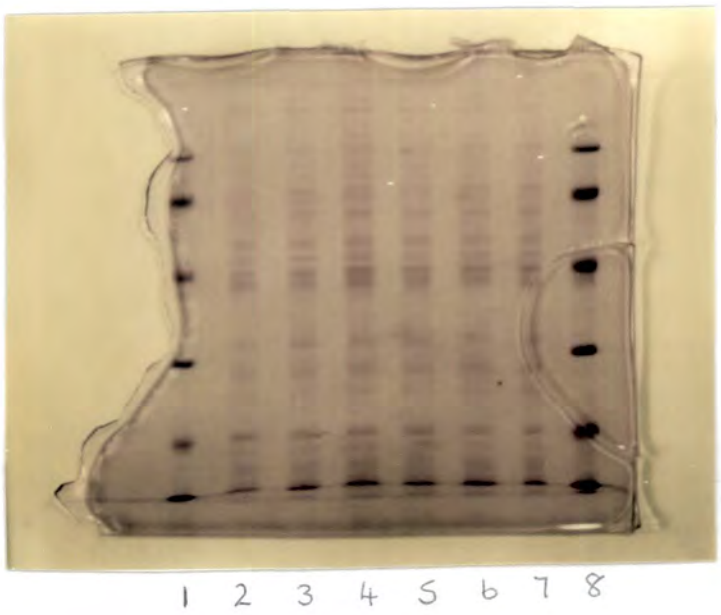

Fig. 29 SDS-polyacrylamide electrophoresis gel showing whole cell proteins. Lanes 1 and 8, low molecular weight standard; lanes 2 and 5, AJ-1; lanes 3 and 6, AJ-1/23 C-2; lanes 4 and 7, AJ-1/23 C-2 F-1.

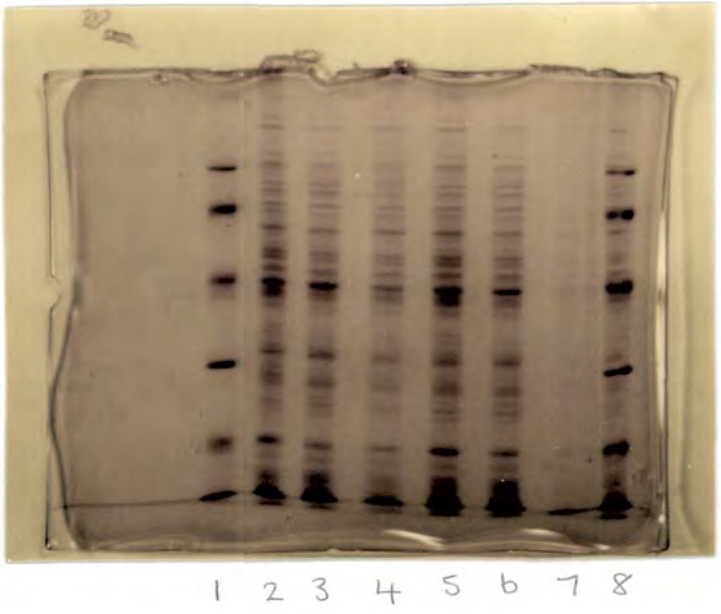

Fig. 30 SDS-polyacrylamide electrophoresis gel showing cell wall proteins. Lanes 1 and 8, low molecular weight standard; lanes 2 and 5, AJ-1; lanes 3 and 6, AJ-1/23 C-2; lanes 4 and 7, AJ-1 C-2 F-1.

3.12. Measurement of zeta potential

Measurements were made at the Luton factory of Coulter Electronics, Ltd. and at ICI Chemicals and Polymers, Runcorn Heath.

The DELSA 400 system has not so far been much used for micro-organisms and a number of problems were encountered. The concentration of particles in the suspension is crucial and a suitable dilution was very time-consuming to obtain. Relatively high levels of some of the broth components also caused difficulties; deposits of silver chloride were produced on the silver electrodes from the sodium chloride present in the medium. The refractive index and viscosity of the medium were assumed to be the same as for water, as it was not possible to measure these independently. Other parameters which need to be controlled for accurate measurements to be made are the pH and ionic strength of the sample.

The results from the Luton visit shown in Figs. 31 - 33 and Table 16 were from samples which varied slightly in pH and the sample with extra $MgSO_4$ would have had a higher ionic strength than the other two. It was in an attempt to reproduce these results under more carefully controlled conditions that the visit to Runcorn was made but, unfortunately, no more reliable data were generated. The two main aims were to find out whether the bimodal distribution of zeta potential obtained with the flocculating AJ-1/23 C-2 was genuine and whether the change in distribution observed with extra $MgSO_4$ was merely the result of increasing the ionic strength or something more significant.

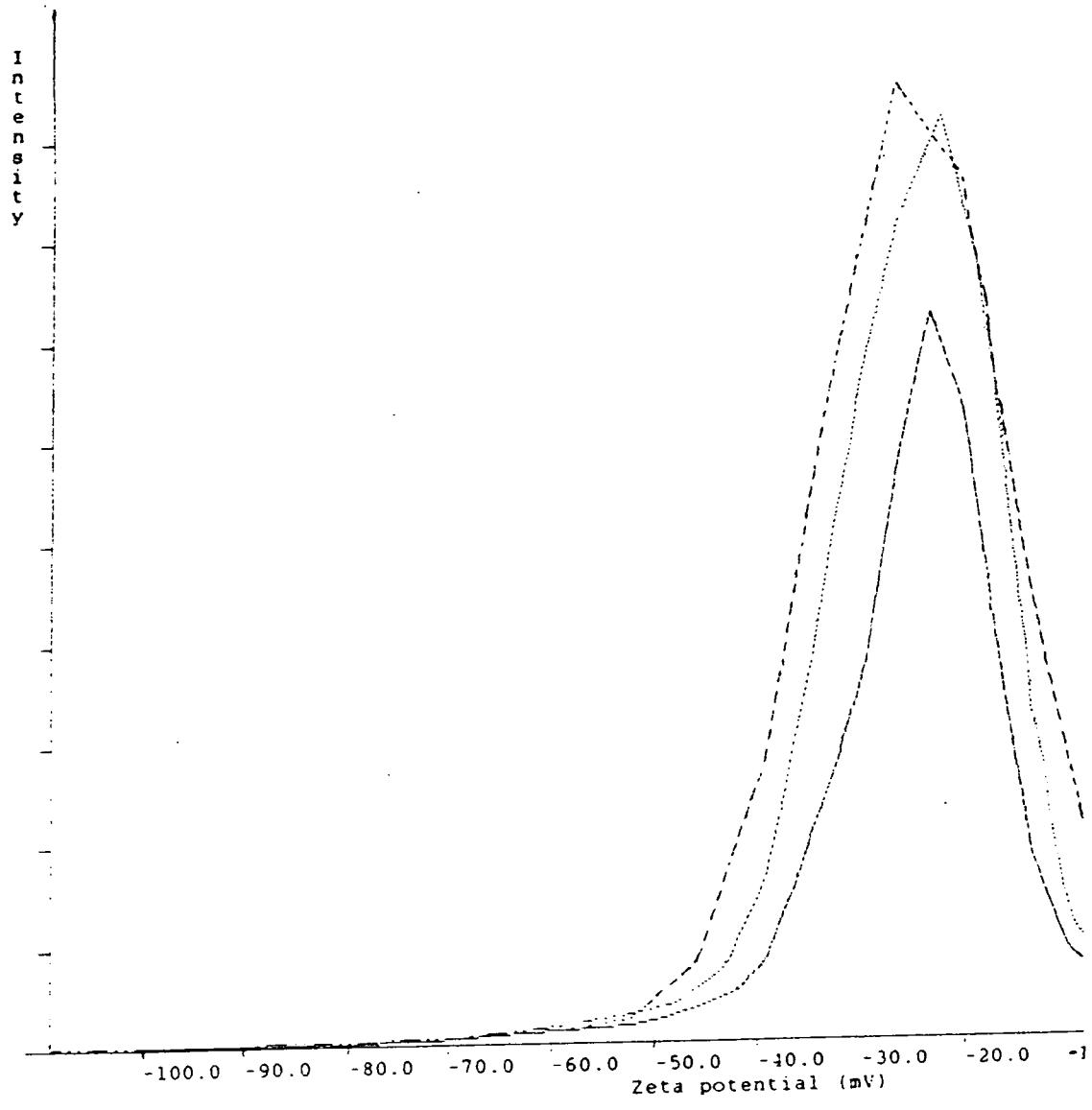

Fig. 31 Zeta potential of AJ-1 in L-broth. Printout from DELSA 400 system. Three separate lines indicate measurements of potential made at three different detection angles. Figs. 32 and 33 from same source; four measurements made for Fig. 33.

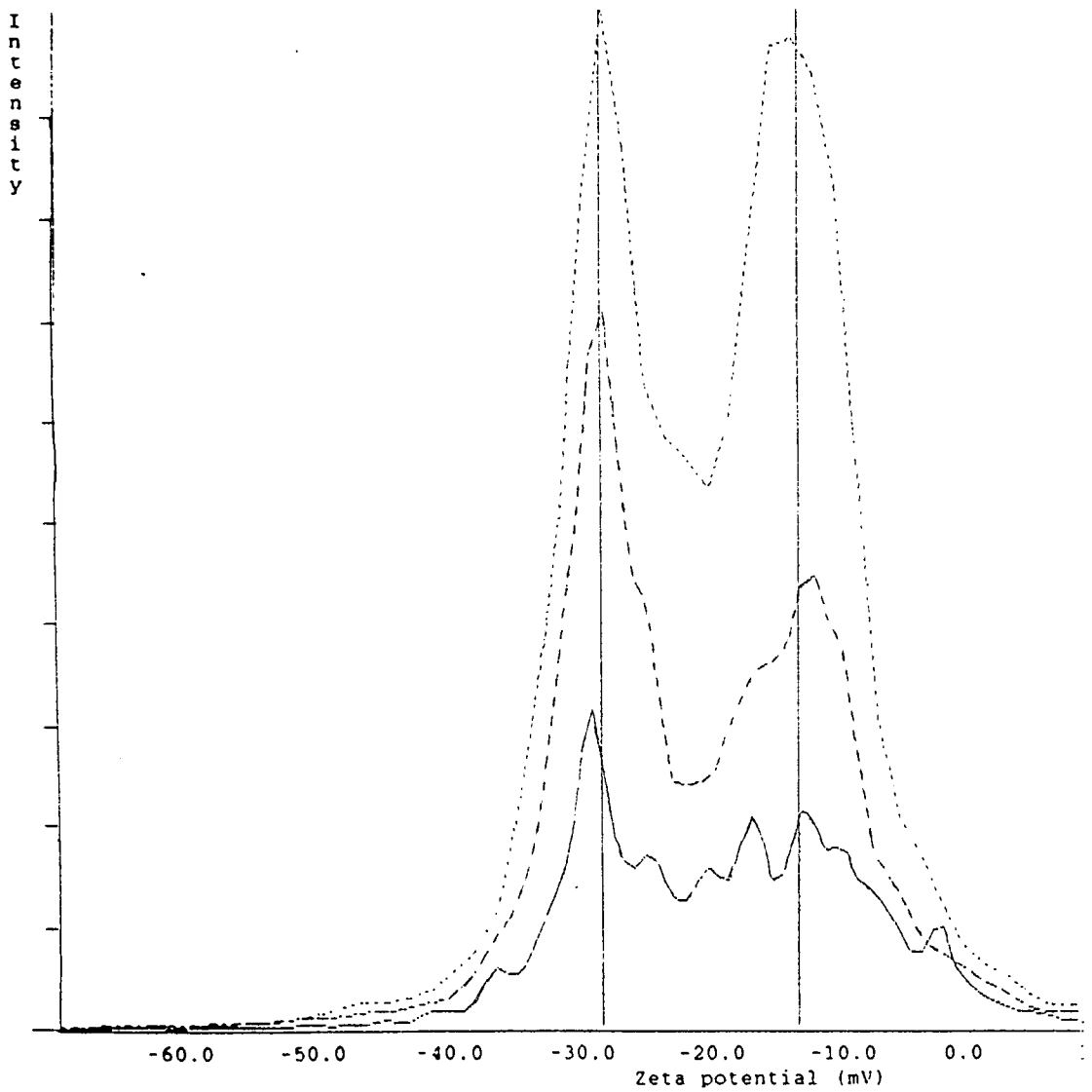

Fig. 32 Zeta Potential of AJ-1/23 C-2 in broth

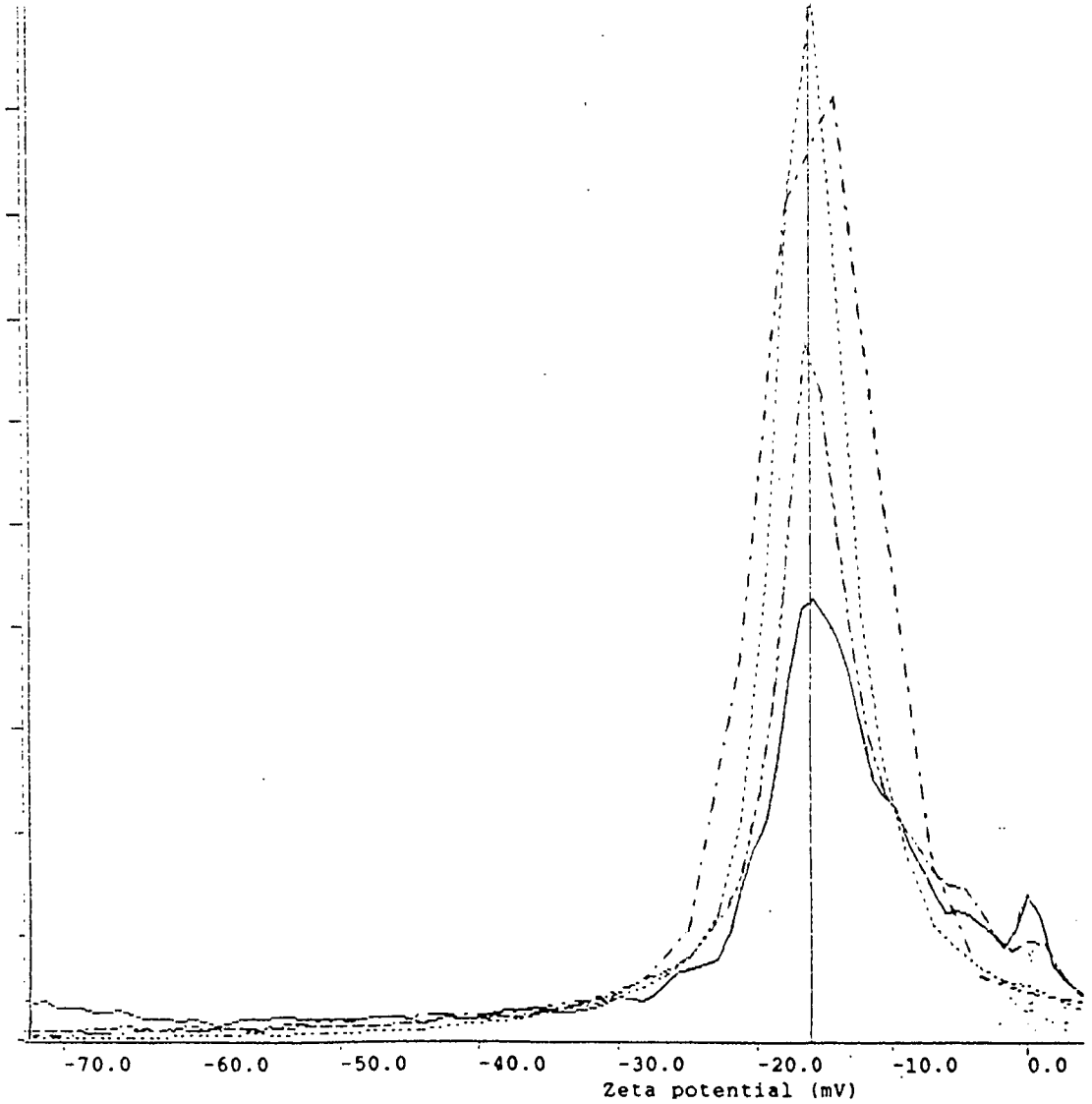

Fig. 33 Zeta potential of AJ-1/23 C-2 in broth with additional magnesium sulphate

Table 16 Zeta potentials of three different cultures

Culture	Zeta potential (mV)
AJ-1, L-broth	-23.6
AJ-1/23 C-2, L-broth	-21.6 and -10.9
AJ-/23 C-2, L-broth + $MgSO_4$	-16.1

3.13. Investigation of floc strength

L-broth cultures of AJ-1/23 C-2 were subjected to shear (see Methods) for varying lengths of time and the effect on particle size distribution is recorded in Table 17.

Fig. 34 shows the particle size distribution after 23 minutes stirring and Fig. 35 the distribution after 75 minutes stirring.

Short periods of stirring actually encouraged flocculation although after 75 minutes most of the larger particles had been broken down. Considerable variation was observed when the experiment was repeated, the flocs breaking down after only 23 minutes in one case and remaining stable for three hours of stirring in another. There was no time to investigate this variation further but it is possible that the age of the culture plays a part; the strongest flocs were from a 15-hour culture and the weakest from a 24-hour batch.

When the flocs had been broken up, the culture was placed on an orbital shaker and left for several hours to see if the flocs would re-form. Fig. 36 shows the particle size distribution after 2 hours and Fig. 37 after 6 hours. Clearly, floc formation is reversible.

3.14. Uptake of magnesium and calcium by cells

Analysis of the magnesium content of L-broth before and after growth of cells was made to try to establish how much of the magnesium was available for cell uptake. Results are shown in Table 18.

Table 17 Particle size of AJ-1/23 C-2 after stirring for varied lengths of time

Time (min)	Median size (μm)	90th percentile of distribution (μm)
0	1.66	20.95
23	2.64	27.83
43	2.17	23.19
63	1.77	9.93
75	1.55	9.40

Table 18 Decrease in magnesium content of broth following cell growth

Strain	Medium	Initial A_{600}	Original Mg^{2+} (ppm)	Final Mg^{2+} (ppm)	% decrease in Mg^{2+} per A_{600} unit
AJ-1	L-broth	1.965	1.3	0.2	43.1
AJ-1	L-broth				
	+ $MgSO_4$	3.726	32	27	4.2
AJ-1/23 C-2	L-broth	2.262	1.3	0.4	30.6
AJ-1/23 C-2	L-broth				
	+ $MgSO_4$	3.672	32	9	19.6

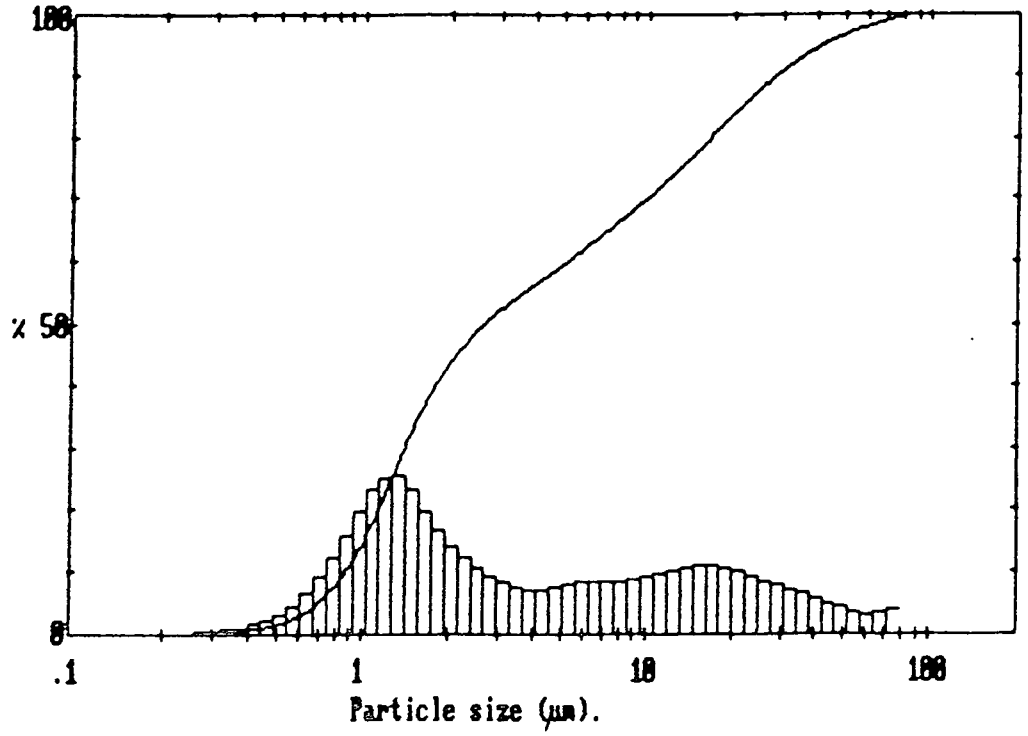

Fig. 34 Particle size distribution of AJ-1/23 C-2 after stirring for 23 min. to break up flocs

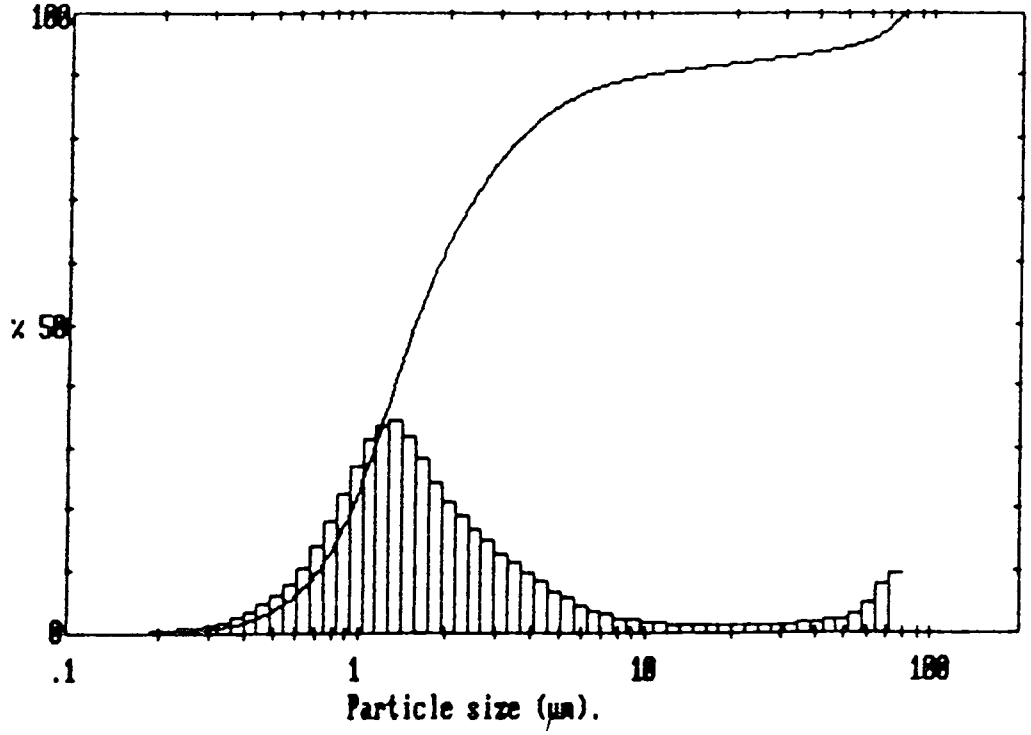

Fig. 35 Particle size distribution of AJ-1/23 C-2 after stirring for 75 min. to break up flocs

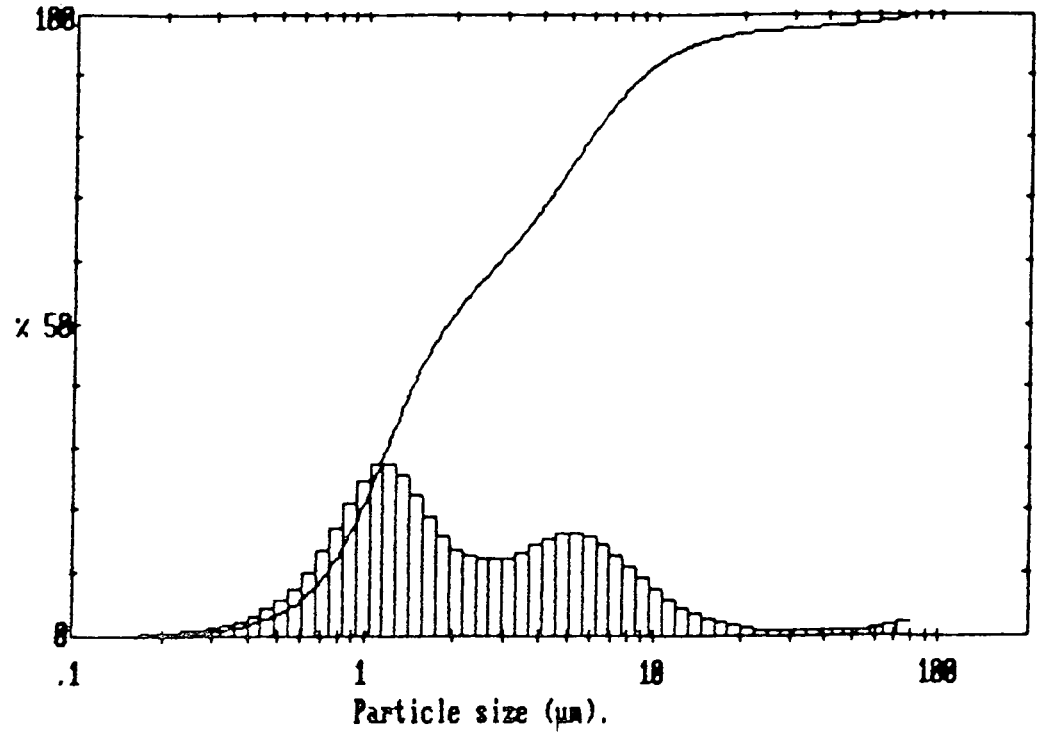

Fig. 36 Particle size distribution of AJ-1/23 C-2 with the flocs broken up, after 2 hr on shaker, showing floc re-formation

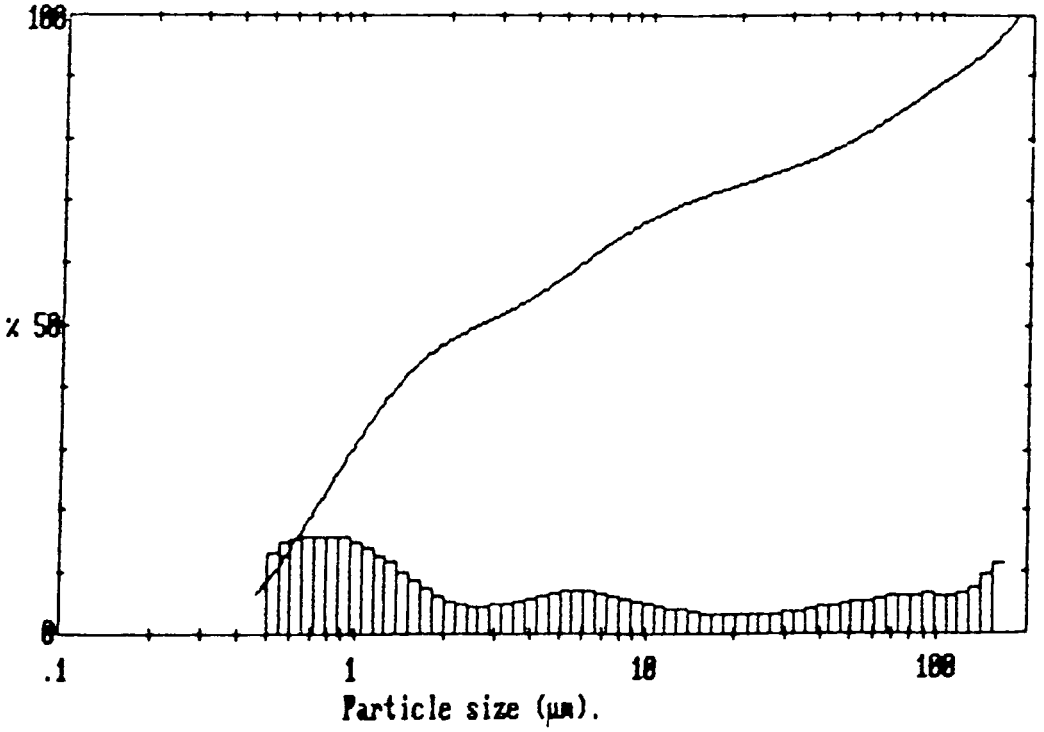

Fig. 37 Particle size distribution of AJ-1/23 C-2 after a further 4 hr on shaker, showing floc re-formation

Although the final results for the two strains in L-broth alone differ considerably, because the original Mg^{2+} level was so low this is probably not a significant difference. When Mg^{2+} is plentiful, however, AJ-1/23 C-2 takes up much more than does AJ-1, indicating that it has a higher requirement for Mg^{2+} . The reliability of this comparison is increased by the very similar initial optical densities of these two cultures.

There is no evidence to suggest that the magnesium in the L-broth itself is unavailable to the bacteria as the final concentration, particularly with AJ-1, is tending towards zero.

Changes in calcium level were also investigated but, as the initial concentration was less than 3 ppm, the differences were not regarded as being significant.

Discussion

4.1 Variation in flocculation

Autoflocculation in *Pseudomonas putida* appears to be under genetic control. Over a four-month period, it has been shown to be a consistent feature of some strains, provided some selection pressure is applied, and not of others. The measured amount of flocculation in flocculating strains was, however, very variable, although in AJ-1, and in inhibiting environments such as Seed 2 medium, flocculation was consistently low. The population of AJ-1/23 C-2 was possibly genetically mixed, containing a proportion of non-flocculating cells. This hypothesis is supported by the particle size distribution of Fig. 15, in which there is still a number of cells between 1 and 3μm in size, and would also be worth investigating on the basis of the bimodal zeta potential of AJ-1/23 C-2, which could be either a charge effect or the result of different sizes of cell in the population. Some workers have demonstrated, however, that, at sufficiently high cell concentrations, a dynamic equilibrium exists between flocs and single particles such that, in conditions suitable for flocculation, not all the cells will flocculate (Ash, 1979). Taking single-colony isolates from the stock plates to inoculate the shake flasks would enable the degree of genetic variation to be examined.

The sedimentation test for measuring flocculation is simple to carry out but in practice can have serious limitations. Optical density depends on both particle concentration and particle size. It seems likely that, in some cases at least, cells were reproducing in the sedimentation tube and compensating for other cells aggregating as flocs, thus an incorrect value for flocculation would be recorded. With certain cultures, flocs were visible to the naked eye but were relatively few,

especially when the growth rate was low, and so may not have been included in a sample at all. Where AJ-1/23 C-2 was flocculating in L-broth, large flocs in a sample were seen to sediment almost immediately in the spectrophotometer cuvette, thus giving a falsely low initial A_{600} reading for the culture. Some of the variation in flocculation discussed above may have resulted from these practical problems. With the addition of flocculating agents such as aluminium sulphate or Zetag, where there was a reaction with the broth, the flocs stayed suspended and no quantitative measurements were possible. Results from the Malvern particle size analyser were more reliable but this instrument was only acquired towards the end of the project period. As far as possible, however, the conditions for operating the sedimentation test were uniform and the results given are means of at least three samples in almost every case.

4.2 Phases of growth

Flocculation was only really measurable via the sedimentation test towards the end of log phase or in stationary phase of growth. Neither increase in cell concentration nor cell death appeared to contribute to the process (see Section 3.3). Some metabolic changes in the cells may, however, occur with age and this was not investigated; there was some evidence of floc strength decreasing as the cultures aged.

4.3 Environmental factors

Environmental factors were shown to play a major role in flocculation. It seems that AJ-1/23 C-2 should be described as a flocculating strain of *Pseudomonas putida* only if the growth medium is also clearly defined. Standard Luria broth made with Oxoid tryptone T enables flocculation to occur but it is possible to manipulate the broth composition and prevent flocculation. In all cases, the level of Mg^{2+} proved to be critical and this was also shown to be true for Seed 2 minimal medium. There is the possibility that Ca^{2+} may have a smaller but similar effect

- time did not permit further investigation of this or other trace elements present in the media.

Divalent cations frequently cause flocculation in suspensions as a result of charge neutralisation or, especially in the case of calcium, the formation of bridges. It was surprising, therefore, to discover the opposite effect in this instance. One earlier worker has cited magnesium ions as inhibitory to flocculation of an activated sludge bacterium, which could have been a pseudomonad (Tezuka, 1967), but there is no suggestion as to how the mechanism might operate.

4.4 Standard flocculating agents

Aluminium sulphate flocculates both flocculating and non-flocculating strains of *P. putida* and the cationic polyelectrolytes Zetag 76 and Magnafloc LT22 also flocculate AJ-1. This is as would be expected with negatively charged particles. More work needs to be done with cultures grown in S2 to try to establish how much of the measured flocculating effect with these agents was due to interactions with the complex medium , especially the peptones.

The fact that mannan did not flocculate AJ-1 indicates that *P. putida* lacks the type of receptor found in that other Gram-negative bacterium *E. coli* and in yeasts. The iso-electric point (pH producing a large increase in flocculation) seemed to be similar for both AJ-1 and AJ-1/23 C-2 (see Fig. 20) and within the normal range for Gram-negative bacteria.

4.5 Flocculation in non-flocculating strains: the cause of flocculation

It proved possible to induce flocculation in both AJ-1 and the parent of the flocculating strain, AJ-1/23, by reducing the Mg^{2+} content of S2 medium. This medium then allows only very slow growth but does lead to flocculation. Higher A_{600} values reached by AJ-1 in L-broth with extra magnesium compared with standard L-broth show that growth in normal L-broth is magnesium-limited. This

suggests that the basis for flocculation in AJ-1/23 C-2 is magnesium depletion.

Nutrient-limitation as a cause of flocculation would explain why early growth stages of cultures show little or no flocculation. Magnesium does not become a limiting factor until the cell concentration has reached a certain level; one study found that 0.02mM Mg^{2+} allowed the same growth rate as 0.5mM up to an A_{600} of 0.6 (Nicas and Hancock, 1983).

4.6 Role of magnesium in micro-organisms

Magnesium is required by bacteria for ribosomal synthesis, as a cofactor for many enzymes, including alkaline phosphatase and some enzymes of the glycolytic pathway, for use of ATP and in the structure of the cell wall. In Gram-negative bacteria, divalent cations, probably mainly Mg^{2+}, play an important structural role in the outer membrane by forming bridges between phosphate groups of both lipopolysaccharides and phospholipids. In *E. coli* , magnesium was found to compose approximately 0.5% of the dry weight of the cells (Mandelstam *et al* , 1982).

The experiments described in Section 3.8 show that magnesium is taken into the cells and not simply adsorbed on to the surface. Flocculation in magnesium-depleted conditions cannot be reversed by external application of soluble magnesium, indicating that some metabolic process is involved.

The difference in size produced during magnesium-depleted growth occurred in all three strains investigated (AJ-1, AJ-1/23 and AJ-1/23 C-2) and appears to be a separate phenomenon from flocculation. The fact that increase in cell size results from magnesium deprivation explains why there was no change in morphology following addition of $CaCl_2$ to AJ-1 (Fig. 23) – only increasing the magnesium level would alter cell dimensions. The bacteria already have adequate calcium for their needs. Other workers have described filamentous cells produced in nutrient-limiting conditions, including magnesium-limitation (Tempest *et al* ,

1965). Magnesium plays a part in cell division and at low concentrations cell division is inhibited, resulting in much elongated cells. Specific proteins affecting cell shape have been identified in *E. coli* (Spratt, 1975) where it was suggested that a different protein is required for making peptidoglycan for the cross walls from that for the side walls. This could perhaps form a basis for the role of magnesium deprivation in that lack of cross wall synthesis would tend to lead to filamentous growth.

The question may then arise as to whether flocculation is merely a result of cells failing to divide properly. This phenomenon, termed flocculent growth, does occur in some micro-organisms lacking sufficient energy for separation after division but can be rejected here on the basis of the microscopic evidence and the fact that the flocs can re-form after being broken up; this would not be possible with flocculent growth.

4.7 The mechanism of flocculation

The mechanism of flocculation operating in *Pseudomonas putida* is not at all clear but several possibilities can be considered. Section 4.9 below gives suggestions for future work which could lend support to one or more of the following hypotheses.

Involvement of cations suggests at first a charge neutralisation effect but this would need to operate in reverse – high Mg^{2+} will encourage flocculation in *E. coli* and *Serratia marcescens*, for example (Cumming, 1987). The zeta potential might be more negative for AJ-1 than for AJ-1/23 C-2 but, as mentioned previously,the evidence for this is so far very unreliable. Polymer bridging, in which the free ends of wall polymer molecules become attached to other cells, cannot be the whole explanation either, as this mechanism is usually destroyed when flocs are broken up and with *P. putida* the flocs were able to re-form. Flagella are not visible in the electron micrographs, nor are pili, but a different method of preparation

might provide clearer evidence of surface structures.

Figs. 9 and 13 show evidence of secreted material between the cells of AJ-1/23 C-2 which could, either alone or in conjunction with a cationic substance, be trapping the cells and causing flocculation. Analysis of both protein and lipid content of flocculating and non-flocculating strains showed no qualitative differences in either (Sections 3.10 and 3.11) but it is not possible to draw conclusions about the relative amounts of the various compounds. This still leaves polysaccharides to be investigated; as mentioned previously, secreted polysaccharides have been implicated in some types of flocculation.

Magnesium deprivation, especially in chemostat culture, has been the subject of a number of studies over the past twenty years. Work on *P. aeruginosa* has shown several distinct changes in cell wall structure resulting from magnesium-limitation; these include increases in total carbohydrate, phosphatidylglycerol and free fatty acids and decreases in phosphatidylcholine and phosphatidylethanolamine (Kenward *et al* , 1979). Sutherland (1985) also found enhanced polysaccharide production but emphasised the difficulty in distinguishing between wall polysaccharides and secreted ones, the wall polymers possibly being excreted or lost into the medium as the cells age. Membrane proteins may also be affected. Nicas and Hancock (1983) describe induction of synthesis of an outer membrane protein, H1, resulting from growth of *P. aeruginosa* in magnesium-depleted conditions.

Magnesium-limited pseudomonads, grown in both batch and chemostat culture, have been shown to be resistant to the lytic effects of EDTA and to the antibiotic polymyxin B (Kenward *et al* , 1979). The mechanism is thought to involve replacement of Mg^{2+} in the outer membrane by some other cations, such as polyamines, which cannot be removed by EDTA and prevent attachment to the phosphate sites in the cell wall by polymyxin. The bacteria are thus rendered resistant to attack.

Changes in many of the components of the cell wall are therefore known to occur with magnesium deprivation, resulting from metabolic changes within the cell and so developing over a period of time. This agrees with the findings of Section 3.8. Presumably it is an alteration of this type in the bacterial outer membrane which leads to flocculation.

There may be a relatively straightforward chain of events in which Mg^{2+} depletion leads to increased production and secretion of polysaccharide which causes the cells to stick together. The genetic basis for this could be via a change in synthesis of either a particular enzyme or a wall protein involved in permeability. The electron micrographs in Figs. 12, 13 and 25 show a much rougher surface for cells in the flocculating system (Fig. 13) than for the other cells. Roughness could be associated with increased permeability (to polysaccharides or other substances), increased hydrophobicity or a greater tendency to adhere because of larger surface area. Any of these could lead to flocculation.

Although many species of bacteria have been shown to secrete polymers, the ecological significance of this is often not yet understood (Wrangstadh *et al*). An attempt at explanation has been made, however, for some of the other changes induced by magnesium-depletion. In their natural environment, where growth conditions are often unstable and seldom ideal, nutrient insufficiency is likely to be the most common extreme to which bacteria are exposed. Maximum growth rate is rarely achieved and the organisms must modify their behaviour to cope with sub-optimal conditions and still compete effectively with other species. This may involve decreasing the rate at which plentiful nutrients are taken up, to achieve a balance within the cell, or induction of some high-efficiency uptake system for the growth-limiting nutrient. Novel outer membrane proteins may act to "scavenge" the surroundings selectively for the depleted substrate, the biochemical flexibility of the cell wall enabling a rapid response to be made to environmental

conditions. Any increase in the negative charge on the cell surface, for example, would help in the adsorption and possible subsequent uptake of cations.

While no differences in protein were detected between the flocculating and non-flocculating strains of *P. putida* , there could be some other scavenging mechanism in the outer wall whose induction leads directly or indirectly to flocculation. As with the EDTA- and Polymyxin-resistant cells, Mg^{2+} at its limit of availability may be replaced by other cations from the medium. If ions of larger radius were to replace the Mg^{2+} so that they protruded further from the outer membrane, they might interact with cell wall polymers to form bridges and lead to flocculation. The secreted material visible in Figs. 9 and 13 may or may not be important.

Whatever the actual mechanism, the difference in behaviour between flocculating and non-flocculating strains could be related to differing requirements for magnesium. If AJ-1/23 C-2 has a higher Mg^{2+} requirement, conditions would become limiting much earlier than with other strains. The results in Section 3.14 tend to support this hypothesis.

4.8 Possibility of using flocculation in cell separation

Floc strength measurements showed that the size of the flocs was actually increased by initial periods of stirring because, for particles of less than 1μm diameter, moderate shear stresses considerably increase the collision frequency of the cells and hence increase flocculation (Ash, 1979). Stability of flocs during small-scale centrifugation was also confirmed. This suggests that the flocs would stand up quite well to downstream processing with its attendant shear and turbulence effects. It was evident throughout the work that separation of cells from medium by centrifuging was more efficient with AJ-1/23 C-2 than with AJ-1, even when sedimentation tests did not give very high flocculation values.

It proved possible to induce flocculation in other strains apart from AJ-1/23

C-2 by lowering the magnesium concentration in the medium, so it may not be necessary to use AJ-1/23 C-2 in production. If the genes for autoflocculation in this strain could be identified and isolated, however, they could perhaps be transferred to other strains in the future by genetic engineering techniques.

Autoflocculation occurs in growth-limiting conditions which would not be the best for fermenter culture. Neither would it be ideal to grow the cells in a flocculated state as cells in the centre of the flocs could be deprived of oxygen and nutrients. This suggests a two-stage fermentation; initial growth of *Pseudomonas putida* should be carried out under carbon-limiting, rather than magnesium-limiting, conditions, followed by a lowering of the magnesium concentration to induce flocculation once enough dehalogenase has been produced.

If a whole cell preparation is to be used commercially rather than an enzyme extract, it may be important to be able to reverse flocculation in order to provide sufficient surface area for enzyme-substrate interaction. The cells could then be immobilised on a suitable support, as has already been done with *P. putida* containing active hydantoinase (Chevalier *et al* , 1989).

4.9 Suggestions for further work

More use could be made of the Malvern particle size analyser for assessing flocculation in view of the drawbacks of the sedimentation method. This could be particularly valuable in a chemostat situation where the effect of changing the magnesium level in the medium could be monitored on a continuous basis.

Several possible mechanisms for flocculation have been suggested. The electron micrographs should be repeated to make sure the cell wall details and the presence of secreted material appear again and are not merely artefacts. Identification of the secreted material should be carried out; if it is truly secreted it should be separable from the cells by centrifugation, when it could be recovered in the supernatant and

analysed. The effects of adding such supernatant to non-flocculating cultures of *P. putida* could then be investigated. A repeat of Cousland's work (unpublished) on floc destruction by cellulase and proteinase K could also be carried out.

The importance of charge effects and any possible alteration in surface charge brought about by magnesium limitation require more work to be done on the zeta potentials of the different strains. Hydrophobicity studies on flocculating and non-flocculating strains would also be of interest.

The role of calcium ions in flocculation and their relationship to magnesium has been given only passing attention and deserves further study. Removal of calcium from the medium by addition of a chelating agent such as EDTA would be a straightforward procedure but perhaps not sufficiently specific. The role of Ca^{2+} might more accurately be established by making up particular media with the calcium salts omitted. It is important, however, for calcium and other trace elements, and for magnesium at very low levels, that measurements of concentration are as reliable as possible and this needs particularly clean glassware if the results are to be repeatable.

There are clearly major differences between shake flask culture and the same organisms grown in a chemostat or large-scale fermenter and it is not possible to extrapolate results from one to the other. In this instance especially, where flocculation depends so critically on medium composition, it is important that the same types of experiment as have been carried out with batch culture are repeated with continuous culture and the correct fermentation medium. Only then can it be established whether or not the process of flocculation can be a useful application in the fermenter growth of *Pseudomonas putida* .

Summary

The project showed that flocculation in the strains of *Pseudomonas putida* under study is controlled by the magnesium level in the medium, such that flocculation is induced in conditions of magnesium-deprivation. Other trace elements in the medium may also play a part. The mechanism of flocculation has not been established but probably involves alterations in the structure and biochemistry of the bacterial outer membrane induced by a lower than normal internal magnesium concentration. No difference was found in the lipid or protein content of flocculating and non-flocculating strains but some evidence for secretion of polymer material, possibly polysaccharide, was seen in flocculating systems and this may also play a part in cell aggregation. A bridging mechanism of some kind therefore seems more likely as an explanation for flocculation than charge neutralisation effects.

Apart from causing flocculation in particularly magnesium-sensitive strains, magnesium-depletion also resulted in altered morphology in both flocculating and non-flocculating strains of *P. putida* . It seems that the biochemical changes causing elongation of the cells may be a completely separate phenomenon from those linked to flocculation.

As far as downstream processing is concerned, the project showed that flocculation does improve the separation of cells from medium by centrifugation and that the flocs may well be strong enough to warrant the use of flocculation in the commercial production of *Pseudomonas putida* .

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