A MOLECULAR BIOLOGICAL CHARACTERISATION OF *MICROCYSTIS AERUGINOSA* KüTZ. EMEND ELENKIN

by

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SUMMARY

Microcystis aeruginosa Kütz. emend Elenkin is a cyanobacterial species which is sometimes found to be toxic. The most common toxin is a hepatotoxin but other toxins are also produced.

The purpose of this study was to study the possibility of plasmid involvement in toxin production by *M. aeruginosa*. Curing experiments gave no clear answer to this question. The lysis of cells grown in tap water- and deionised water-based media was studied. Both types of cells were refractile to common methods of lysis, with growth conditions having a profound effect on this refractility. A grinding method gave the highest yield of DNA but this did not exceed 25 %. Relatively high levels of nuclease were found but these could be inhibited by the use of diethyl pyrocarbonate or EDTA. A single contaminating bacterial species was found, which was not identified. The bacterium produced Slow Death Factor and did not contain plasmid DNA.

Mass lyses, using methods tested in small scale lyses, yielded plasmid DNA from *M. aeruginosa* WR70. The mol % G+C of the different DNA's was determined, with the plasmid and *M. aeruginosa* having similar mol % G+C's (39,57 and 39,25 respectively). The bacterium had a G+C content of 60,54 which was similar to that found for a second band in lysates of *M. aeruginosa* (61,89). The latter DNA's were probably from the same organism. Electron microscopy of the plasmid DNA revealed the presence of two plasmids which had molecular masses of 2,05 Md and 5,02 Md respectively.

From this study no definite conclusion can be drawn as to whether or not plasmids are involved in the toxicity of *M. aeruginosa* WR70, but the finding that strain WR70 contains at least one plasmid which is not present in the non-toxic strain PCC 7005 may be an indication of this possibility.

Suggestions for future research are discussed.

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SAMEVATTING

Microcystis aeruginosa Kütz. emend Elenkin is 'n cyanobakteriespesie wat soms toksies is. Die mees algemene toksien is 'n lewer-toksien maar ander toksiene word ook aangetref. Die doel van hierdie studie was om die moontlikheid van plasmiedbetrokkenheid by die toksisiteit van *M. aeruginosa* te bestudeer. Die metodes wat gebruik is, naamlik elimineringstudies en plasmied isolasie, word beskryf.

Die resultate van elimineringstudies word bespreek. Geen verklaring oor plasmiedbetrokkenheid by toksisiteit kon uit hierdie resultate gemaak word nie.

Selle is gekweek in media wat opgemaak is met of kraan- of gedeioniseerde water. Albei seltipes is moeilik liseerbaar. Groeitoestande is bevind om 'n groot verskil te maak in die liseerbaarheid van selle. 'n Maalmetode het die meeste DNA vrygestel, maar selfs met hierdie metode is nie meer as 25 % van die sel-DNA vrygestel nie.

Die nukleaseaktiwiteit van *Microcystis* is bestudeer. Relatiewe hoë vlakke is gevind maar die gebruik van of di-etiel piro-karbonaat of EDTA het 'n inhiberende effek op hierdie aktiwiteite gehad.

'n Enkel kontaminerende bakterie-spesie is gevind, wat nie geidentifiseer is nie. Die bakterie het 'n toksien geproduseer en nie 'n plasmied bevat nie.

In grootskaalse liseringtoetse is 'n plasmied in *Microcystis* gevind. Die DNA-basissamestelling van die plasmied (39,57) was na aan dié van *Microcystis* (39,25), terwyl die bakterie 'n mol % G+C na aan dié van 'n tweede band in gradiente gehad het (60,54 en 61,89 onderskeidelik). Dit is dus moontlik dat hierdie twee DNA's van dieselfde organisme afkomstig is, d.i. die bakterie. Twee plasmiede is gevind, met molekulêremassas van 2,05 Md en 5,02 Md.

Geen uitspraak oor plasmiedbetrokkenheid by die toksisiteit van *M. aeruginosa* WR70 kan gemaak word nie, maar die bevinding dat WR70 minstens een plasmied het wat nie in die nie-toksiese tipe, PCC 7005, gevind is nie dui op hierdie moontlikheid.

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ABBREVIATIONS USED IN THIS REPORT

SDS	Sodium dodecyl sulphate
AL	Autolysin preparation extracted from *Microcystis aeruginosa*
FDF	Fast Death Factor (the toxin of *M. aeruginosa*)
SDF	Slow Death Factor
SSC	Saline sodium citrate
TE	Tris-EDTA buffer
EDTA	Ethylene diamine tetraacetic acid (sodium salt)
DEP	Diethyl pyrocarbonate
DTT	Dithiothreitol
PEG	Polyethylene glycol

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ERRATA

Please take note of the following corrections:

Page	Paragraph	Line	Correction
v	1	6	plasmiedisolasie
v	5	2	geïdentifiseer
v	6	4	gradiënte
v	6	6	molekulêre massas
v	7	3	for "tipe" read "stam"
9	3	2	discovered
10	4	4	add "cyanophage" before "LPP-1"
14	4	1	add "have" after "been"
18	2	2	Rippka
20	2	17	chromosomal
21	2	14	concomitant
34	2	2	for "3.3" read "3.4"
43	1	1	for "future" read "subsequent"
43	3	2	for "future" read "subsequent"
45	4	2	for "unquantitated" read "unquantified"
50	4	1	replace second "of" with "to"
51	2 (title)		for "LII" read "LIII"
51	5	2	implied
53	1	2	implied
53	3	3	disturb
53	6	1	add "of" after "wall"
65	6	2	yields
67	1	8	removed
67	4	1	add "(DTT)" after "dithiothreitol"
68	3	5	bands
70	1	6	should read "lanes 2-6 and 8"
75	Table	Last column	%G + C
77	2	4	add "plasmid" after "2 Md"
78	4	1	involvement
79	4	1	giving
85-89	for "J. Mol. Biol." read "J. mol. Biol." and for "Mol. Gen. Genet." read "Mol. gen. Genet."		
86	add "Bukhari, A.I., Shapiro, J.A. and Adhya, S.L., 1977. DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory." after "Brown, P.J."		
100	1	8	EDTA. Fe 1,2 mg/ℓ (as Fe)

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CHAPTER 1

INTRODUCTION

The toxicity of freshwater cyanobacteria, first reported by Francis (1878), has become a subject of ever-increasing importance, especially in countries such as South Africa which already have, or are faced by, serious water shortages as well as eutrophication problems. An indication of this importance was the decision by the US Environmental Protection Agency to sponsor a conference at Wright State University, Dayton, Ohio (29 June to 2 July 1980). The conference, "The Water Environment: Algal Toxins and Health", dealt with algal toxins in general, with especial reference to freshwater cyanobacterial toxicity.

There are numerous reports on freshwater cyanobacterial toxicity (reviewed by Schwimmer and Schwimmer, 1969; Gentile, 1971; Gorham and Carmichael, 1979). About 12 species from nine genera have been reported toxic, but three species, *Microcystis aeruginosa*, *Anabaena flos-aquae* and *Aphanizomenon flos-aquae* are most often reported toxic (Gorham and Carmichael, 1979; Carmichael, 1980; Gorham and Carmichael, 1980).

In South Africa only two of the 12 species have been found to be toxic, viz. *Nodularia spumigena* and *M. aeruginosa* (Scott, Barlow and Hauman, 1980). *N. spumigena* has been found to be toxic in only one location but toxic *M. aeruginosa* has a widespread distribution and may even occur throughout the country (Eloff, 1980; Scott, Barlow and Hauman, 1980). To date *Anabaena flos-aquae* and *Aphanizomenon flos-aquae*, which is very rare, have not been found to be toxic in South Africa (Scott, Barlow and Hauman, 1980). This finding is as yet inexplicable.

Environmental factors causing or influencing the toxicity of *M. aeruginosa* have received some attention (Gorham, 1964; Brown, 1974; Eloff and van der Westhuizen, 1980; Runnegar and Falconer, 1980; Sirenko, 1980) but to date very little has been done to study the genetics of toxin production.

The aim of this project was to study the possibility of plasmid involvement in toxin production by *M. aeruginosa* strain WR70.

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Two approaches were used in an attempt to answer this question. These were:

(a) Studying the toxicity of *Microcystis* grown in the presence of various agents known to eliminate plasmids or select for plasmid-free cells in bacteria.

(b) Attempting to isolate and characterise plasmids from *Microcystis*.

This report deals with results of curing experiments, lysis studies and the characterisation of DNA isolated from *Microcystis* cultures. An attempt is made to correlate the results of the lysis studies with various cell wall models.

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CHAPTER 2

LITERATURE REVIEW

2.1 Cyanobacterial toxicity

Toxic freshwater cyanobacteria were first described by Francis (1878), who reported livestock deaths in Australia as a result of a bloom of *Nodularia spumigena* on a lake. Frequent reports have since appeared from various countries with similar climatic and geographic conditions (Schwimmer and Schwimmer, 1968). More than 12 species from nine genera have been reported toxic, but most reports deal with *Microcystis aeruginosa*, *Anabaena flos-aquae* and *Aphanizomenon flos-aquae*, each with its own specific toxin type (Gorham and Carmichael, 1979). *M. aeruginosa* appears to be the most widely distributed species (Carmichael, 1980) and is also most frequently reported toxic (Gentile, 1971).

In South Africa reports on freshwater cyanobacterial toxicity have been restricted to two species only, viz. *M. aeruginosa* (syn. *M. toxica*) (Steyn, 1945; Louw, 1950; Toerien, Scott and Pitout, 1976) and *N. spumigena*, which has thus far been found to be toxic in only one location (Scott, Barlow and Hauman, 1980). *Anabaena flos-aquae* is a common bloom-forming organism in dams in the Transvaal (P.J. Ashton, 1978, personal communication) but has not yet been reported toxic (Scott, Barlow and Hauman, 1980).

Livestock deaths in South Africa attributable to *M. aeruginosa* have been reported since 1914 (Schwimmer and Schwimmer, 1968). The first report of a direct link between *M. aeruginosa* (classified as *M. toxica* by Stephens but considered the same species (Scott, 1974)) and livestock deaths was that of Steyn (1945).

Toxic blooms of *M. aeruginosa* have been reported from the Orange Free State, Transvaal and the coastal regions of the Cape Province (Eloff, 1980; Scott, Barlow and Hauman, 1980). This is a widespread distribution but it is highly likely that even more

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areas will be found if a thorough survey is undertaken. It is of interest that all reports thus far have involved *M. aeruginosa* forma *aeruginosa* and not forma *flos-aquae* (Toerien, Scott and Pitout, 1976; Scott, Barlow and Hauman, 1980).

Toxic and non-toxic strains of *M. aeruginosa* have been isolated and cultured (Hughes, Gorham and Zehnder, 1958; Scott, 1974; Gorham and Carmichael, 1979), making more detailed studies possible. Much of this work has been on strain NRC-1 (Hughes, Gorham and Zehnder, 1958). The strain used in the present studies was isolated from a bloom on Witbank Dam (Scott, 1974).

Various types of toxin have been described in *Microcystis*: a hepatotoxin, which is most common (Steyn, 1945; Ashworth and Mason, 1946; Elleman, Falconer, Jackson and Runnegar, 1978); a diarrhoea-producing toxin (Aziz, 1974); a toxin resembling anatoxin type c in symptoms and mode of action, with separable hepatotoxic and neurotoxic activities (Gorham and Carmichael, 1979) and a neurotoxin (Gorham and Carmichael, 1979, Carmichael, 1980).

The most common symptoms and pathology of mammalian intoxication by *Microcystis* have been described (Ashworth and Mason, 1946; Konst, McKercher, Gorham, Robertson and Howell, 1965; Tustin, van Rensburg and Eloff, 1973). In all cases the major and often only lesions were observed in the liver (Konst *et al.*, 1965; Foxall and Sasner, 1980). Gross enlargement of the liver is usually observed and sometimes a doubling of the normal liver weight is found at the time of death (Toerien, Scott and Pitout, 1976). Death is within 4 h, whence the name Fast Death Factor (FDF)(Bishop, Anet and Gorham, 1959). The exact mode and site of action of this hepatotoxin is as yet unknown but indications have been found that it affects liver cell membranes (Foxall and Sasner, 1980). The possibility that it may have a lectin-type activity on red blood cells has been reported (Carmichael, 1980), as has anti-cholinesterase activity (Kirpenko, Lukina, Sirenko, Orlovskii and Peskov, 1976).

Mammals appear to be the only species affected by the hepatotoxin, with fish and wildfowl apparently unaffected (Gorham, 1964)

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although there are reports to the contrary (Schwimmer and Schwimmer, 1968).

Louw (1950) reported the isolation of a toxic alkaloid from *M. toxica* but this was not confirmed by other workers. A peptide which produced all the hepatotoxic symptoms reported for natural bloom material was isolated from strain NRC-1 (Bishop, Anet and Gorham, 1959). The finding that the hepatotoxin is a peptide has been confirmed by workers in the U.K. (Rabin and Dabre, 1975), Australia (Elleman *et al.*, 1978) and South Africa (Toerien, Scott and Pitout, 1976). Similar but not identical compositions for a toxin from strain NRC-1 were found independently by Bishop, Anet and Gorham (1959), Murthy and Capindale (1970) and Rabin and Dabre (1975). This variability in toxin composition from a supposedly homogenous culture may be explained by findings that *Microcystis* strains may contain between two (Toerien, Scott and Pitout, 1976) and four (D.P. Botes, 1980, personal communication) toxic components. This situation is similar to that found amongst species of *Bacillus* producing peptide antibiotics by a non-ribosomal mode of synthesis (Katz and Demain, 1977).

The factors influencing and controlling toxin production, whilst not totally understood, have received a fair amount of attention. This has mainly been to optimise conditions for maximal production of material for studies on toxin structure and mode of action.

In *Anabaena flos-aquae* it has been found that bacteria may inhibit toxin production to a degree (Carmichael and Gorham, 1977) and this is probably significant in nature. Ornithine decarboxylase activity has been found in 84 % of the bacteria found in association with *Microcystis* in nature (Eloff and van der Westhuizen, 1980) and might thus inhibit a toxin similar to that isolated by Bishop, Anet and Gorham (1959).

The first studies aimed at optimisation of *M. aeruginosa* toxin production were those of Gorham (1964), who found maximal production after 4 days at 25 °C i.e. late log phase. This was confirmed by Eloff and van der Westhuizen (1980) who also observed

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that slowly growing cells were more toxic than fast growing cells. In nature cells are likely to be growing fastest at the beginning of a bloom and then slow down as the bloom becomes established, the stage when cases of livestock poisoning are normally reported.

The effect on toxicity of variation in concentrations of various salts was studied by Brown (1974) who found that, with exceptions, any variation in concentration caused an increase in toxicity. She hypothesized that this could be the result of:

(a) metabolic upset which caused the cells to be less dense so that there was no real increase in toxicity,

(b) stress on a cell causing an increase in toxicity or

(c) stress conditions forcing a change in the type of toxin.

Runnegar and Falconer (1980) found that the LD_{100} at 29 °C was about four times higher than that at 18 °C. In contradiction to the findings of Brown (1974) they found that growth in two different media (BG-11 and modified ASM, which have different salt concentrations) had no significant effect on toxin levels.

It has been suggested (Sirenko, 1980) that the toxicity of *M. aeruginosa* is an inducible property which is expressed or repressed under the influence of agents such as the exogenous metabolites of species antagonists. This would mean that toxicity levels and appearance are under control of environmental factors only.

Some doubt existed as to the origin of the hepatotoxin as it has proved difficult to obtain axenic cultures of *M. aeruginosa* (Gorham, 1964). This was resolved by the findings of Gorham (1964) that hepatotoxic activity was associated with the algal-rich and not the bacterial-rich fraction after differential centrifugation and those of Eloff and van der Westhuizen (1980) that the bacterial flora of cultures of different origins was very different. It appears that the bacteria do produce a neurotoxin which causes slow deaths (SDF) (Bishop, Anet and Gorham, 1959).

Kumar and Gorham (1975) studied the effect of acridine dyes, streptomycin and chloramphenicol on the toxicity of *Anabaena*

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flos-aquae NRC-44-1 and found no decrease in the amount of toxin produced. The lack of effect of the acridine dyes was taken to indicate that the toxicity of this strain is not extrachromosomally determined.

In nature the toxicity of *M. aeruginosa* varies widely and rapidly (Gorham, 1964). Using single cell isolation techniques two non-axenic clones were obtained from strain NRC-1, one toxic and the other not (Gorham, 1964). This was taken to indicate genetic heterogeneity of toxin production. Later work has indicated that the original culture of NRC-1 was heterogenous and that the two clones were morphologically different (Gorham and Carmichael, 1980). The non-toxic species appears to have become dominant in the culture of NRC-1 studied by Stanier, Kunisawa, Mandel and, Cohen-Bazire (1971) who found that it had a mol % G+C of 66,3 which is in the range for organisms classified as *Synechococcus* sp. Twenty eight clonal isolates from a toxic clonal culture of NRC-1 were all toxic (Eloff and van der Westhuizen, 1980), indicating that the results of Gorham (1964) were due to species heterogeneity of the culture and not genetic heterogeneity for toxin production. Strain NRC-1 has been toxic since its original isolation in 1958, indicating genetic stability of toxin production or alternatively that if toxin production is lost in some cells in a culture it can be efficiently reintroduced. Rabin (1976) has hypothesized that the variability of toxin production in nature is for genetic and not physiological reasons.

As a prophage could only be induced from toxic strains of NRC-1 using mitomycin C it was suggested that toxicity was as a result of phage conversion (Vance, 1977), similar to that found in for instance *Clostridium botulinum* type C (Eklund, Poysky, Reed and Smith, 1971) or *Corynebacterium diphtheriae* (Barksdale and Arden, 1974).

No correlation between the presence/absence of gas vacuoles and toxicity was found although non-toxic strains lost their gas vacuoles faster than did toxic strains (Eloff and van der Westhuizen, 1980).

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2.2 Genetic phenomena in cyanobacteria

This subject has been well reviewed by Ladha and Kumar (1978) and Doolittle (1979). Some aspects which are of importance to this study justify elaboration here.

2.2.1 Cyanobacterial DNA

The order *Chroococcales* (in which *Microcystis* is classified) is an extremely heterogenous group with a wide range in mol % G+C of the genome (Edelman, Swinton, Schiff, Epstein and Zeldin, 1967). This has been confirmed by Stanier *et al.* (1971), who found that the two strains in their collection classified as *M. aeruginosa* were very different and were placed in two different morphological groups (viz. IA and IIC). The difference in morphology was also reflected in a large difference in mol % G+C, with strain PCC 6911 (supposedly strain NRC-1 and classified by them in group IA) having a mol % G+C of 66,3 and strain PCC 7005 (placed in group IIC) a mol % G+C of 45,4.

The first thorough study of a cyanobacterial genome was that of Roberts, Klotz and Loeblich (1977) who found that *Agmenellum quadriplicatum* had a genome of approximately 3,9 x 10^9 d with the DNA content per cell being of the order of 8,6 x 10^9 d . This indicates a multicopy genome. Herdman, Janvier, Rippka and Stanier (1979b) extended this work to cover 128 strains and species. They found a range of 1,6-8,6 x 10^9 d for genome sizes, representing four classes with class midpoints of 2,2; 3,6; 5,0 and 7,4 x 10^9 d, which suggests that the genomes had arisen by fusion of an ancestral genome with a size of 1,2 x 10^9 d . Unicellular cyanobacteria had a genome size range of 1,6-2,7 x 10^9 d (i.e. similar to the bacteria). Strain PCC7005 (considered *M. aeruginosa* by Stanier *et al.*, 1971) had a genome of 2,06 x 10^9 d, a kinetic complexity of 8,6 x 10^6 d and 10,8 % of the genome present as a rapidly renaturing fraction with 26 copies of this fraction per cell (Herdman *et al.*, 1979b).

Methylated DNA is present in the DNA of *Plectonema boryanum* and possibly in the DNA of other cyanobacteria as well

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0,1 % (v/v) sarkosyl followed by an osmotic shock treatment with the lysozyme leads to good lysis of a number of Gram-negative strains normally refractile to the action of lysozyme. The wash probably acts to remove the lipopolysaccharide layer whilst the osmotic shock will drive the lysozyme through the layers to its place of action as proposed by Witholt, Van Heerikhuizen and De Leij (1976).

Weinbaum, Rich and Fischman (1967) found that it was also possible to obtain spheroplasts (the rounded cell forms found after lysozyme treatment. Their rounded shape is as a result of the removal of the rigid layer(s) which give the cell its characteristic shape) by treating *E. coli* with trypsin followed by phospholipase, indicating that it is not only the murein that gives the cell its shape.

Various methods have been reported to remove the lipopolysaccharide layer, leaving the lower layer(s) accessible to lytic enzymes or treatments. These methods include treatment of cells with aqueous phenol, especially after treatment with papain (De Petris, 1967), a mixture of Triton X-100 and EDTA (Schnaitman, 1971b), 1 % SDS in 0,1 M Tris buffer at pH 8 (Weise, Drews, Jann and Jann, 1970) and aqueous butanol or EDTA (Wilkinson, 1977). The sarkosyl wash used by Schwinghamer (1980) probably has the same function.

The modes of action of various detergents used in lysis have reviewed by Schnaitman (1971a), Filip, Fletcher, Wulff and Earhart (1973) and Helenius, McCaslin, Fries and Tanford (1979). SDS (0,5 %) was reported to solubilise all the membranes of *E. coli*, as does 2 % Trixton X-100 whilst 0,5 % sarkosyl only solubilised the cytoplasmic membrane (Filip *et al.*, 1973). This finding is in contradiction to that of Schnaitman (1971a) who found that Triton X-100 solubilised only the cytoplasmic membrane. Helenius *et al.* (1979) report that SDS is a far harsher detergent than sarkosyl, explaining the findings of Filip *et al.* (1973) in this regard.

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(Leach and Herdman, 1973). As the DNA of *Fremyella diplosiphon* is uncut by the restriction enzymes Bam HI, Hae II and Sal I, and as restriction enzymes are present in a number of cyanobacterial species (Doolittle, 1979), cyanobacteria may have a restriction/modification system similar to that found in bacteria.

2.2.2 Recombination in, and the isolation of plasmids from cyanobacteria

Intraspecific (Bazin, 1968) and intergeneric (Kumar and Tyagi, 1978) transfer of antibiotic resistance markers has been reported among the cyanobacteria. In bacteria such transfer occurs by a process known as conjugation, involving sex pili due to plasmids (Hayes, 1968). Whether a similar mechanism is responsible for antibiotic resistance transfer in cyanobacteria is as yet unknown, but Vaara and Lounatmaa (1980) have described pilus-like structures in *Synechocystis* CB3 with unknown function.

The presence of plasmids (see 2.6) in cyanobacteria was first discoverd in *Anacystis nidulans* by Asato and Ginoza (1973). Since then plasmids have been found in a number of unicellular (Lau and Doolittle, 1979; Van Den Hondel, Keegstra, Borrias and Van Arkel, 1979) and filamentous (Simon, 1978a) strains. Lau and Doolittle (1979), Van Den Hondel *et al.* (1979) and Lau, Sapienza and Doolittle (1980) also found that a number of plasmids in closely related species were homologous according to restriction enzyme analysis. In some cases the species were not closely related but still had homologous plasmids, possibly indicating plasmid transfer (Van Den Hondel *et al.*, 1979). No sign of a plasmid was found in strain NRC-1 (Lau, Sapienza and Doolittle, 1980) whilst Van Den Hondel *et al.* (1979) found a 2 Md plasmid in the non-toxic strain PCC 7005. It is possible that Lau, Sapienza and Doolittle (1980) worked with strain PCC 6911 (Stanier *et al.*, 1971) and not the NRC-1 strain isolated by Hughes, Gorham and Zehnder (1958)(see 2.1). A case of curing of one of the

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plasmids of *A. quadriplicatum* BG-1 by growth in 0,008 % SDS was reported by Lau, Sapienza and Doolittle (1980).

As all plasmids thus far isolated from cyanobacteria have been cryptic (i.e. no function ascribable to them) (Doolittle, 1979) a successful attempt was made to introduce a transposon, Tn 901, into a plasmid of *A. nidulans* to have some way of following its transfer and other activities (Van Den Hondel, Verbeek, Van Der Ende, Weisbeek, Borrias and Van Arkel, 1980).

The ease of loss of gas vacuoles in cyanobacteria led Walsby (1977) to suggest that gas vacuole presence may be plasmid determined as has been found in *Halobacterium* strain 5 (Simon, 1978b). The findings of Eloff and van der Westhuizen (1980) do not support this hypothesis for *M. aeruginosa*.

2.2.3 Transformation and transduction in cyanobacteria.

Shortly after the discovery of antibiotic resistance transfer in cyanobacteria, transformation was described in *A. nidulans* (Shestakov and Khyen, 1970), who found 7,5 x 10^{-4} transformants to erythromycin resistance per 20 μg DNA. Transformation of *A. quadriplicatum* has been described (Stevens and Porter, 1980) as has intergeneric transfer (Devilly and Houghton, 1977). The discovery of transformation has led to a dismissal of the claims for recombination as mere examples of transformation by naturally occurring DNA in the medium but this has not yet been proved (Doolittle, 1979).

The occurrence of lysogeny in cyanobacteria (Cannon, Shane and Bush, 1971) led to the suggestion that transduction may also occur. Transduction of streptomycin resistance by LPP-1 in *P. boryanum* at frequencies ranging from 2 x 10^{-5} - 3,8 x 10^{-10} was reported by Singh and Singh (1972).

The presence of regular structures in toxic *M. aeruginosa* was reported by Barlow (1978). These structures were

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investigated by Payne and Williams (1977) and found to consist of square lattices of rods 24 nm in diameter. It is possible that these structures may represent the intracellular stage of some cyanophage.

2.3 Cell wall structure and composition in bacteria and cyanobacteria

The morphology and chemical composition of the cell wall of *Escherichia coli*, the best studied Gram-negative organism, have been reviewed by De Petris (1967) and Schnaitman (1971b). Cyanobacteria occasionally stain Gram-positive but are generally considered to be members of the Gram-negative group of organisms (Doolittle, 1979). It is thus tempting to draw parallels between the morphology and chemical composition of the cell walls of cyanobacteria and *E. coli*. This, as will be discussed later, has proved fruitful (e.g. Schmidt, Drews, Weckesser and Mayer, 1980).

A four-layered cell wall has been proposed for *E. coli*, with a lipopolysaccharide layer external to this (Schnaitman, 1971b). The outer membrane of the wall is considered to be double-layered, with the outer layer (LIV) being phospholipid bound on either side by protein. The inner layer of the outer membrane (LIII), which is electron-transparent, is thought to be lipoprotein. The next, electron-dense, layer (LII) is the so-called murein or peptidoglycan which is the substrate for lysozyme (De Petris, 1967; Schnaitman, 1971b). Below the murein is the electron-transparent periplasmic gap (LI) which is possibly composed of protein (De Petris, 1967). Below this four-layerd wall is the cytoplasmic membrane.

Frank, Lefort and Martin (1962) were the first to report on the multilayered nature of cyanobacterial cell walls and also found that the murein, which was digested by lysozyme, was chemically similar to that of Gram-negative bacteria. Early work, based on the idea that cyanobacteria were plants, found no evidence of cellulose in the cell walls (Fuhs, 1973). This has been confirmed by Dunn and Wolk (1970) and Fjerdingstad, Fjerdingstad and Fjerdingstad (1979). By 1973 the four-layered nature of cyanobacterial cell walls was accepted as fact, as was the overall

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chemical similarity with Gram-negative bacterial cell walls (Drews, 1973). One major difference is the very much greater thickness of the murein in cyanobacteria (Schmidt *et al.*, 1980), which is classified as type I (Jones and Yopp, 1979). The major membrane protein of cyanobacteria is reported (Golecki, 1977) to have a molecular weight of 43 kd which is close to the 44 kd found in Gram-negative bacteria (Schnaitman, 1970). The lipopolysaccharide has a core structure which appears to be very different to that of Gram-negative bacteria (Schmidt *et al.*, 1980). The lipid A moiety of cyanobacterial lipopolysaccharides is rich in β-hydroxypalmitic acid which is rare in the lipid A of Gram-negative bacteria (Schmidt *et al.*, 1980).

The fine structure of the cell wall of *M. aeruginosa* was studied by Barlow (1978) who found the four-layered cell wall typical of other cyanobacteria (Drews, 1973). She also described "micro-villi-like protruberances" of unknown function on some toxic cells. It may be that these are similar to the pilus-like structures of Vaara and Lounatmaa (1980) and may even function as pili in a conjugation-like process, although at this stage this is unproven.

2.4 Lysis of bacterial and cyanobacterial cells

2.4.1 Lysis of bacterial cells.

Lysozyme, discovered by Fleming (Strominger and Ghuysen, 1967), has been the enzyme most used in lysis studies. The linkage attacked by lysozyme is the β-1-4 link between N-acetyl muramyl and N-acetylglucosaminyl residues, a linkage found in all cells with a normal murein (Strominger and Ghuysen, 1967).

Lysis of *E. coli* and other Gram-negative organisms was first studied in detail by Repaske (1956) who found that it was greatly enhanced in the presence of ethylene diamine tetraacetic acid (EDTA). Repaske (1958) found that Tris buffer enhanced lysis by lysozyme, as did an increase in pH (to pH 8,6). The action of EDTA was presumed to involve its chelating activity, possibly removing interfering metals from the cell wall (Repaske, 1958). Witholt, Van

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Heerikhuizen and De Leij (1976) hypothesized that EDTA destabilises the outer membrane layer (LIV according to Drews, 1973), possibly forming holes through which the lysozyme is forced. They found that lysozyme acted on the inside side of the murein layer and thus had to be forced through three layers before it reached its substrate. They used the influx of water after osmotic shock to force the lysozyme in. Schnaitman (1971b) suggested that the requirement for EDTA indicated that divalent cations play a role in the impermeability of the cell wall. He found that the cell wall layers affected by various agents were: LIV-EDTA; LIII-trypsin (in line with the proposed lipoprotein nature of this layer); LII-lysozyme; the lipopolysaccharide external layer-a mixture of Triton X-100 and EDTA and the cytoplasmic membrane-Triton X-100. The work of Schnaitman (1971b) is of great value when it comes to interpreting results of experiments using the above-mentioned agents. In a study on the lysis of *E. coli* by lysozyme, Metcalf and Deibel (1969) found that the order of addition of reagents made a profound difference, with the best results in their system being obtained when lysozyme was the first to be added. Excessive concentrations of lysozyme proved inhibitory to lysis, possibly because the lysozyme forms an impenetrable layer round the cell which first has to be removed by e.g. detergents before lysis can take place.

Pre-lytic treatment of *Pseudomonas aeruginosa* with acetone is needed to get optimal lysis using lysozyme (Warren, Gray and Bartell, 1955). The acetone probably removes some layer which is inhibitory to lysis, such as the lipopolysaccharide layer. Noller and Hartsell (1961a, b) studied a number of modifications of the standard lysozyme method of lysis of *E. coli* and found best lysis using a mixture of lysozyme, trypsin and butanol. The trypsin and butanol probably act by removing LIV and LIII, leaving LII open to the action of the lysozyme. They also found that heat or pH 10 treatment enhanced lysis by lysozyme (Noller and Hartsell, 1961b). Schwinghamer (1980) reported that a pre-lytic wash with

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The autolysis of *E. coli* was studied in detail by Leduc and Van Heijenoort (1980) who found best results were obtained if the cells were first subjected to a downshock by suspension in water and then an upshock by the addition of sodium acetate (pH 6,5) to 0,5 M . Growth of the cells in rich medium led to better lysis as did the use of cells in the log phase of growth (as has been found using most lysis methods (Witholt, Boekhout, Brock, Kingman, Van Heerikhuizen and De Leitz, 1976)).

The antibiotic penicillin, which acts by inhibiting cross-linkage of peptide side-chains in the murein (Martin, 1966), has been used to produce osmotically sensitive cells which are lysed when subjected to osmotic shock (Ghuysen, 1968; Ledeboer, Krol, Dons, Spier, Schilperoort, Zaenen, Van Larebeke and Schell, 1976). The action of penicillin is limited to growing cells only (Ghuysen, 1968).

The use of Triton X-100 (Miozzari, Niederberger and Hütter, 1978) and dimethyl sulfoxide (DMSO)(Fantes, Roberts and Huetter, 1976) to permeabilise cells for the assay of enzyme activities in the cell also hold promise as a way of exposing for instance the murein to the action of a lytic enzyme.

2.4.2 Lysis of cyanobacterial cells

The fact that cyanobacteria have four-layered cell walls (Drews, 1973) just as Gram-negative bacteria do (Schnaitman, 1971b) leads one to expect that methods which lyse Gram-negative bacteria will also lyse cyanobacteria. This assumption has proved to be valid in practice.

Fuhs (1958) found that *Oscillatoria amoena* was lysed by lysozyme. A certain optimum lysozyme concentration was found to give maximal lysis and increases in concentration had no positive effect (as was found by Metcalf and Deibel (1969) with *E. coli*).

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The findings of Repaske (1956) on an EDTA requirement for the lysozyme lysis of *E. coli* has been confirmed for cyanobacteria by Drews and Meyer (1964) and Mineeva, Semenova and Gusev (1980).

Biggins (1967) conducted a study to define the optimal conditions for spheroplast formation in *Phormidium luridum*. Suspension in a 0,5 M mannitol-0,3 M potassium phosphate buffer (pH 6,8) gave far better results than other buffers and osmotica (Tris, potassium phosphate, glycylglycine, glucose, sucrose, polyethylene glycol). Prolonged incubation (2 to 3 h) was required. Similar results were found with *M. aeruginosa* although a 4 h incubation was required and spheroplast yield never exceeded 70 % (Vance and Ward, 1969). The reason for this finding is unknown but it was found that the higher the optimal growth temperature is, the longer the incubation needed for spheroplast formation. *M. aeruginosa* NRC-1 has been found to be relatively insensitive to lysozyme with spheroplast yields of the order of 50 % being found (Jones and Jost, 1970). It has been found that increasing the cell concentration leads to higher yields of spheroplasts of *A. nidulans* (Gusev, Nikitina and Korzhenevskaya, 1970).

The substrate layer for lysozyme has been found to be LII (Jensen and Sicko, 1971; Lindsey, Vance, Keeter and Scholes, 1971), confirming the close relationship between cyanobacteria and bacteria.

Other enzymes which have been used to lyse cyanobacteria include pectinase (Yoneyama, 1978) and the lytic enzyme LI produced by *Cytophaga* sp. (W.E. Scott, 1979 personal communication). Trypsin was found to have no effect on *M. aeruginosa* (Jones and Jost, 1970).

Methods for the removal of the lipopolysaccharide layer are similar to those used with Gram-negative bacteria, viz. aqueous phenol or SDS (Weise *et al.*, 1970). Golecki and Drews (1974) found that the use of 45 % aqueous phenol at

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68 °C removed not only the lipopolysaccharide layer but LIV and LIII as well.

The mucilage which binds individual *M. aeruginosa* cells together can be removed by treatment with 0,3 % NaOH at 90 °C for 30 min (Reynolds and Jaworski, 1978). As this mucilage layer could interfere with lysis this method may be of use.

The detergents used in lysis experiments have on the whole been the same as those used with bacteria. Craig, Leach and Carr (1969) used a mixture of 2 % SDS and 4 % 4-aminosalicylate, as did Devilly and Houghton (1977), who found that the two detergents have an additive effect. Another detergent mixture which has been used is 1 % Triton X-100 plus 0,2 % sodium deoxycholate (Dmitrieva and Kozlov, 1979).

The chemical similarity of cyanobacterial and Gram-negative bacterial cell walls is also reflected in the sensitivity of cyanobacteria to growth in the presence of penicillin, yielding osmotically sensitive cells (Drews, 1973). This penicillin sensitivity was used by Jones and Jost (1970) for the gentle lysis of *M. aeruginosa*. Lysis was so gentle that the fragile gas vesicles they were studying were isolated intact.

The presence of autolytic and lytic enzymes in a number of cyanobacterial species has been reported by Ingram (1973).

2.5 Methods of DNA isolation

The classical method of isolation is that of Marmur (1961) in which cells are lysed as gently as possible (usually using lysozyme), the DNA deproteinised using isoamyl alcohol-chloroform and phenol, concentrated by some method of alcohol precipitation (ethanol or isopropanol) and dissolved in a suitable buffer. RNAase may be used to remove unwanted RNA but problems may be encountered in getting rid of all traces of DNAase in the RNAase (McCormick, Larson and Maher, 1974). Lysis is usually done with lysozyme but

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2 % SDS at 60 °C has been used (Marmur, 1961; Owen and Lapage, 1976).

The nature of the bonds between protein and DNA, with reference to deproteinisation with phenol, was studied by Kirby (1957). He suggested the use of solutions of salts such as 4-aminosalicylate, azide, trichloracetate, salicylate, sorbate or benzoate to maximise the release of DNA from the contaminating protein.

The MUP method described by Britten, Pavich and Smith (1968) was used with great success by Herdman, Janvier, Waterbury, Ripkka, Stanier and Mandel (1979a) and Herdman *et al.* (1979b) in their study of the DNA of 128 species and strains of cyanobacteria.

Graham (1978) has described a method based on the grinding of cells frozen in liquid nitrogen and their immediate suspension in a deproteinising mixture. This method was described for use with eukaryotic organisms but should also prove of use with prokaryotes.

In all the methods used for DNA isolation, and especially when small components of the total cell DNA (e.g. plasmids) are being sought, the presence of nuclease activity in the extract is a problem. Graham (1978) sought to avoid this by immediate suspension in a deproteinising solution but this approach cannot always be used. The discovery that diethyl pyrocarbonate (DEP) has anti-nuclease activity at concentrations of as low as 0,2 % (v/v) is thus of great use (Ledeboer *et al.*, 1976).

2.6 Plasmids, their isolation and elimination

A plasmid is defined by Clowes (1973) as a stably inherited component of the genome which is physically separate from the chromosome. It is non-essential for normal growth of the host and can usually be gained or lost with no lethal effect on the host.

According to Novick (1969) "...without exception, intact DNA of established plasmids has been found to be in the closed circular duplex configuration." Whilst the general truth of this statement is readily established from the literature, there is a report by

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Hayakawa, Tanaka, Sakaguchi, Otake and Yonehara (1979) on the isolation of a linear plasmid from a *Streptomyces* sp.

A number of criteria have been defined for establishing the plasmid linkage of a gene (Novick, 1969). These are:

(a) lack of genetic linkage to the chromosome
(b) lack of genetic homology with the chromosome
(c) occurrence of conjugal transfer
(d) demonstration of physical autonomy
(e) demonstration of replicative autonomy by methods such as curing.

Criteria d and e are most likely to be useful for cyanobacteria in view of the rudimentary knowledge at present available on the genetics of these organisms (Doolittle, 1979). It must be stressed that criterion e alone is insufficient evidence for plasmid linkage of a gene as other factors such as selective and/or mutagenic effects of the curing agents or other sources of hereditary variation such as point mutations or deletions may be involved (Novick, 1969). Elimination of a character followed by non-isolation of a previously present plasmid may be taken as fair evidence but the possibility of regulatory plasmids with the gene(s) of interest on the chromosome should not be ignored (Hopwood, 1978).

A number of determinants have thus far been found on plasmids such as sex factors (F), antibiotic resistance (R), resistance to heavy metals, toxin production, bacteriocin production (Novick, 1969) as well as antibiotic production in some *Streptomyces* spp. (Hopwood, 1978).

For the isolation of plasmids the basic method used is to gently lyse the cells to release the DNA and then to analyse the DNA in some way for "satellite" components. This analysis can be done in two main ways:

(a) density gradient centrifugation in either caesium chloride (Guerry, Le Blanc and Falkow, 1973) or sucrose (Hughes and Meynell, 1977) gradients or
(b) on agarose gels by electrophoresis (Meyers, Sanchez, Elwell and Falkow, 1976).

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Ethidium bromide is included in the gradients as in its presence covalently closed circular DNA (ccc DNA) is denser than open circles or linear DNA and thus forms a second, denser, band in the gradient. The linear plasmid described by Hayakawa *et al.* (1979) would thus not have been found using this method. The agarose gel electrophoretic method is based on a combination of separation according to size and also according to conformation i.e. circular or linear.

A number of refinements and modifications of this basic method have been published. Some of them are based on the finding that linear DNA is denatured at high temperatures or if the pH is higher than 11,5, whilst cccDNA is relatively unaffected (Clowes, 1973). The finding of Hirt (1967) that undegraded chromosomal DNA is preferentially precipitated in the presence of SDS plus 1M NaCl at 4 °C has also been widely used (e.g. Guerry, Le Blanc and Falkow, 1973; Humphreys, Willshaw and Anderson, 1975). "Permeabilisation" of cells using Brij 58 (Clewell and Helinski, 1969) or phenol (Klein, Selsing and Wells, 1980) so that small plasmids and RNA but not the chromosome are released have also been used. In all these methods the aim is to enrich for plasmid DNA at the expense of chromosomal DNA, which may obscure the presence of plasmids. Alkaline denaturation of chromosomal DNA has been very effective in cases where either large plasmids are being isolated (Currier and Nester, 1976) or the plasmid DNA is closely associated with the chromsomal DNA (Palchaudhuri and Chakrabarty, 1976). Heat treatment of isolated DNA has been used by Van Den Hondel *et al.* (1979) to distinguish cccDNA from other forms on a gel.

Once plasmids are isolated their sizes can be estimated using physical methods such as electron microscopy (Clowes, 1973) or agarose gel electrophoresis (Meyers *et al.*, 1976). They may then be characterised using physico-chemical methods such as restriction enzyme analysis (Thompson, Hughes and Broda, 1974).

The elimination of plasmid DNA from a host by artificial means is often referred to as curing (Clowes, 1973). A large variety of agents, with differing modes of action, have been used to cure bacteria of their plasmids. Some of these are: acridine dyes

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(Hirota, 1960), SDS (Tomoeda, Inuzuka, Kubo and Nakamura, 1968; Sonstein and Baldwin, 1972a), other surface-active agents (Sonstein and Baldwin, 1972b), ethidium bromide (Rubin and Rosenblum, 1971), chloramphenicol (Mitchell and Kenworthy, 1977), urea (Tomoeda, Kokubo, Nabata and Minamikawa, 1970) and growth at elevated temperatures (Novick, 1969).

It is suggested that acridine dyes act by interfering with replication of the plasmid (Yamagata and Uchida, 1969) whilst SDS is thought to act on the membrane (Sonstein and Baldwin, 1972a), possibly via the sex pili (Adachi, Nakano, Inuzuka and Tomoeda, 1972). There is some conflict as to whether SDS has a selective effect (Adachi *et al.*, 1972) or not (Sonstein and Baldwin, 1972a). Acridine dyes are usually considered to have no effect on integrated plasmids and prophages (Hirota, 1960) but there is a report (Eklund *et al.*, 1971) on the curing of the prophage induced toxicity of *Clostridium botulinum* type C. The prophages in *C. botulinum* type C responsible for toxicity are however known to be in the non-integrated, pseudolysogenic state (Eklund *et al.*, 1971). Detergent curing of *C. botulinum* type A has been described (Takumi, Kinouchi and Kanata, 1980), with concomittant changes in the cell wall structure. Pseudolysogeny may also be involved here.

Williams Smith (1953) found that chloramphenicol caused no induction or curing when tested with lysogenic strains of *Salmonella*. This is in contrast to reports on the curing (at fairly low frequencies) of a plasmid-determined property in *E. coli* using chloramphenicol (Mitchell and Kenworthy, 1977).

2.7 Conclusions

From a review of the available literature it was evident that the mechanisms of control of toxin production by the cyanobacteria are poorly understood. In view of the possible harmful effects of these organisms to humans it was felt that a genetic study of one of them, *M. aeruginosa*, might be of use.

The variations in toxin amounts, as well as the variability of presence/absence of toxicity, gave rise to the idea that a plasmid(s) might be involved in toxin production.

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The literature on plasmids was reviewed and two approaches were thought suitable for answering this question. These were:

(a) Curing studies

(b) Plasmid isolation

As cell lysis is an important part of the plasmid isolation procedure, the literature on this subject, as well as that on factors which might affect lysis, such as cell wall structure, was also reviewed.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Organism and culture conditions

The strain of *Microcystis aeruginosa* forma *aeruginosa* used in these studies was isolated from a toxic bloom on Witbank Dam in 1974 (Scott, 1974) and is kept in the culture collection of the NIWR as strain WR 70. This strain has lost its large colonial form and is usually single-celled but under certain conditions small colonies are formed in liquid media. Gas vacuoles are present, as is the case in nature. This is in contrast to findings that the ability to form gas vacuoles is often rapidly lost after isolation of a strain from nature, especially if the strain is non-toxic (Eloff, 1980).

A batch culture system was used for mass cultivation, with the cultures for curing experiments being grown in small volumes in flasks. The medium used for all cultures was that of Volk and Phinney (1968) with some minor modifications (Barlow, 1978). This medium results in a higher yield of cells than the BG-11 medium of Stanier *et al.* (1971) (W.E. Scott, 1980, personal communication).

As preliminary experiments with the batch culture system indicated that the use of tap water for media had no negative effect on growth and indeed enhanced the final yield of cells, tap water was initially used. It was however found that growth in tap water medium led to the loss of colonial form so media were later prepared using deionised water. Under these conditions colonies form, which is closer to the situation in nature and thus considered preferable (W.E. Scott, personal communication). A breakdown in the deionising system, leading to a drastic drop in water quality (W.E. Scott, personal communication), made a comparison with earlier results on tap water grown cells possible.

The gas vacuoles of the cells were collapsed by a rapid pressure change (Wolk, 1973) before harvesting by centrifugation.

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Difficulties have thus far been encountered with the isolation and maintenance of axenic cultures of *M. aeruginosa* (Gorham, 1964; Vance, 1966; Eloff, 1980). The cultures used in these studies were not axenic but were microscopically examined for bacterial contamination and discarded when appearing heavily contaminated-greater than 1 % by count (W.E. Scott, 1980, personal communication). As the objective of the present study was to investigate the possible involvement of plasmids in the toxicity of *Microcystis*, it was necessary to ensure that any plasmids isolated were in fact from *M. aeruginosa* and not from contaminating bacteria. A culture of the contaminant was made by inoculating a loopful into Nutrient Broth 2 medium (Oxoid), incubated at room temperature overnight, lysed with SDS and the released DNA analysed for the presence of plasmids by ethidium bromide-caesium chloride density gradient centrifugation (Guerry, Le Blanc and Falkow, 1973) and agarose gel electrophoresis (Meyers *et al.*, 1976). It is realised that this isolation method is selective, so a similar analysis of the bacteria in the supernatant fluid of a culture of *M. aeruginosa* was carried out.

3.2 Chemicals and enzymes

All chemicals and enzymes except autolysin (AL) were obtained from the following commercial sources:

BDH, Boehringer Mannheim, Sigma, Merck and Miles.

AL was isolated, using the method of Ingram (1973) from a freeze-dried sample of a toxic bloom of *M. aeruginosa* f. *aeruginosa*, collected from Hartbeespoort Dam on 20 December 1978. As this was a sample from a bloom which was already well established it is to be expected that bacterial numbers would be high (this was not checked) and that the AL preparation would therefore have bacteriolytic activity as well. No attempt was made to concentrate the AL preparation and the purification attempts made were to remove a substance(s) which caused smears on agarose gels when the AL extraction was made at high EDTA concentrations.

3.3 Curing experiments

Four sets of experiments, using agents curing plasmids in bacteria (see 2.6), were performed.

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Test agents, dissolved in distilled water, were added to 2 d old cultures which were incubated at 20±1 °C in daylight with no aeration.

In the first set of experiments pre-adaptation of cultures by growth at low concentrations of the test agents and incubation for three months before testing was used. In the third cultures were transferred to fresh medium for two weeks before testing after six weeks growth in the test media. This was done to obtain a maximum yield of cells and also to test whether curing, if it had taken place, was stable. In sets 2 and 4 cultures were grown in the presence of the agent for three months.

At the end of the incubation period cells were harvested by centrifugation, disrupted by sonication and injected intraperitoneally into mice, using one mouse per culture. Cultures were considered toxic if the mice died within 4 h and had the swollen livers symptomatic of intoxication with the hepatotoxin (Toerien, Scott and Pitout, 1976).

3.4 Lysing methods

The generally used method for lysing micro-organisms using an enzyme consists essentially of the following:

(i) pelleting of cells by centrifugation, followed by resuspension in a buffered osmoticum e.g. sucrose in Tris

(ii) addition of lysing enzyme e.g. lysozyme

(iii) addition of EDTA leading to spheroplast formation (Repaske, 1956)

(iv) lysis of spheroplasts by the addition of a detergent e.g. SDS.

Initial experiments using this "general" method gave very poor lysis, even poorer than that reported (Vance and Ward, 1969; Jones and Jost, 1970). Systematic variations in the method, as well as numerous additional approaches, were therefore studied. Experimental details are provided in Chapter 4 (4.2 and 4.3).

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3.4.1 Tests of various lysing methods

In order to find optimal lysis conditions small scale tests were done, using microlitre volumes. Typical volumes used were: enzyme solution-20µℓ; sucrose solution-10µℓ; EDTA solution-20µℓ; detergent solution-50µℓ for a total volume of 100 µℓ. These were added to the pellets from approximately 100 µℓ of a concentrated culture sample, representing a freeze-dried weight of approximately 1 mg per assay, thus making direct comparisons of lysis efficiencies on gels possible. The standard incubation times used were: 1 h with the enzyme, sucrose, EDTA mixture and then 30 min with the detergent. In studies where detergents only were tested incubation times of 1 to 1,5 h were used. Incubation was always at 37 °C unless otherwise indicated.

3.4.2 Large scale lyses

The lysis methods used were:

(a) for tap water grown cells: lysozyme, SDS and AL, SDS in the above general method

(b) For deionised water grown cells:

(i) AL, sarkosyl in the above general method;
(ii) sarkosyl only at 65 °C in the presence of 0,2 % (v/v) DEP;
(iii) the method described by Graham (1978) with the cells being ground using a pestle and mortar and
(iv) a modified Graham method using 0,15 M Na p-aminosalicylate as the suspending fluid (as recommended by Kirby, 1957).

The large volumes necessitated concentration of DNA as an essential step prior to analysis on gradients. In cases where SDS or sarkosyl was used as the detergent addition of NaCl to a final concentration of 0,5 M and overnight storage at 4 °C was used to precipitate the cell debris and chromosomal DNA (Hirt, 1967). This step was not used when the method of Graham (1978) was used as in this case total cellular DNA was studied. Two methods of DNA concentration

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were used in the course of these studies. These were the PEG method of Humphreys, Willshaw and Anderson (1975), which was developed for large scale plasmid isolation, and precipitation using ethanol (Marmur, 1961) with the Graham method. The precipitated DNA was suspended in a suitable volume of 0,01 M Tris-0,001 M EDTA (TE) buffer, pH 8 (Guerry, Le Blanc and Falkow, 1973). Deproteinisation using phenol and/or chloroform-isoamyl alcohol (24:1) (Marmur, 1961) was followed by phase separation by centrifugation and extensive dialysis of the aqueous phase against TE. When the Graham method (standard and modified) was used, as well as with normal deproteinisation, the interface material was always re-extracted in 0,15 M salicylate (modified Graham method) or TE (other lysis methods) and this fluid was added to the deproteinised lysate for further deproteinisation.

3.5 Assay for lysis

As the aim of this study was a study of the DNA of *M. aeruginosa*, release of DNA was taken as a measure of the efficiency of lysis. Release of the blue pigment, phycocyanin C, which is found in all cyanobacteria (Wolk, 1973), and which is often taken as a reliable indication of lysis (Crespi, Mandeville and Katz, 1962) was rarely observed so that this could not be used.

Agarose gel electrophoresis of lysates (Meyers *et al.*, 1976) was used for the detection of DNA. Two buffer systems were used: Tris-acetate (Hayward and Smith, 1972) and Tris-borate (Koekman, Doms, Klapwijk and Schilperoort, 1979), the latter allowing higher potential differences across the gel without the excessive heat generation associated with Tris-acetate buffer. The higher potential differences allowed faster runs (3 h vs 9 h). The standard gel concentration was 0,7 % (Meyers *et al.*, 1976). Most runs were done on horizontal gels (20 cm x 15 cm x 0,5 cm) although a few runs were done on vertical gels (20 cm x 15 cm x 0,5 cm) or on tube gels (10 cm x 0,5 cm). A 0,1 volume of a 25 % (m/v) sucrose-0,05 % (m/v) bromophenol blue solution was added to each sample before loading onto the gel.

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In some cases ethidium bromide was incorporated in the gels at a final concentration of 0,5 µg/mℓ but gels were normally stained after electrophoresis in a 0,5 µg/mℓ ethidium bromide solution for 30 min. Gels were illuminated with long wave UV light and photographed on Polaroid 665 film with a Polaroid SS65 camera fitted with a red filter.

3.6 Quantitation of results

Densitometric scans of photographic negatives were made and the peak areas of DNA bands on the negative calculated. From these areas a relative scale was adopted to serve as a measure of the amount of DNA released. With experience it was possible to judge DNA yields visually, without having to resort to scanning and measurement. It is realised that this is a subjective method but experience showed its acceptability as increases or decreases of an order great enough to be of interest and use were readily visible, especially because standardised conditions and a reference treatment were used.

3.7 Density gradient analysis

Throughout these experiments caesium chloride gradients were used. Two types of rotor, viz. the Beckman types 65 (at 35 000 rpm) and VTi65 (at 50 000 rpm) were used. The VTi65 rotor, by the nature of its construction, allowed shorter runs to be made (12 to 14 h vs 72 h for the 65 rotor) with no loss in sharpness of bands or decrease in separation distance. For the VTi65 rotor the volumes used were as follows: 4 mℓ lysate, 4 g CsCl and 200 µℓ of a 10 mg/mℓ solution of ethidium bromide. After the run bands of DNA were visualised by illumination with long wave UV light and the band(s) of interest collected with a needle and syringe. Ethidium bromide was removed from the fractions thus collected by exhaustive extraction with 20 x SSC saturated isoamyl alcohol and the fractions were then dialysed against TE (with frequent changes) for at least 24 h before use in any experiment. Deproteinisation of samples before centrifugation was usually necessary as, if this step was omitted, it was sometimes difficult to extract bands from the centrifuge tube without severe mixing. This was especially the case when the VTi65 rotor was used.

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3.8 Electron microscopy

The purified fractions (obtained as described in 3.7) were prepared for electron microscopy by the method of Coetzee and Pretorius (1979).

The molecular masses of the plasmids were determined by measuring their length relative to that of plasmid RP4 (Clowes, 1972). A molecular weight of 36 Md was assumed for RP4 (Bukhari, Shapiro and Adhya, 1977).

3.9 Characterisation of the isolated DNA's

The mol % G+C of the DNA's was determined in two ways: by thermal denaturation in 0,1 x SSC, using a Cary 210 spectrophotometer (Marmur and Doty, 1962; Mandel and Marmur, 1968) and by buoyant density centrifugation (Schildkraut, Marmur and Doty, 1962). The spectral ratios method of Ulitzur (1972) was also used to estimate the mol % G+C.

3.10 Determination of the nature of the material in gradient bands

RNAase was used to prove that bands were indeed DNA and not RNA. In agreement with the results of McCormick, Larson and Maher (1974) the use of a 10 min boiling period (Marmur, 1961) was not sufficient to destroy the DNAase contamination of the RNAase. It was found that overnight incubation at 85 °C destroyed sufficient DNAase activity for a solution of RNAase prepared in this way to be used to distinguish between RNA and DNA without excessive degradation of the DNA. Gradient fractions were incubated with RNAase solution (5 mg/mℓ) for 30 min before loading on a gel.

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 The effect of various agents on the toxicity of *Microcystis aeruginosa*

The aim of these experiments was to investigate the possibility of plasmid involvement in the toxicity of *M. aeruginosa*, using agents known to cure plasmids in bacteria.

The results of the four sets of experiments are given in Table 1. In sets 1 and 2 the mass of material injected was not measured but was approximately double the LD_{100}.

The results of set 1 seem to indicate that the toxicity of *M. aeruginosa* is either coded for by a plasmid or other extrachromosomal element or is under the control of a factor which can be eliminated by the use of various agents. Growth was slow at high concentrations of these agents so that pre-adaptation at low concentrations and a long incubation period were used. All the cultures produced large amounts of slime.

The action of acridine orange was taken as evidence for a plasmid rather than a prophage as suggested by Vance (1977) as it has been found (Hirota, 1960; Jacob and Wollman, 1961) that acridine dyes have very little effect on integrated plasmids (e.g. the Hfr factors) and phages (e.g. λ). Curing of prophages, possibly in the pseudolysogenic state, by detergents has been reported (Takumi, Kinouchi and Kanata, 1980) so that the action of SDS cannot be taken as definite proof of a plasmid. The effect of streptomycin and chloramphenicol can however be taken as proof in the light of the results of Williams Smith (1953) on the lack of effect of these agents on prophage induction.

It is of interest that the effective concentrations of agents found were relatively low as compared to the doses used with bacteria. No explanation for this finding is readily available.

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TABLE 1. Effect of curing agents on the toxicity of *M. aeruginosa*, as determined by intraperitoneal injection of extracts into mice.

Set	Agent	Concentration (µg/mℓ)	Culture volume (mℓ)	Mass injected (mg wet mass)[a]	Toxicity[b]
1	Acridine orange	1	100	N.D.	N.G.
	"	0,5	100	N.D.	-
	"	0,1	100	N.D.	+
	"	0,01	100	N.D.	+
	SDS	1 000	100	N.D.	N.G.
	"	100	100	N.D.	-
	"	10	100	N.D.	-
	Streptomycin	10	100	N.D.	N.G.
	"	1	100	N.D.	-
	"	0,1	100	N.D.	-
	"	0,01	100	N.D.	+
	Chloramphenicol	10	100	N.D.	-
	"	1	100	N.D.	-
	"	0,1	100	N.D.	+
	Control		100	N.D.	+
2	SDS	10	3 000	N.D.	+
	Control		3 000	N.D.	+
3	SDS	1 000	100	N.D.	N.G.
	"	100	100	414,6	-
	"	10	100	495,7	-[c]
	Ethidium bromide	0,1	100	N.D.	N.G.
	"	0,01	100	768,0	-
	"	0,001	100	795,2	+[d]
	Control		100	845,4	-
	"		100	473,9	-
4	SDS	100	3 000	473,0	-
	Control		3 000	N.D.	+

[a]N.D. = injected mass not determined
[b]N.G. = no growth (level of test agent toxic to *M. aeruginosa*)
- = non-toxic
+ = toxic
[c]Mouse died after approximately 18 h, probably because of Slow Death Factor
[d]Liver grossly enlarged and dark red in colour

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Curing of a cyanobacterial plasmid by growth in the presence of 80 µg/mℓ SDS has been reported (Lau, Sapienza and Doolittle, 1980).

An attempt to culture a large volume of cured *M. aeruginosa* (set 2) was unsuccessful. The slime production noted with set 1 was absent but this may have been as a result of the large volume used. A number of explanations for the lack of curing, in contrast to that found with set 1, may be put forward:

(a) No curing took place in set 1 as too little material (i.e. less than the LD_{100}) was injected.

(b) The cultures used in set 1 also contained a non-toxic strain or a contaminant which was selected for by the various treatments. The cultures of set 1 appeared unialgal on microscopic examination so that the presence of a contaminant is unlikely but the presence of a non-toxic strain cannot however be excluded. The culture used in set 2 was from a fresh single-cell isolation (W.E. Scott, 1980, personal communication) so that the likelihood of a non-toxic strain still being present was small. A process of selection would explain the fact that cultures were toxic at the lower concentrations in set 1 but this could also be explained in terms of the level required for curing of a plasmid.

(c) Curing took place in set 1 but the culture used in set 2 produced another toxin type which was not plasmid-determined and which was repressed in the presence of the plasmid. The lack of a selective and sensitive assay for the different toxin types will make this hypothesis difficult to test.

There are at least two ways to interpret the results of set 3:

(a) Curing took place. The non-toxicity of the controls contradicted this.

(b) Toxicity was inducible by environmental factors. The non-toxicity of the controls was thus explicable.

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The difference in growth conditions used for sets 1, 2 and 3 may also have played a role in the differences found between these sets.

The result of set 4 (another large scale culture using a long incubation period as used with set 1 and 2), especially in conjunction with those of sets 1 and 2, once again indicated that curing had taken place but that a higher concentration of SDS was required for curing.

It should be noted that the controls for sets 1, 2 and 4 were all toxic.

When the results of all four sets were considered together it was difficult to decide, from the evidence presented, whether curing had indeed taken place i.e. that toxicity was plasmid-determined or whether the treatments merely interfered with the expression of chromosomal toxicity genes, which were induced in the presence of low concentrations of ethidium bromide.

Isolation of a plasmid from a toxic strain which was not present in cured strains, together with cell-free synthesis of the toxin(s) using this plasmid as template would be a convincing demonstration of plasmid involvement in toxicity.

4.2 Lysis studies on tap water grown cells

Major differences were found between tap and deionised water grown cells. The results obtained are therefore treated separately, starting with the aforementioned. Freeze-dried and fresh cells were studied and this distinction is retained in this section.

4.2.1 Small scale lyses to find optimal methods and conditions

In view of the large number of experiments (approximately 600), they were classified according to similarities of experimental procedure, rather than treating each experiment separately. This gave eight groups which are graphically summarised in Fig. 1. Even so, for the sake of brevity, not

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all results are presented but the selection was such as not to omit any observation of potential significance.

The overall strategy of research was to follow the "general method" (see 3.3) but varying selected parameters indicated for each group (Fig. 1). Results for freeze-dried cells are shown by broken lines and those for fresh cells by solid lines. References relevant to a treatment are given numerically in Fig. 1.

For the sake of clarity and brevity results are not discussed extensively in terms of previous work. As literature references pertaining to procedures or reagents found by others to give rise to satisfactory lysis of their test organisms are given (Fig. 1), elaboration on divergence or agreement of the present experiments with respect to previous observations is limited.

4.2.2 Group 1: Effects of hydration medium on the lysis of freeze-dried cells (Fig. 1, distr. 1)

These experiments showed that the hydration medium used prior to the lysis procedure had a major effect on the lysis of freeze-dried cells (0 for water, 100 for EDTA pH 7). This observation seemingly is unprecedented but a possible explanation may be a combination of EDTA destabilisation of the cell wall (Witholt, Van Heerikhuizen and De Leij, 1976) and a pH effect, as supported by the observation for sucrose (pH 7 = 0; pH 8 = 75) as hydration medium. In all subsequent experiments hydration was in 0,25 M EDTA, pH 7, for 15 min as no major effect was observed when incubation was prolonged for up to as much as 72 h .

4.2.3 Group 2: Replacement of sucrose (Fig. 1, distr. 2 and 3)

Sucrose replacement was tested in conjunction with three enzymes: AL, L1 and lysozyme. None of the substituting agents exhibited a drastically improved effect which also indicated that nuclease contamination of sucrose (R. Schilperoort and A. Ledeboer, 1980, personal communication) was insignificant under the present circumstances.

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FIGURE 1. The amount of DNA released from fresh and freeze-dried cells, grown in tap water. DNA release is given in arbitrary units, relative to the amount released from freeze-dried cells using the general method (see below).
Results for fresh cells are presented in solid lines and in broken lines for freeze-dried cells.

The general method of lysis used was:

a) Approximately 1mg (freeze-dried mass) of cells were suspended in 100μℓ 0,25 M EDTA, pH 7, for 15 min and then pelleted. This hydration step was omitted when fresh cells were used.
b) The pelleted cells were resuspended in 10μℓ 25% sucrose in 0,05 M Tris, pH 8.
c) 20μℓ of 0,5 M EDTA, pH 8, was added.
d) 20μℓ of a 5mg/ml enzyme solution (indicated) was added and the mixture incubated for 1 h at 37°C.
e) 50μℓ of a 5% detergent solution was then added and incubation continued for 30 min. After addition of dye solution the mixture was loaded into the wells of an agarose slab.

The variations on this basic method studied, were:

i) Hydration medium (distr. 1) (enzyme used was L1)
ii) Replacements for sucrose (distr. 2 and 3)
iii) The EDTA requirement (distr. 4)
iv) Enzyme efficiencies (distr. 5 to 8)
v) Detergent efficiencies (distr. 9 to 11)
vi) Washes of cells before the lytic procedure (distr. 12 and 13)
vii) Other treatments differing from the basic method (distr. 14 and 15)
viii) Osmotic shock treatments (distr. 16)

Relevant references for the different methods and variations used are indicated below each experiment.

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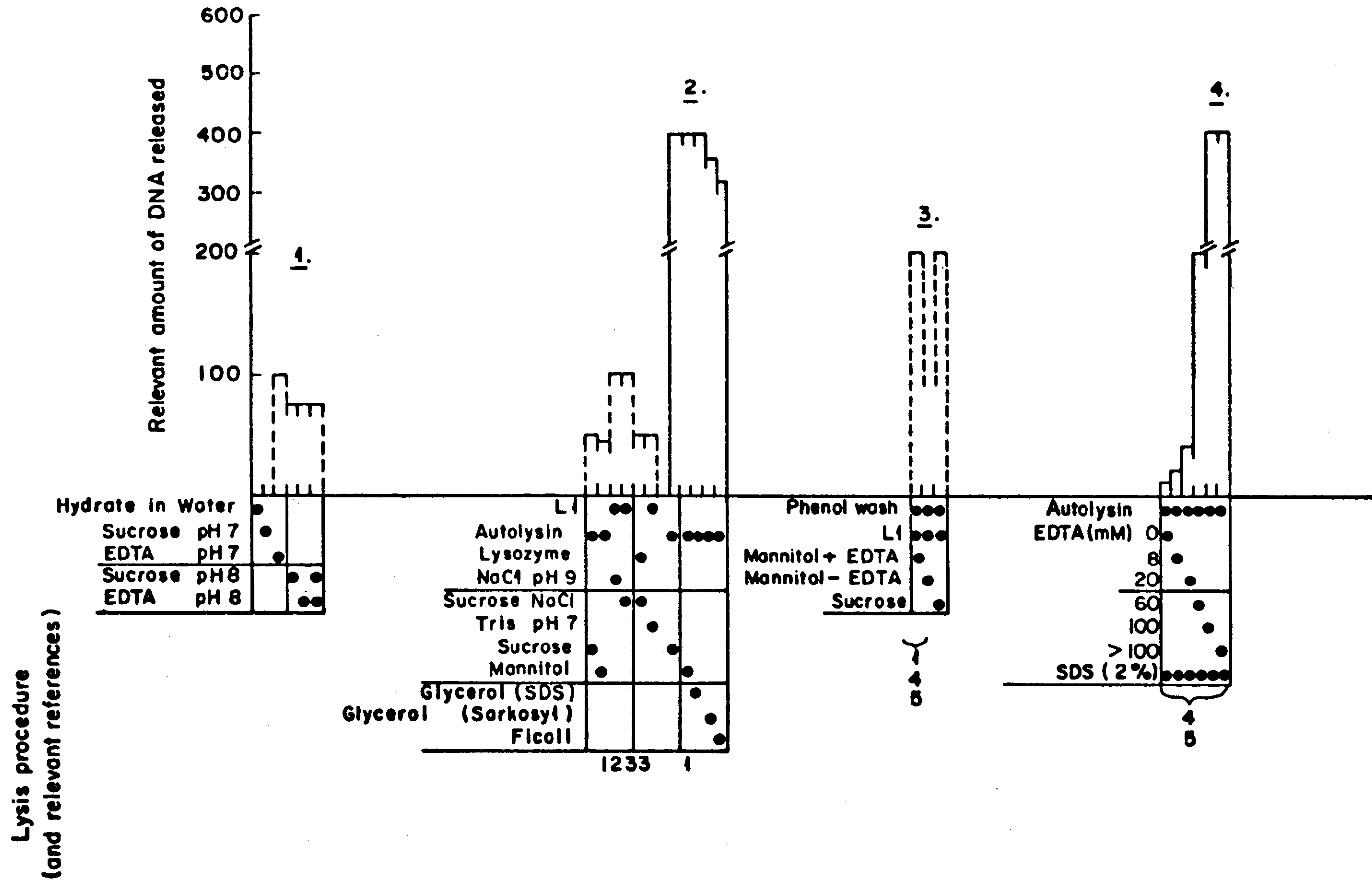

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Fig. 1 (cont.)

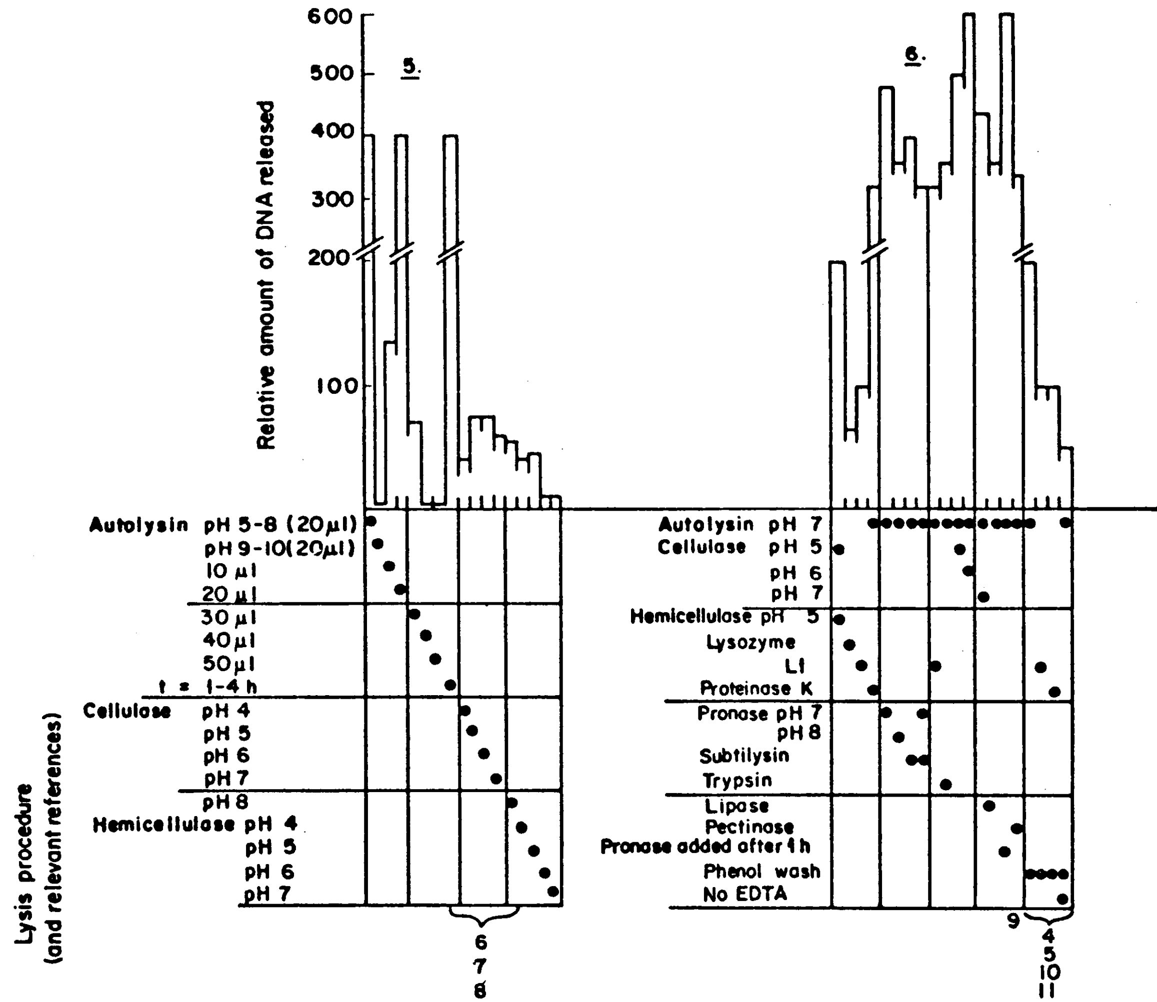

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Fig.1 (cont.)

Relative amount of DNA released

600
500
400
300
200
100

7.

8.

Lysis procedure (and relevant references)

L 1
Lysozyme, 37° C
Lysozyme, 0° C
Autolysin, 1 h
L1, pH 10
Pectinase
Amylase
Subtilysin
Thermolysin
Amyloglucosidase
Proteinase K.
Lipase
Pronase
Cellulose
Lysozyme added after 1 hr
Phenol wash
1 M KCl, 80, 5 min., wash

9
10
11

L 1
Autolysin, 1 h
Proteinase K
Lipase
Pronase
Cellulase
Trypsin
RNase
Protease Neutral
Pronase added after 1 h
2 h exposure
Pellet resuspended, 1 h
Pellet resuspended, 1 h
3 h exposure
4 h exposure

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Fig. 1 (cont.)

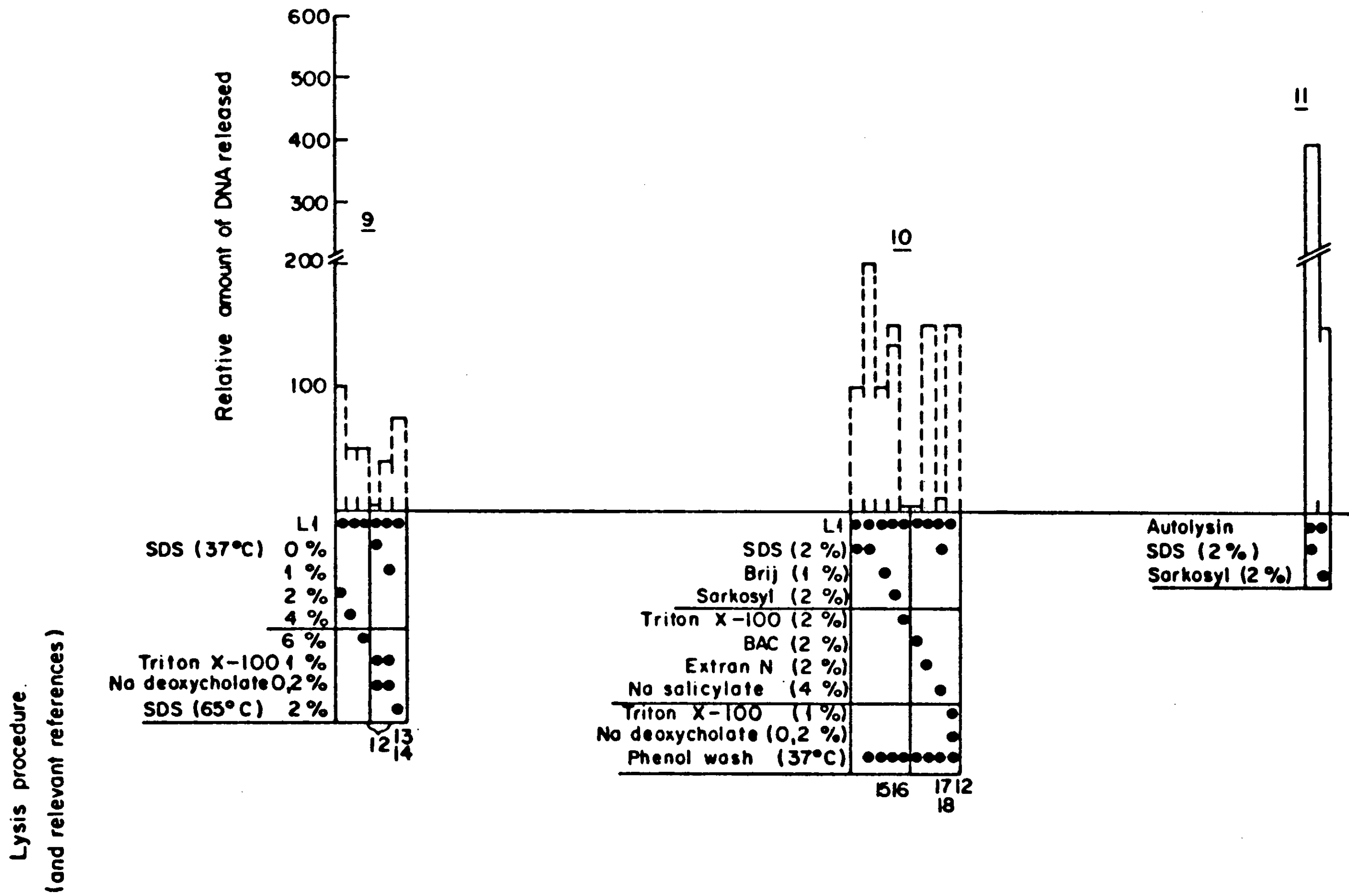

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Fig. 1 (cont.)

Relative amount of DNA released

600
500
400
300
200
100

12.

13.

Lysis procedure
(and relevant references)

L 1
65°C SDS
Butanol
Toluene
5 M NaCl
37°C SDS
Sarkosyl
Pronase
Triton X-100
Trypsin
Resusp. in trypsin - EDTA
Lipase
Papain
DNase
Resusp. in Tris pH 9 (+ EDTA)
Resusp. in Tris pH 9 (- EDTA)
Resusp. in Tris pH 9 (+ EDTA) + sucrose

19 11 3 21 22
20

L 1
Autolysin
Lysozyme
Sarkosyl
Pronase
Trypsin
Papain
DMSO
Methanol
EDTA pH 7
EDTA pH 8
Tris pH 9
NaOH 2,5 % (pH 8)
NaOH 5,0 % (pH 8)
1 M KCl, 80°C, 5 min.
1 M KCl, 80°C, 10 min.
Phenol 60°C
Phenol 0-37°C
Acetone
Protease neutral
Proteinase K

11 11 11 10 10 11 11
23 23

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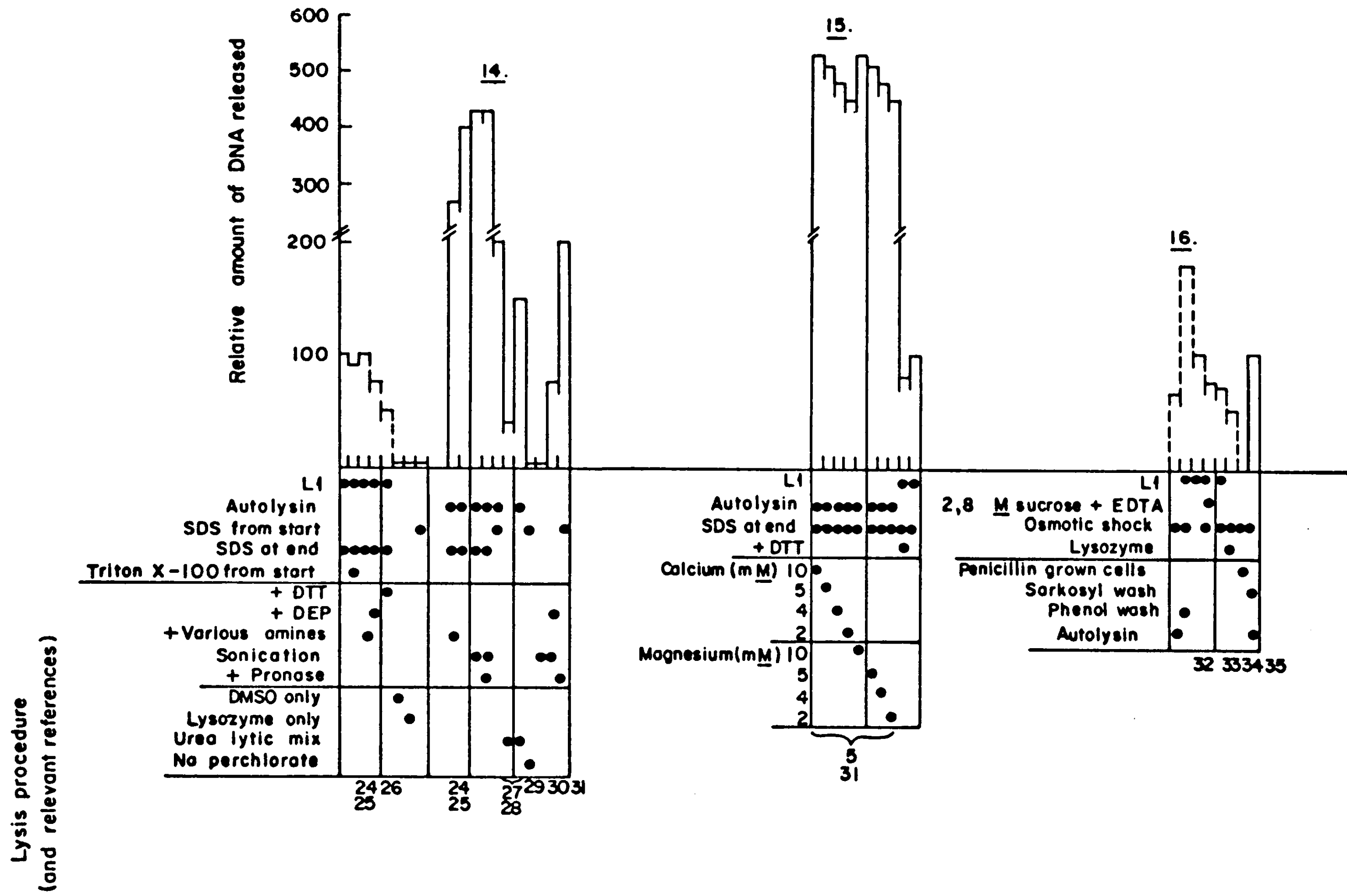
Fig. 1 (cont.)



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Consequently 25 % sucrose in 0,05 M Tris was used in future studies. In accordance with Repaske (1958) Tris slightly increased DNA yields (results not shown).

Two observations in relation to NaCl were of interest. Firstly, it could replace sucrose and secondly, EDTA was not required when NaCl was used (results not shown, yield also 100), contrary to Repaske (1956). There was however an EDTA requirement when cells had been washed with phenol (distr. 3).

4.2.4 Group 3: Effect of EDTA on the efficiency of AL (Fig. 1, distr. 4)

A very clear requirement for EDTA was seen, saturating at 100 mM . This concentration was used in all future experiments with AL. The EDTA requirement could have been for two reasons:

(a) destabilisation of the cell wall (Repaske, 1956; Repaske, 1958; Witholt, Van Heerikhuizen and De Leij, 1976) or

(b) inhibition of nuclease activity (Marmur, 1961).

A small amount of lysis in the absence of EDTA was seen. This was expected as the cell wall might not be homogenous in strength.

4.2.5 Group 4: Efficiency of various enzymes and enzyme combinations

(a) Fresh cells (Fig. 1, distr. 5 and 6)
High levels of lysis were obtained using AL on its own (400, distr. 5). Concentration and pH dependencies were striking, the latter being in agreement with Ingram (1973).

Cellulase (75 at pH 5 and 6) and hemicellulase (45 at pH 5)(distr. 5) and lysozyme (65) and L1 (100)(distr. 6) were decidedly less effective in comparison. These results confirm those of Vance and Ward (1969) and Jones and Jost (1970) with respect to *M. aeruginosa* and

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lysozyme and Lindsey *et al.* (1971) with respect to lysozyme and *A. nidulans*. They were contrary to those of Dunn and Wolk (1970), Fuhs (1973) and Fjerdingstad, Fjerdingstad and Fjerdingstad (1979) with respect to cellulase and cyanobacteria and to those of W.E. Scott (personal communication) as to the effect of L1 on *Anabaena* sp. It should however be noted that contamination of the cellulase by an unknown enzyme, giving rise to the above observations, could not be excluded (C.P. Wolk, 1980, personal communication).

Combining AL with pronase or cellulase increased DNA yields (600 for both, distr. 6) whereas other combinations proved slightly inhibitory (e.g. AL + proteinase K 320; AL + trypsin 350). Increases due to cellulase were ambiguous in view of the above but those due to pronase might have been the result of removal of obstructing structure(s) by one enzyme to favour the action of the other. Some credibility for this notion stemmed from the kinetics observed (475 for AL plus pronase vs 600 for AL with pronase added after 1 h-distr. 6).

(b) Freeze-dried cells (Fig. 1, distr. 7 and 8)

L1 proved most effective (100, distr. 7) contrary to the case with fresh cells (distr. 5 and 6). Exposure to L1 for up to 4 h did not increase DNA yields (distr. 8) but repeated re-exposure of pellets from lysates yielded up to 70 % of the original release (distr. 8). Even then pellets remained, indicating that the low yields for freeze-dried cells were due to the general refractility of the cells.

No combination of L1 with other enzymes enhanced DNA yields and decreases might in fact be significant (distr. 7 and 8).

Heat treatment, proven successful in other cases (Noller and Hartsell, 1961b), had no effect on the lysis

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of *M. aeruginosa* (distr. 7) except for subtilysin (150) whose yield fell short of maximum levels obtained by other means.

The most striking observation was that prewashing cells with phenol enhanced DNA release by L1 (200 vs 100) and confirmed previous observations with freeze-dried cells (distr. 3) and fresh cells (distr. 6). It was also in agreement with the work of De Petris (1967) and Weise *et al.* (1970) who found that phenol removed the lipopolysaccharide layer. The inhibitory effect of a phenol wash on DNA release by AL (200 vs 400) (distr. 6) suggested a difference in the mode of action of AL and L1.

Comparison of distributions 5 and 6 with 7 and 8 showed that L1 worked on fresh and freeze-dried cells with equal but low efficiency. It also suggested that freeze-drying affected the target of AL such that lysis was only 10 % efficient compared with fresh cells (DNA yields of 40 and 400, distr. 5 and 7 respectively).

4.2.6 Group 5: Efficiency of various detergents following enzyme treatment (Fig. 1, distr. 9 to 11)

These experiments were mostly directed at improving lysis of freeze-dried cells. Preliminary unquantitated studies (results not shown) indicated that the effects of detergents were similar with both types of cell.

The marked superiority of SDS could readily be observed, in line with the findings of Helenius *et al.* (1979) that SDS is a very powerful denaturant. An optimal concentration of 2 % was found (distr. 9) and this concentration was used in the majority of experiments.

As a phenol wash before lysis was found to give maximal lysis of freeze-dried cells, most detergent studies were done on phenol-treated cells (distr. 10).

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The results found with Triton X-100 (distr. 10) were in agreement with those found for *E. coli* (Schnaitman, 1971a) but the poor lysis (relative yield in brackets after agent) found with other detergents and mixtures was contrary to that observed by others: Marmur (1961), Stanier *et al.* (1971) and Owen and Lapage (1976) used SDS at 65 °C (75) with satisfactory results; a SDS-sodium salicylate mixture (10) was used by Craig, Leach and Carr (1969) as well as Devilly and Houghton (1977) who found that the effects of SDS and salicylate were additive; Dmitrieva and Kozlov (1979) found that a mixture of Triton X-100 and sodium deoxycholate (0) was best for the release of the chromosome of *Anabaena variabilis*.

4.2.7 Group 6: Pre-exposures (washes) of cells before enzymatic treatment (Fig. 1, distr. 12 and 13)

The aim of these studies was to find either substances removing cell wall layers inhibiting lysis (De Petris, 1967; Schnaitman, 1971b; Drews, 1973) or making them permeable to subsequently applied agents (e.g. DMSO - Fantes, Roberts and Huetter, 1976; Triton X-100 - Miozzari, Niederberger and Hütter, 1978; toluene - Serrano, Gancedo and Gancedo, 1973). Intact cells were exposed to the test agent, washed by centrifugation and exposed to appropriate enzymes in the general lysing procedure.

For freeze-dried cells potentiation by phenol for L1 as lysing enzyme was confirmed (200 vs 100) (distr. 13) and only for papain was a synergistic effect with phenol found (250), in agreement with De Petris (1967), although papain alone (distr. 12) did not potentiate the effect of L1. These observations for phenol plus papain were mimicked for AL as lysing enzyme (100 vs 50) and in addition pronase and proteinase K seemingly also enhanced DNA release by AL (100 vs 50). The latter phenomena were however of little practical significance in view of the relative inefficiency of AL on freeze-dried cells.

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No greatly significant potentiation of lysis of fresh cells was observed.

4.2.8 Group 7: Other treatments (Fig. 1, distr. 14 and 15)

Some experiments which deviated from the general method with respect to the sequence in which reagents were added (e.g. SDS or Triton X-100 added prior to osmoticum), omission of some reagents or inclusion of substances with specific functions (e.g. DEP against nucleases), etc. are presented here. Enhanced DNA release was obtained for neither fresh nor freeze-dried cells (distr. 14).

Some concentration dependant enhancement of yield by calcium and magnesium (525 at 10 mM vs 400 at 0 mM) was found with fresh cells and AL (distr. 15).

4.2.9 Group 8: Osmotic shock treatments (Fig. 1, distr. 16)

These results indicate that osmotic shock in conjunction with a number of chemical treatments did not produce DNA yields in excess of those found in the absence of osmotic shock.

4.2.10 Discussion

The objective of this study was to find a chemical lysing procedure for *M. aeruginosa* which would facilitate detection of a plasmid(s). Harsh physical treatments were not used in view of their destructive potential for plasmids.

The main conclusion was that the strain of *M. aeruginosa* (WR 70) used in these studies was essentially refractile to all commonly used methods of lysis, including some which have been developed for use with other refractile organisms. In view of subsequent work in which harsh physical procedures were used refractility could be quantified. For freeze-dried cells the best yield of DNA was approximately 3,3 % and approximately 10 % for fresh cells. These were at best upper limits in terms of total DNA content of the cell

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population as the 100 % point could not be unequivocally established.

The question arose whether these low yields were due to low release per individual cell or from a small lysable subpopulation of the total population of cells. The preliminary assessment was that it was the former. Evidence in support of this was the fact that pellets were invariably found upon completion of lysing procedures and subsequent centrifugation. These were hardly different in size from pre-lysis pellets and, in addition, light microscopy revealed cyanobacterial structures not significantly different in appearance and quantity from untreated samples.

A second conclusion was that L1 worked with equal but low efficiency on fresh and freeze-dried cells, whereas AL proved two- to three-fold more efficient on fresh cells only. This was taken to indicate that freeze-drying adversely affected the target of AL but not that of L1. In view of L1 consisting of proteases, β-1-3 glucanases (endo- and exo-), chitinases, lipases and isoamylases (BDH Biochemical Product Information) and the exact activity of AL being unknown, their action is still not understood. As for L1, the fact that proteinase K in conjunction with lipase gave rise to lysis comparable to that of L1 alone after a phenol wash (distr. 7) might give some indication of its pertinent activity, in accordance with the findings of Weinbaum, Rich and Fischman (1967) on spheroplast formation in *E. coli*.

The most noteworthy findings may be summarised in relation to previous work (where applicable):

For both fresh and freeze-dried cells:

(a) Lysozyme was not very effective, (distr. 2, 6, 7), as was found by Jones and Jost (1970).

(b) SDS was the most efficient detergent (distr. 10 and 11) as would be expected from the finding (Helenius *et al.*, 1979) that SDS was an efficient denaturant.

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(c) Osmotic shock procedures were highly inefficient (distr. 16). This was in marked contrast to the results of Jones and Jost (1970).

(d) No enhancement of lysis was found using the mannitol-phosphate buffer of Biggins (1967) (distr. 2 and 3).

For freeze-dried cells only:

(a) The hydration medium used was critical and under certain conditions (water or sucrose pH 7) no lysis was found (distr. 1).

(b) A pre-wash with phenol, especially after papain treatment, greatly enhanced lysis when L1 is used (distr. 7).

(c) For L1, the most efficient enzyme, the pH optimum observed (pH 8, results not shown) was that for optimal proteolysis (BDH Biochemical Product Information).

(d) Optimal lysis of freeze-dried cells required:

 (i) Hydration of cells in 0,25 M EDTA, pH 7 for at least 15 min.

 (ii) A papain wash followed by a phenol wash.

 (iii) Use of L1 as the enzyme in the general method with SDS as the detergent.

For fresh cells:

(a) AL, the best enzyme, had a definite EDTA requirement (distr. 4).

(b) The pH 5 to 8 optimum for AL extracted from *Microcystis* (distr. 5) was similar to that found for the autolysin of *A. quadriplicatum* (Ingram, 1973).

(c) In line with the findings of Ingram (1973) a drop in activity was found when AL was combined with certain proteases (distr. 6).

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(d) No wash was found to increase lysis unlike the phenol wash used with freeze-dried cells.

(e) The combination of cellulase plus AL or addition of pronase to the AL lysis mixture after an hour gave maximal lysis (distr. 6). The lysis found using cellulase was probably as a result of some or other contaminating activity in the cellulase preparation.

(f) Calcium and magnesium enhanced lysis by AL (distr. 15) contrary to observations that Mg stabilises cell walls against enzymatic lysis (Schnaitman, 1971b; Witholt, Van Heerikhuizen and De Leij, 1976; Leduc and Van Heijenoort, 1980; BDH Biochemical Product Information). Costerton, Ingram and Cheng (1974) however reported that high Mg^{2+} levels might have an effect on the wall which was similar to that of EDTA.

(g) Exposure of a culture of penicillin for 5 h prior to harvesting did not seem to have any positive effect on enzymatic lysis (results not shown) and the cells were also not osmotically sensitive (distr. 16).

(h) Optimal lysis of fresh cells required:

  (i) AL followed by pronase (pH 7) after 1 h, with 100 mM EDTA present throughout, as the enzymatic treatment in the general lysing method.

  (ii) Use of SDS as the detergent.

In studies directed at visualising a plasmid on agarose gels no evidence for a plasmid was ever found, notwithstanding the fact that controls of plasmid-bearing bacteria always revealed plasmids.

However the bacteria used were comparatively easy to lyse. The methods developed in this section were scaled up and used to isolate sufficient DNA for caesium chloride-ehtidium bromide density gradient analysis.

A four-layered cell wall with an external layer of lipopolysaccharide is common to all models of Gram-negative and

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cyanobacterial cell wall structure (see 2.3 and Fig. 2). From knowledge of the composition of these layers as well as on the mode of action of various agents, predictions could be made as to the results of various experiments. The validity of some of these predictions are discussed below, with reference to the different layers:

(a) The lipopolysaccharide layer, LIV and LII.

(i) Most treatments removing the lipopolysaccharide layer (e.g. phenol) also remove LIV and LIII (e.g. Golecki and Drews, 1974) so that these three layers will be dealt with together. The action of phenol found by Golecki and Drews (1974) implied that the murein was then the only layer stopping DNA release and as lysozyme acts on this layer (Frank, Lefort and Martin, 1962; De Petris, 1967; Schnaitman, 1971b) its lysing capacity should be potentiated. This was not found, but the release by L1 was doubled (Fig. 1, distr. 7). Two explanations for this may be proposed: (a) that the phenol did not remove all the layers expected, leaving an inhibitory layer or (b) the murein of *M. aeruginosa* is modified so as to be refractile to the action of lysozyme (Ghuysen, 1968). The great thickness of the murein (Drews, 1973) may also play a role (see also (b)(ii) below).

(ii) In view of the proteinaceous nature of LIII (De Petris, 1967; Costerton, Ingram and Cheng, 1971; Schaitman, 1971b), proteases should increase the action of phenol (as found by De Petris, 1967). This was so when papain was used.

(iii) Other treatments reported to remove these layers had little effect on lysis.

(b) The murein.

(i) The lack of effect of a phenol wash on the action of lysozyme implied that the murein was not the layer retaining DNA in *Microcystis*.

(ii) The number of activities present in L1 made it difficult to decide which was responsible for lysis but

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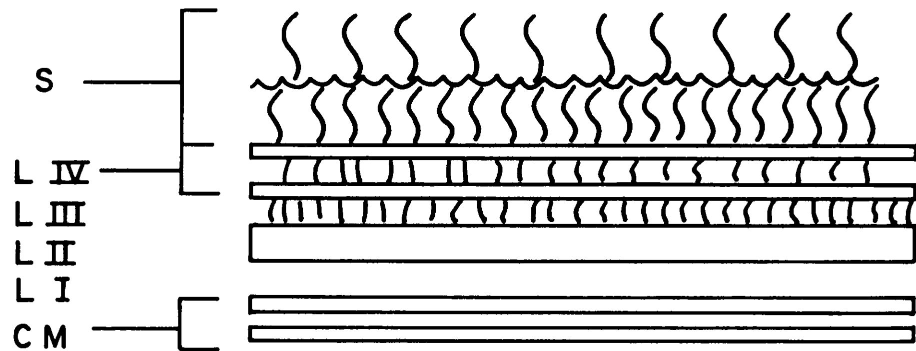

Figure 2. Diagrammatic representation of the cell wall of a cyanobacterium (From Jost, 1965).

S Sheath and lipopolysaccharide layer

LI-LIV Layers of the cell wall

CM Cytoplasmic membrane

LII is the murein or peptidoglycan

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experiments using single activity enzymes (lipase and proteinase K) again impled that the murein was not the only or main layer inhibiting DNA release but that a protein and a lipid layer (or a lipoprotein layer i.e. LIII) were the responsible components (cf. Weinbaum, Rich and Fischman, 1967).

(iii) The lack of effect of growth in penicillin on lysis had the same implication.

One major difference found between the models and the present studies was the enhancement of AL lysis by magnesium (e.g. Witholt, Van Heerikhuizen and De Leij, 1976). There is however a report (Costerton, Ingram and Cheng, 1974) that elevated Mg^{2+} levels can distrurb the integrity of the outer membrane (the lipopolysaccharide layer, LIV and LIII in the nomenclature used in this study). This would therefore explain the above finding.

In conclusion:

(a) Similarities and differences with respect to present models exist.

(b) As these experiments were not specifically designed to study cell envelope structure, no clear picture on this subject emerged. Results found, e.g. inhibitory effects, could be of use in a study on cell wall structure.

(c) The structure and composition of the cell wall *M. aeruginosa* require elucidation and such knowledge may facilitate the achievement of more complete lysis.

4.3 Lysis studies on *M. aeruginosa* strain WR 70 grown in good and poor quality deionised water and on a Vaal Dam isolate grown in deionised water.

During the course of this study two changes in mass cultivation conditions occurred. Firstly, deionised water rather than tap water was used for media. The second was due to a breakdown in the deionising system, leading to water with an ion content of tap water (W.E. Scott, 1980, personal communication). As the two types

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of cells responded differently to lysing procedures some results of these lysis experiments are of interest.

4.3.1 Deionised water grown cells

(a) Varying enzymes, detergents and EDTA in the general lysing procedure (Fig. 3, distr. 1 and 2).

These results (dist. 1) indicated that lysozyme, AL, L1 and pronase in conjunction with SDS gave comparable yields of DNA (approximately 400). This yield corresponded well with that for fresh cells grown in tap water, using AL (400))Fig. 1, distr. 5). However the fact that all enzymes acted with similar efficiency was in sharp contrast to previous results (4.2.5, Fig. 1, distr. 5 to 8).

In conjunction with 5% sarkosyl DNA release, ranging from 600 to 800 for all enzymes, was enhanced as compared to release with SDS (Fig. 3, distr. 1) and a concentration dependance for sarkosyl was suggested. This finding was contrary to what had been observed for tap water grown cells where SDS proved most effective (5.6; Fig. 1, distr. 10). Other treatments (Fig. 3, distr. 1) had no significant effect, except for alkaline sarkosyl whose inhibitory effect was apparent.

A slight EDTA dependence was suggested (Fig. 3, distr. 2) but this was not as strong as was previously observed (4.2.4; Fig. 1, distr. 4).

(b) Lysis with detergents alone (Fig. 3, distr. 3).

Considerable lysis (1000) was observed when cells were directly exposed to low concentrations of detergent without any pre-treatment (Fig. 3, distr. 3). This was not observed with tap water cells (Fig. 1, distr. 14).

Sarkosyl proved most effective, with slight concentration dependence and some DEP enhancement but

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FIGURE 3. The relative amount of DNA released from cells grown in deionised water, using various lysis methods. Units of DNA release are as defined for figure 1.

The basic enzymatic method of lysis was:

a) Cells were pelleted and then resuspended in 10µℓ 25% sucrose in 0,05 M Tris, pH 8.

b) 20µℓ of 0,5 M EDTA, pH 8, was added.

c) 20µℓ of a 5mg/ml enzyme solution was added and the mixture incubated at 37°C for 1 h.

d) 50µℓ of a 5% detergent solution was added and incubation continued for a further 30 min.

When detergents alone were used for lysis the cells were directly resuspended in 100µℓ of a 5% solution of the relevant detergent.

The variations on the basic method studied, were:

i) Variations in enzymes and detergents (distr. 1 and 2)

ii) Use of detergent only (distr. 3)

iii) Effects of washes and other treatments (distr. 4 and 5)

iv) Use of the method described by Graham (distr. 6)

In distribution 7 the results of lysis assays on cells grown in poor quality deionised water (see text) are compared with results of lyses of cells grown in tap water (both fresh and freeze dried) and deionised water.

A strain of *M. aeruginosa* isolated from Vaal Dam was also studied. This strain was grown in deionised water and then freeze-dried. The lytic responses of this strain, together with those of WR70 grown in tap and deionised water, are shown in distribution 8.

Relevant references for the various method are indicated below each experiment.

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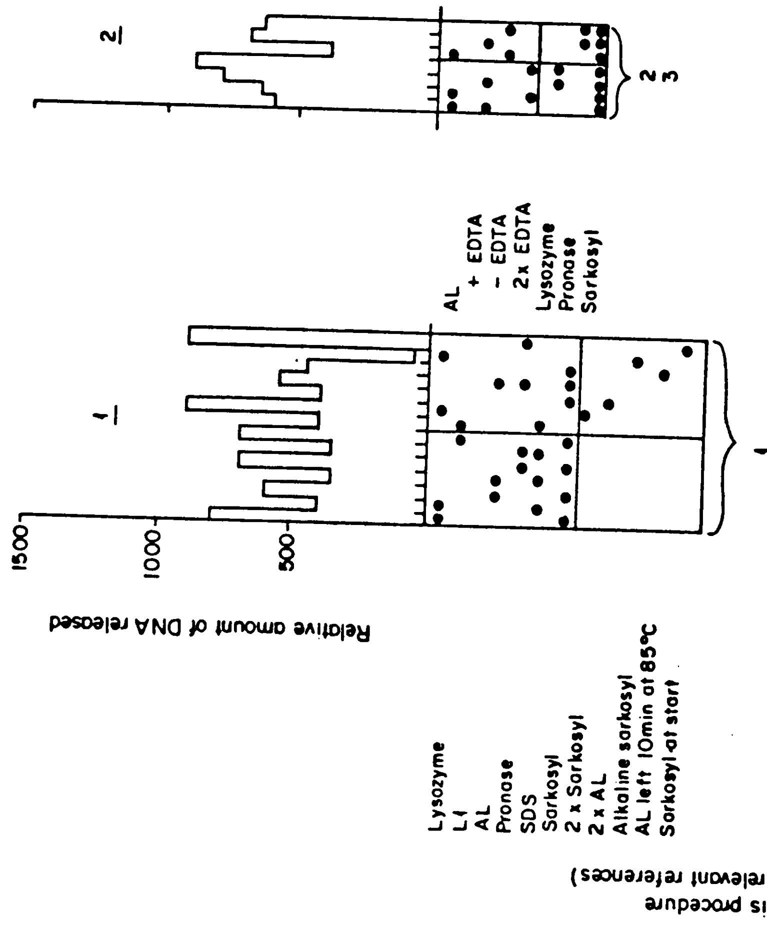

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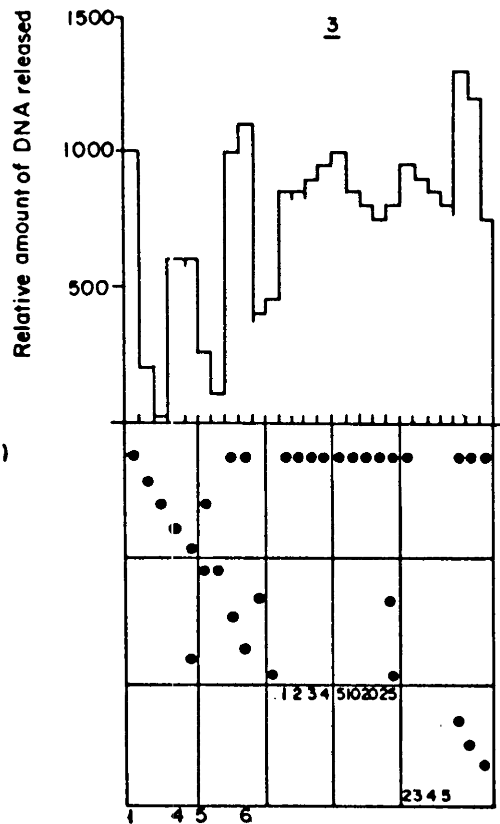
Fig. 3 (cont.)



Lysis procedure
(and relevant references)

Sarkosyl (30 min to 180 min)
Alkaline sarkosyl
Triton X-100
SDS-salicylate
Sarkosyl-salicylate
Na deoxycholate
SDS 30 min
0° to 37°C
DEP
SDS 180 min
% Sarkosyl
65°C
85°C
+ DMSO
Wash and resuspend

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Fig. 3 (cont.)

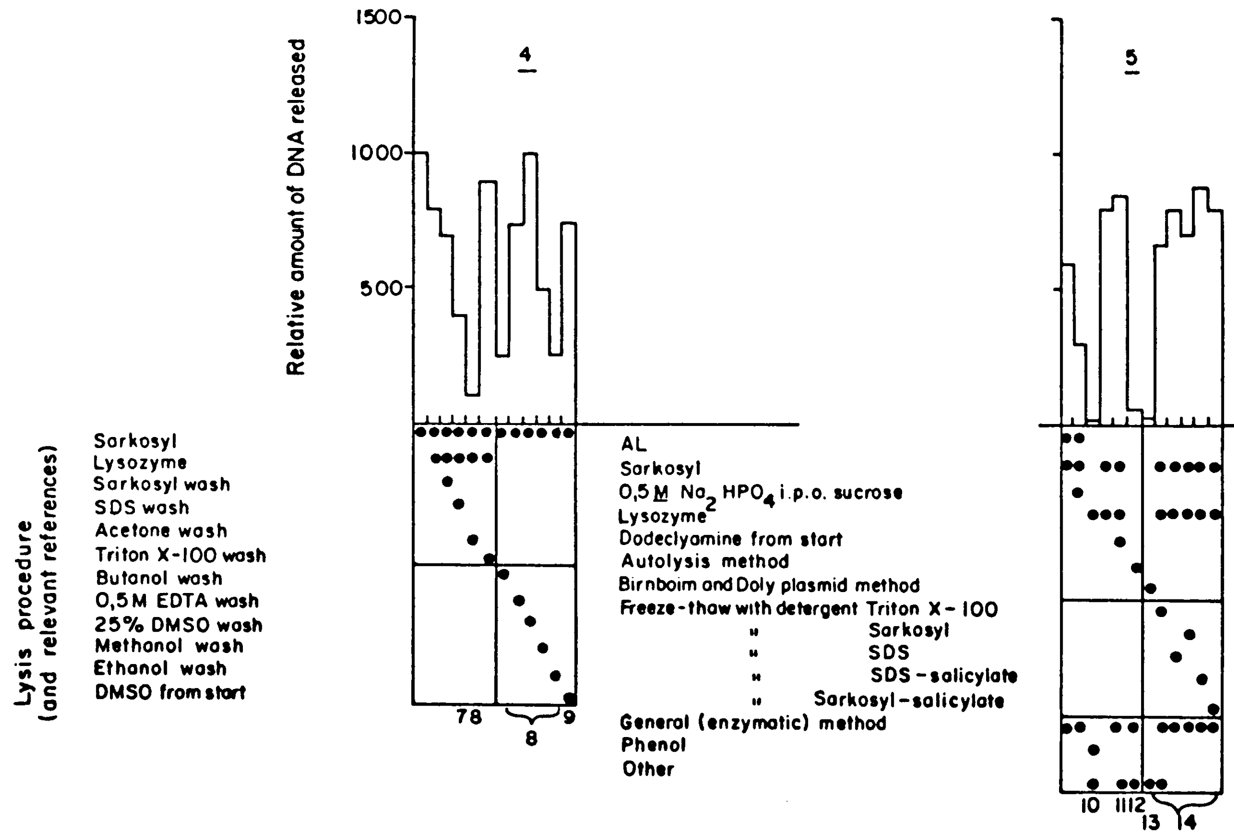

Fig.3 (cont)

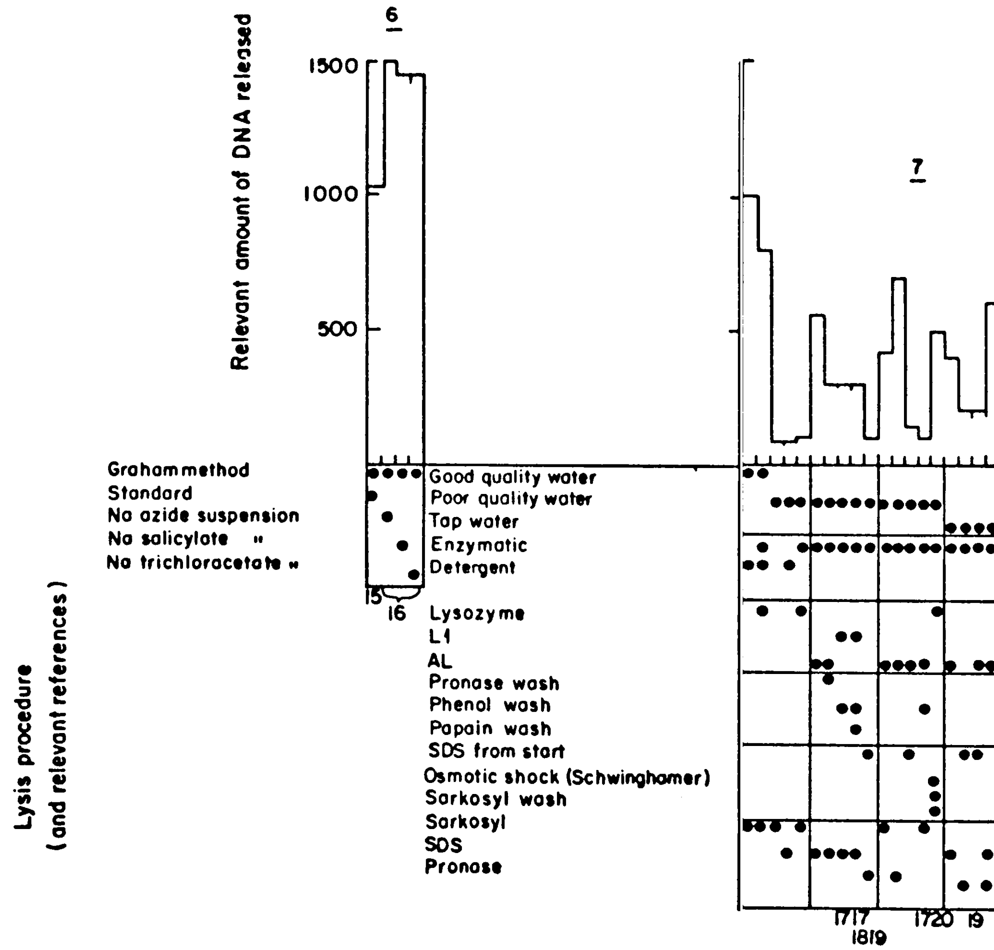

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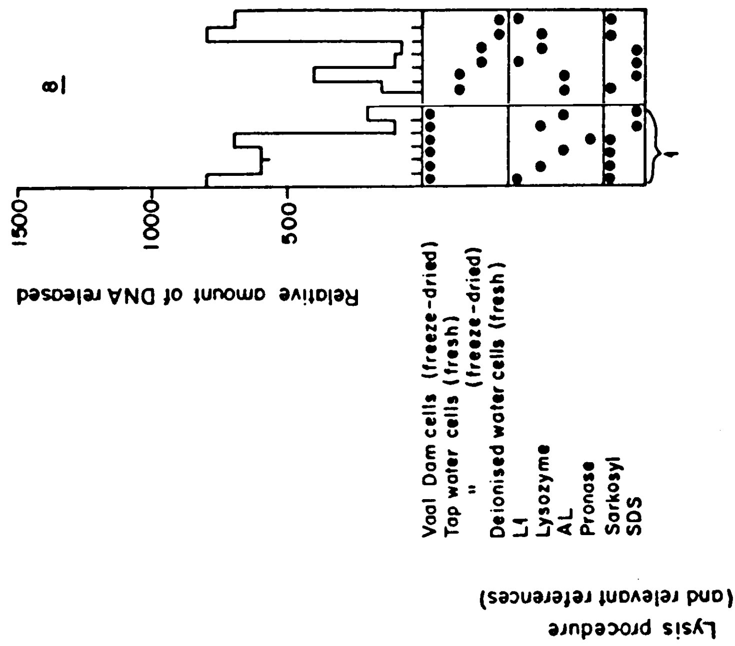
Fig.3 (cont.)



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no exposure time dependence was observed (Fig. 3, distr. 3). A temperature optimum for lysis of 65 °C was found (1300).

Experiments involving combinations of detergents and/or other reagents did not improve lysis (Fig. 3, distr. 3).

(c) Effects of washes and other treatments (Fig. 3, distr. 4 and 5).

Prewashing cells and subsequent addition of detergent or employment of the general lysing procedure did not enhance DNA release (Fig. 3, distr. 4).

Of interest was the effect on release of freezing cells in detergent, thawing them and then lysing using the general method with sarkosyl (Fig. 3, distr. 5).

Other methods used by the authors indicated were poor in comparison, although slight enhancement in the presence of dodecylamine was observed (850 vs 800)(Fig. 3, distr. 5).

(d) The method of Graham (1978)(Fig. 3, distr. 6)

This method, involving the grinding of frozen cells and immediate suspension in a deproteinising solution gave the highest yields (1500) of DNA observed in the course of these studies, especially when combined with the salt solutions recommended by Kirby (1957). Notwithstanding objections as to its potential mechanical destructiveness to plasmids, this method was also subsequently used on a large scale.

4.3.2 Poor quality deionised water grown cells (Fig. 3, distr. 7)

The first point to observe is that these cells were not as easily lysed as were deionised water grown cells (e.g. 100 vs 800 for the general method using lysozyme and sarkosyl). Levels of lysis were however slightly higher than those

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found with tap water grown cells (e.g. 560 vs 400 using the general method with AL and SDS).

Overall, the lysing pattern observed was closer to that found with tap water grown cells than to that of deionised water grown cells, with some exceptions e.g. the slightly higher levels of lysis.

4.3.3 Studies on a strain of *M. aeruginosa* isolated from Vaal Dam, grown in deionised water and freeze-dried (Fig. 3, distr. 8)

Distribution 8 has results for cells grown in tap water (fresh and freeze-dried) and deionised water for comparison.

It can readily be seen that the Vaal Dam cells resembled deionised water grown cells far more than they did tap water grown cells when it came to ease of lysis. There was however the difference that L1 appeared to be better than lysozyme (800 vs 600), which was also found with tap water grown cells (see 5.5).

On the whole the refractility of freeze-dried cells found in studies using tap water grown cells (4.2) was not seen when freeze-dried Vaal Dam cells were used. The superiority of SDS as detergent (4.2.6 but see 4.3.1(a)) was not seen with Vaal Dam Cells and the use of sarkosyl as detergent yielded about six times more DNA than was found using SDS (600 vs 100 for lysozyme).

4.3.4 Discussion

These studies suggest an important role for culture conditions in the ease of lysis of *Microcystis*, especially when compared with the results obtained with tap water grown cells (4.2). In spite of a 250 % increase in DNA yields over that obtained earlier, total lysis was still not found.

The most significant findings were:

(a) The highest yields were obtained when a modification of the method of Graham (1978) was used and it is unlikely

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that grinding in a pestle and mortar will be sufficient to disrupt all the cells. Microscopic examination revealed that about 75 % of the cells were still intact.

(b) Sarkosyl was the most efficient detergent (Fig. 3, distr. 1 and 3), contrary to earlier results (see 4.2.6) and also to the results of Filip *et al.* (1979).

(c) Lysis by sarkosyl and SDS in the absence of enzymatic treatment (Fig 3, distr. 3) was also contrary to previous results with SDS (Fig. 1, distr. 14). A possible explanation is that the integrity and/or composition of the cell envelope is affected by growth in deionised water, making it more permeable.

(d) In contrast with earlier results (see 4.2.5) lysozyme was the most efficient enzyme (Fig. 3, distr. 1) but yields were not as high as those obtained using sarkosyl only.

(e) a slight rise in DNA yield was found when dodecylamine was used (Fig. 3, distr. 5), in contrast to earlier findings (Fig. 1, distr. 14) and again emphasised the influence of growth conditions on ease of lysis.

With respect to models of cell wall structure (see 2.3, 4.2.10), a number of comments can be made:

(a) The greater efficiency of sarkosyl as compared with lysozyme followed by sarkosyl (Fig. 3, distr. 1 and 3) again implies that the layer inhibiting DNA release is not the murein. It is possible that the lysozyme blocks passages in the murein, stopping free access of sarkosyl to its target.

(b) The results of Filip *et al.* (1973) on the action of sarkosyl, i.e. that it solubilises the cytoplasmic membrane only, could explain the present results if it is assumed that the murein has holes in it which are sufficiently large to allow sarkosyl free access to the cytoplasmic membrane. The very much lower efficiency of SDS could possibly be explained in terms of its

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greater denaturating capability i.e. that the solubilised outer membranes form a layer around the cell which inhibit further action.

4.4 Nuclease activity in *M. aeruginosa*

Plasmid isolation in *Serratia marcescens HY* (Timmis and Winkler, 1973) and *Pseudomonas cepacia* 4G9 (Williams, Yeggy and Markovetz, 1980) have been hampered by high levels of nuclease activity. In spite of precautions, yields of plasmid DNA remained low and in some cases no plasmid DNA at all could be found in plasmid-bearing strains (Williams, Yeggy and Markovetz, 1980). Such cases necessitate the isolation of nuclease deficient mutants for plasmid studies (Timmis and Winkler, 1973).

A. nidulans, the only member of the order *Chroococcales* studied by Norton and Roth (1967), was found to have low levels of nuclease activity. However, the fact that in hundreds of small scale agarose gel experiments not a single convincing indication of plasmid DNA in *M. aeruginosa* was observed (4.2 and 4.3), necessitated control experiments as to the level of nuclease activity in this organism.

4.4.1 Nuclease activity in preparaions of AL

Two observations suggested nuclease activity in AL preparations. These were:

(a) the EDTA requirement for its action (4.2.4) which could possibly mean that there was nuclease activity which was inhibited by EDTA and

(b) the fact that use of large volumes of AL resulted in a drop in DNA yiels (Fig. 1, distr. 5; Fig. 3, distr. 1) which could be due to DNAase contamination of the AL. Also, AL preparations were found to degrade phage 5006 M DNA (Fig. 4(a)), with EDTA lowering the amount of degradation (Fig. 4(a)).

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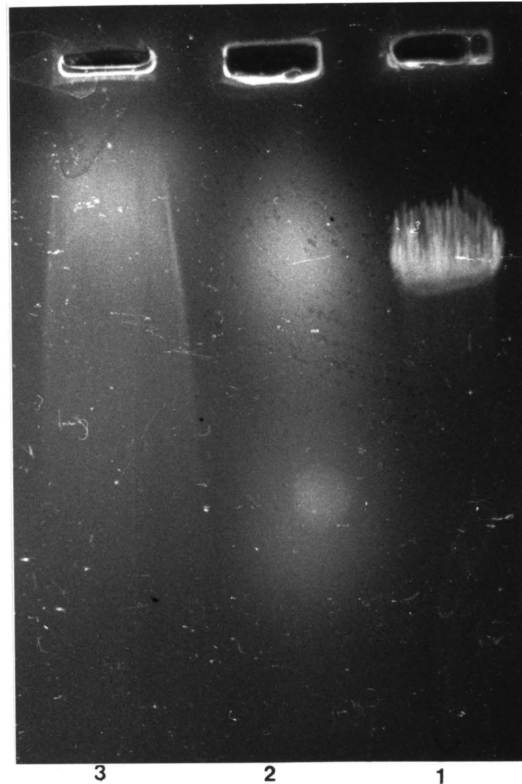

FIGURE 4a. Photograph of an agarose gel showing the effect of AL preparation on DNA from phage 5006 M. Lane 1: 5006 M DNA; Lane 2: 5006 M DNA + AL; Lane 3: 5006 M DNA + AL + 0,25 M EDTA.

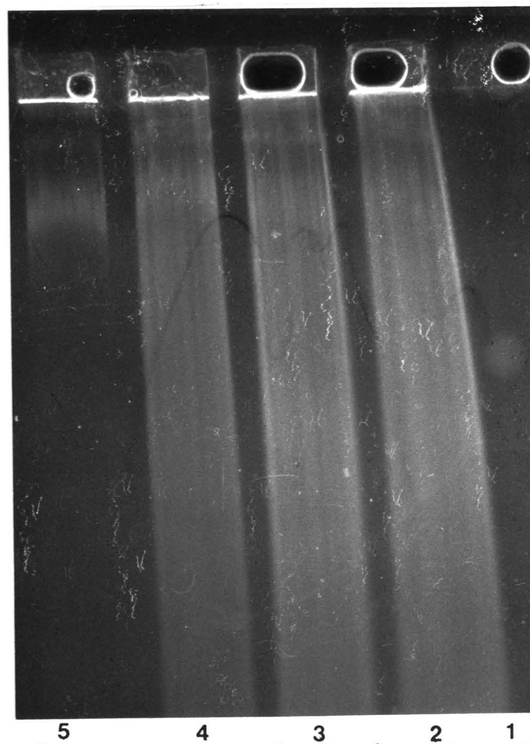

FIGURE 4b. Photograph of an agarose gel showing the effect of various treatments on the smearing substance in an AL preparation extracted using 0,5 M EDTA. Lane 1: AL after ethanol precipitation; Lane 2: Untreated AL; Lane 3: AL after heat treatment; Lane 4: AL after $(NH_4)_2SO_4$ precipitation; Lane 5: AL extracted in 0,05 M EDTA (or after ethanol precipitation) used to lyse *Microcystis*

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The use of 0,5 M EDTA in the AL extraction medium was therefore studied. This AL preparation however caused smears on agarose gels which in turn obscured discrete DNA bands in lysates (Fig. 4(b)). Smearing was not seen when AL was extracted using lower EDTA concentrations (results not shown) and was probably partly attributable to degraded DNA from cells lysed to prepare the AL. Ethanol precipitation remove the smearing DNA from the AL whilst ammonium sulphate precipitation and heat treatment had no effect (Fig. 4(b)). The lytic activity of AL was unaffected by the ethanol precipitation (results not shown). Part of the smearing was probably also due to the high salt concentration as this has been found to cause smearing (M.C. van Dijken, unpublished observation).

The above results imply that nuclease activity which can be inhibited by EDTA was present in AL preparations.

4.4.2 Nuclease activity in lysed *Microcystis*

Three observations indicated that significant levels of nuclease activity were present in *M. aeruginosa*. These were:

(a) Dithiothreitol led to lowered yields of DNA when L1 was used (Fig. 1, distr. 14 and 15). This could have been due to DTT protection of nucleases.

(b) The higher yields found in the presence of DEP (Fig. 3, distr. 3).

(c) The higher yields found with sarkosyl lysis at 65 °C (Fig. 3, distr. 3).

Use of the Brij lysis method (Clewell and Helinski, 1969) with *Microcystis* released no DNA (Fig. 5) so that experiments similar to those done by Williams, Yeggy and Markovetz (1980), aimed at revealing the presence of nucleases, could be performed with *Microcystis* lysates and added phage 5006M DNA.

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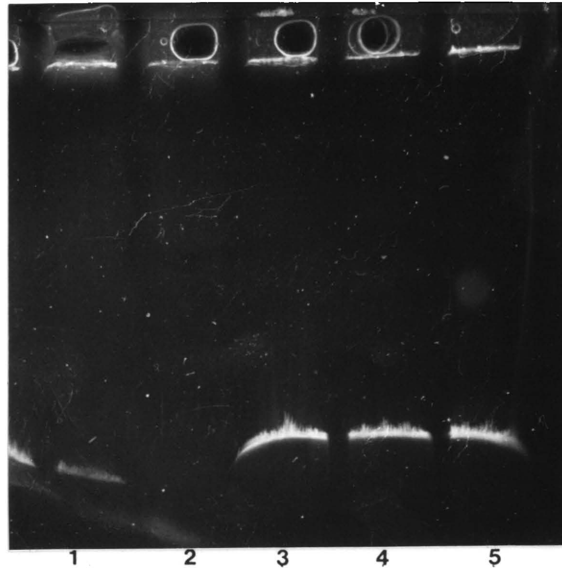

FIGURE 5. Photograph of an agarose gel showing the nuclease activity of Brij lysates of *M. aeruginosa* WR70 tested using phage 5006 M DNA. Lane 1: Brij lysate + 5006 M DNA; Lane 2: Brij lysate; Lane 3: 5006 M; Lane 4: Brij lysate + 5006 M DNA + DEP (0,2 % v/v); Lane 5: Brij lysate + 5006 M DNA + 0,25 M EDTA.

Untreated lysates degraded the added DNA with a lowering of degradation in the presence of DEP or EDTA (Fig. 5), as was found by Williams, Yeggy and Markovetz (1980).

It thus appeared that *Microcystis* may have significantly higher levels of nuclease activity than that reported for another unicellular cyanobacterium (Norton and Roth, 1967).

As for sequence specific endonucleolytic activity, no evidence was found in a crude extract, using the extraction buffer of Murray, Hughes, Brown and Bruce (1976) (results not shown). There was however such a background of randomly cut DNA that it was unlikely that discrete band would have been seen. The possibility of sequence-specific endonuclease activity in *Microcystis* can therefore not be eliminated.

4.4.3 Conclusion

Evidence for the presence of relatively high levels of nuclease activity in *Microcystis* was found. This activity could be inhibited by DEP and EDTA.

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4.5 Studies on the bacteria isolated from *Microcystis* cultures

The difficulties encountered by Gorham (1964) in his attempts to obtain axenic cultures of *Microcystis* still exist as evidenced by the number of publications dealing with this subject (e.g. Vance, 1966; Stanier *et al.*, 1971; Nuruzzaman, 1977; Pretorius, 1977, Eloff, 1980). This means that bacteria are likely to be present in any culture of *Microcystis*, as was the case in this study (see 3.1).

Although their influence on the results of the present DNA studies was expected to be small in view of the criteria established for *Microcystis* cultures (see 3.1) it was deemed important to assess this potenial influence.

4.5.1 Identity of the contaminant

Both methods of isolation of the bacterial contaminants (see 3.1) revealed the presence of only one bacterial species, a Gram-negative motile rod. The bacterium did not belong to the following: *Enterobacteriaceae*, the non-fermentative *Acinetobacter* group, the non-fermentative *Pseudomonas* group, the *Alcaligines faecalis* group and was not a known human pathogen. This conclusion was based on standardised routine tests performed at the Institute of Pathology, University of Pretoria. No further attempt was made to identify the bacterium as tests revealed no sign of a plasmid (4.5.3) and it was also non-toxic (4.5.4).

4.5.2 Lytic response of the contaminant

Cultures of the contaminating bacterium were studied for their response to various lysing procedures used for *Microcystis*. In all cases high levels of lysis (95 to 100%) were found, irrespective of the method employed. One very interesting result was that if lysozyme was used (with sarkosyl) the DNA band hardly moved on the agarose gel, whilst it moved much further when other lysis methods were used (Fig. 6). This finding was useful in demonstrating the presence of excessive bacterial contamination of *Microcystis* cultures as the retarded band was only seen when

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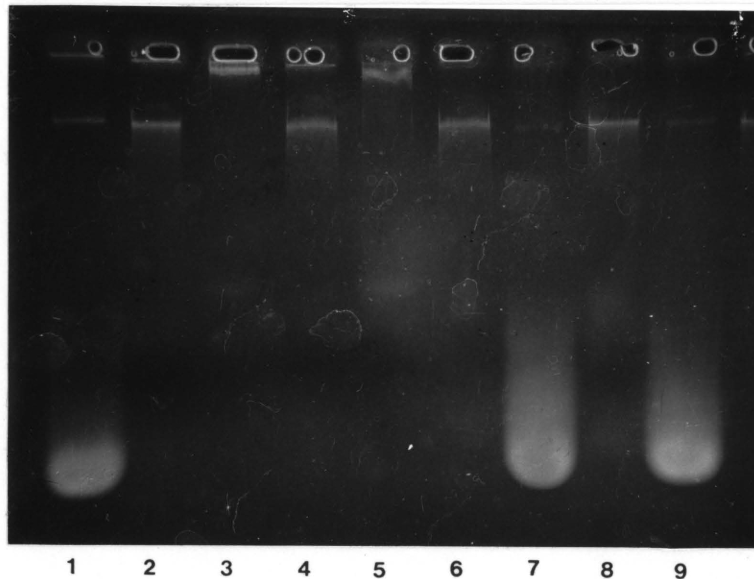

FIGURE 6. Photograph of an agarose gel showing the lytic response of the contaminating bacterium isolated from cultures of *M. aeruginosa* WR 70. Lanes 1, 2, 4, 5-9: Various methods not involving lysozyme (Lanes 1, 7 and 9 had SDS in the lysis mixture. Note the rapidly moving material which is not released by other detergents. Note also the faintness of the upper band in comparison with lanes 2-6, 8 which had sarkosyl as the lysing detergent.)
Lane 3: Lysozyme lysis of cultured contaminant, with sarkosyl as detergent. Note band and DNA which has hardly moved in the gel.
Lane 5: Bacteria in supernatant fluid of WR 70 culture, lysed using the general procedure with lysozyme and sarkosyl.

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bacterial numbers exceeded 1 % of the total count (by microscopic examination).

4.5.3 Small scale tests for the presence of plasmid DNA

Using all the methods developed for plasmid isolation and visualisation on a gel (see 2.6), a plasmid was never found. In controls using plasmid-bearing strains of *E. coli* these methods revealed plasmids in far smaller cell volumes than those used to test the contaminant.

4.5.4 Toxicity of the contaminant

The cells from a 2 d old 100 mℓ culture of the contaminant were harvested by centrifugation, disrupted by sonication and intraperitoneally injected into a mouse. Death occurred after approximately 18 h, with all the symptoms associated with the Slow Death Factor (SDF) (Bishop, Anet and Gorham, 1959), suggesting that the bacterium was not responsible for the fast deaths produced by *M. aeruginosa* WR 70.

4.5.5 Conclusions

It was established that a single bacterial species was associated with *M. aeruginosa*, that it could readily be cultivated, that it could be lysed easily and that it produced SDF.

Although the identity of the bacterium was not established, it was excluded from belonging to a number of species.

Small scale experiments suggested that it does not carry a plasmid.

4.6 Large scale isolations of DNA from *M. aeruginosa* and the contaminating bacterium and the characterisation of these DNA's

4.6.1 Results of mass lyses

Altogether 12 large scale lyses of WR 70 and two of the contaminant were performed. Using ethidium bromide-caesium

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chloride gradient analysis bands were found in nine cases with WR 70 and in both cases with the contaminant.

(a) Number of bands found in gradients

The results of gradient analyses are given in Table 2.

TABLE 2. Results of mass lyses of *M. aeruginosa* WR 70 and the contaminant, as shown by gradient analysis

Culture	Lysis procedure Method	Section where described	Number of bands in gradient runs of large scale lysates
Tap water WR 70	Lysozyme, SDS	3.4	2
"	AL, SDS	4.2.5(a)	0
"	"	"	2
"	"	"	2
Deionised water WR 70	AL, sarkosyl	4.3.1(a)	2
"	"	"	0
"	"	"	0
"	Sarkosyl only	4.3.1(b)	2
"	Graham (std.)	4.3.1(d)	2
"	"	"	3
"	Graham (modif.)	4.3.1(d)	2
"	"	"	2[a]
Contaminant	SDS only	4.5.2	1
"	"	"	1

[a] A very faint third band was seen but this disappeared on recentrifugation in an attempt to improve the separation of the bands.

The complete absence of bands in some cases in inexplicable but nuclease activity could not be excluded as no special precautions were taken to inhibit this activity until sarkosyl only was used for lysis.

It is of interest that the DNA band for the contaminant was found in CsCl gradients at a position corresponding to the lower of the two bands normally found with WR 70 lysates, i.e. this band may be of bacterial origin. No satellite bands were found with the contaminant i.e. no plasmid DNA, whilst the lowest band found in WR 70 lysates corresponded in position with that expected for ccc DNA.

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(b) Nature of bands found in gradients

Whilst it was unlikely that the bands were RNA this had to be confirmed. Incubation of gradient fractions with RNAase, followed by agarose gel electrophoresis was used to check if samples were digested by RNAase. In all cases no digestion was found whilst RNA controls were completely digested. The bands were therefore DNA.

4.6.2 Electron microscopy of isolated DNA's

(a) Nature of DNA

In all cases where one or two bands were found in a gradient (with WR 70 and the contaminant) no ccc DNA was found and the DNA appeared to be random in length i.e. probably of chromosomal origin. The difficulty experienced in getting rid of all the ethidium bromide from these fractions had given an indication that they were chromosomal DNA as this is not found with plasmid DNA (M.C. van Dijken, 1981, personal communication).

In the single instance where three bands were found (with the lowest at the position expected for a plasmid) the upper two bands were once again linear whilst the lowest band contained both supercoiled (ccc) and open circular DNA i.e. the forms found with plasmid DNA. Two sizes of circle were found (Fig. 7).

(b) Electron microscopic determination of plasmid molecular masses

The molecular masses of the plasmids were determined relative to the plasmid RP4 (36 Md - Bukhari, Shapiro and Adhya, 1977).

Forty six small plasmids and 14 large plasmids were measured. This figure reflects the relative abundance of these plasmids in the preparations examined. A single circle of 9,6 Md was seen but this might have

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been an artifact. There are two possible explanations for the amounts of the two plasmids:

(i) small plasmids have higher copy numbers than do large plasmids (Clowes, 1972);

(ii) large plasmids will be more sensitive to breakage as a result of shear forces, even in the supercoiled state. The method of lysis used was harsh so that the shear forces would have been great.

The molecular masses of the two plasmids were 2,05 Md and 5,02 Md.

4.6.3 Determination of the mol % G+C of the isolated DNA's

The results of these determinations are presented in Table 3.

Differences were noted between the mol % G+C values obtained using different methods. A possible explanation for these differences might be the presence of non-DNA material, to which the spectral ratios and thermal denaturation methods are especially sensitive (Ulitzur, 1972 and Marmur and Doty, 1962, respectively).

Additional sources of possible error with the thermal denaturation method were incorrect buffer concentrations and

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TABLE 3. Mol % G+C of DNA's isolated from *M. aeruginosa* and contaminant cultures, determined using three methods

	Method of determination and mol % G+C				
Band	Spectral ratios[a]	Buoyant density		Thermal denaturation	
	% G+C	Density (g/cm^3)	% G+C	Tm (°C)	% g+C
1	48,5	1,6994[b]	39,25	70,7[c]	41,0
2	65,5	1,7212[b]	61,89	82,9[c]	70,8
3 (plasmid)	42,3	1,6997[d]	39,57	-	-
Contaminant	-	1,7199[b]	60,54	83,1[e]	71,3

[a] *E. coli* DNA (mol % G+C = 50; Marmur and Doty, 1962) was used for the reference curves. The 245 nm:270 nm ratios were used.

[b] *E. coli* DNA with a bouyant density of 1,7100 g/cm^3 (Schildkraut, Marmur and Doty, 1962) was used as reference. Equation 15 of De Ley (1970) was used to convert densities to mol % G+C.

[c] Salmon sperm DNA with a mol % G+C of 41 (Schildkraut, Marmur and Doty 1962) was used as reference. The equation of Mandel and Marmur (1968) for 0,1 x SSC was used to calculate mol % G+C values.

[d] Band 2 DNA was used as reference. Both the plasmids had the same density.

[e] *E. coli* DNA was used as reference.

inaccurate temperature calibration. As problems were encountered when a standard DNA was included during melting, an absolute measure of Tm was not possible.

For these reasons the mol % G+C values obtained from buoyant density measurements were considered most reliable and are the values used in this report.

There was relatively good agreement between the mol % G+C found for band 2 DNA and the DNA of the contaminant and it would thus appear that they represented DNA from the same organism i.e. the bacterium. The correspondence found in the position of these DNA's in ethidium bromide-caesium chloride gradients was consistent with this proposal.

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The DNA found in band 1 must therefore have been from *M. aeruginosa*, with a mol % G+C of 39,25 (from buoyant density). The mol % G+C found for strain PCC 7005 (ATCC 27153), which is supposed to be *M. aeruginosa* (Stanier *et al.* 1971) is 45,4 (Stanier *et al.*, 1971; Herdman *et al.*, 1979a). The explanation for this difference is unknown but strain 7005 might not be *Microcystis* as it does not have gas vacuoles and was originally thus classified on the mistaken identification of refractile granules as gas vacuoles (Stainer *et al.*, 1971; Rippka, Deruelles, Waterbury, Herdman and Stanier, 1979). It should be noted that the mol % G+C found for *Microcystis* in this study would place it in the morphologically different *Synechococcus* genus of Rippka *et al.* (1979) and not the *Synechocystis* group in which strain 7005 is classified (Herdman *et al.*, 1979a).

The mol % G+C for the plasmids was very close to that of *Microcystis* (39,57 and 39,25, respectively). As no sign of a plasmid was found in lysates of the contaminant (4.5.3 and 4.6.1(a)), the plasmids were thus probably of cyanobacterial origin.

4.6.4 Discussion

The DNA of *M. aeruginosa* was isolated and analysed in ethidium bromide-caesium chloride gradients. In some cases no DNA was found, even though standard lysis methods were used. Nuclease activity could not be excluded as a possible explanation for this finding.

In all cases where DNA was found in *Microcystis* lysates a second band was found in the gradients. This DNA had a mol % G+C which was similar to that found for the DNA isolated from the contaminant (61,89 and 60,54 respectively) and was thus probably of bacterial origin.

No sign of a plasmid was found in lysates of the bacterium, confirming earlier results (4.5.3).

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The mol % G+C found for *M. aeruginosa* WR 70 (39,25) was not very close to that reported for strain PCC 7550 (45,4 - Stanier *et al.*, 1971). No reference could be found for the mol % G+C of another toxic *Microcystis* strain so that the absolute significance of this difference is unknown, although it may reflect a difference between toxic and non-toxic strains (or species).

Two plasmids were found in lysates of *M. aeruginosa* WR 70, with molecular masses of 2,05 Md and 5,02 Md respectively. The small plasmid was more abundant, as would be expected. A 2 Md has been isolated from the non-toxic strain PCC 7005 (Van Den Hondel *et al.*, 1979). It is tempting to speculate that the 5,02 Md plasmid is involved in the toxicity of strain WR 70, although proof of this will require the non-isolation of this plasmid from a cured strain of WR 70.

The small sizes of the two plasmids means that, if cyanobacteria have the same type of replication control as that found in bacteria (Clowes, 1972), they will be present at high copy numbers. Proof of this will require a more efficient and more gentle method of lysis than that used in this study.

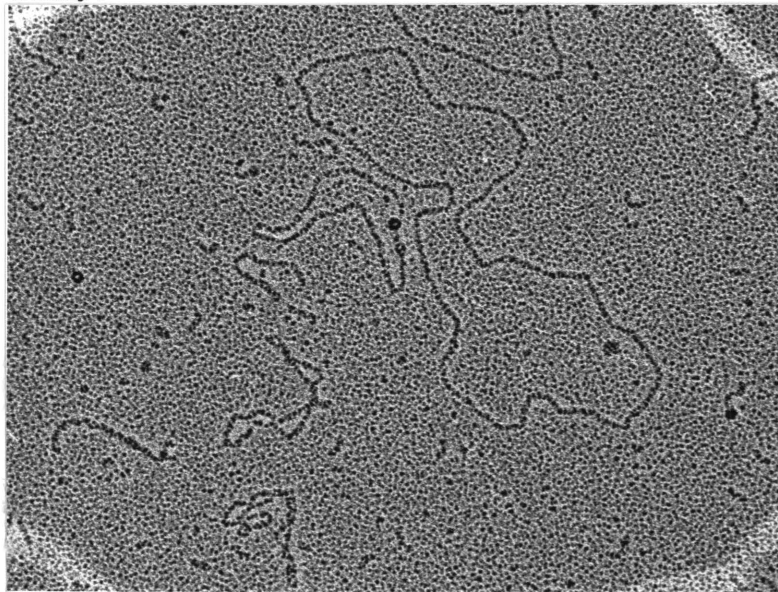

Figure 7. Electron microscope photograph of plasmids isolated from *M. aeruginosa* WR 70.

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CHAPTER 5

GENERAL DISCUSSION

In the course of a review of the literature on the toxicity of cyanobacteria (see 2.1) it was noted that there is very little knowledge on the genetics of toxin production for any of the toxic species.

Prophages have been implicated in the toxicity of *M. aeruginosa* (Vance, 1977) but no confirmation of this work could be found.

The rapid changes in the toxicity of *M. aeruginosa* in nature, both in amount produced as well as presence/absence (Gorham, 1964) led to the hypothesis that plasmid(s) rather than prophages may be involved in toxicity. As plasmids have been found in a large number of species, both unicellular and filamentous (see 2.2.2), this could be possible. All cyanobacterial plasmids thus far isolated have been cryptic (Doolittle, 1979).

Two approaches were used to study the question of plasmid invovement in the toxicity of *M. aeruginosa*. These were:

(a) A study of the effect on toxicity of agents curing plasmids or selecting for plasmid-free cells in bacteria. The results of this approach were not unequivocal (see 4.1) as in some cases toxicity was lost but not in others. A number of explanations can be put forward for these results, e.g. selection of a non-toxic strain present as a contaminant, injection of too low doses in the toxicity tests, changes in toxin type or inducibility of toxin production but no definite answer as to which of these is correct could be found. The finding of Lau, Sapienza and Doolittle (1980) that growth in the presence of 80 μg/mℓ SDS cured *A. quadriplicatum* BG-1 of only one of its three plasmids may be significant in explaining these results.

(b) Attempting to isolate and characterise plasmid(s) from a toxic strain of *M. aeruginosa* (WR 70), as well as from a non-toxic strain (either obtained by curing or isolated from nature). Results for this approach, using strain WR 70 (4.2 and 4.3) and a toxic Vaal Dam strain (see 4.3.3) form the bulk of this study.

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Problems were encountered with the gentle lysis of this strain, which were not unprecedented (e.g. Jones and Jost, 1970). A study of lysis conditions was therefore carried out in an attempt to find the optimal method.

A significant difference in the ease of lysis, as well as in conditions required for optimal lysis, was found between fresh and freeze-dried cells. This was contrary to reports (e.g. Wolk, 1973) that lyophilisation may improve the lysis of cells.

The hydration medium used with freeze-dried cells was found to be critical, as use of water or sucrose pH 7 led to no DNA release.

The enzymes givin optimal lysis were different for the two types of cells, viz. L1 for freeze-dried cells and AL for fresh cells. The action of L1 was potentiated by pretreatment of cells with phenol, especially after treatment with papain (in agreement with the results of De Petris, 1967). No potentiation of lysozyme action by phenol was found. Golecki and Drews (1974) and others have found that phenol treatment removes LIV, LIII and the lipopolysaccharide layer. If the cell wall of *Microcystis* is of the same pattern as that found for other cyanobacteria (Jost, 1965; Drews, 1973), which appears to be the case (Barlow, 1978), it is to be expected that the murein would be exposed by phenol treatment and lysozyme should therefore lyse these cells.

The combination of lipase and proteinase K was found to be as efficient as L1 with freeze-dried cells, after phenol treatment, which implied that the layer(s) inhibiting DNA release contain lipid and protein. This was in agreement with the findings of Weinbaum, Rich and Fischman (1967) for *E. coli*. SDS was found to be the most efficient detergent, in agreement with the work of Filip *et al.* (1973) and Helenius *et al.* (1979). An attempt was made to correlate the results of lysis studies with predictions according to various models of cell wall structure (see 4.2.10) but no clear picture or correlation emerged. Clarification calls for studies specifically aimed at this problem.

The results for cells grown in deionised water based media (4.3.1) were in some ways contradictory to those found with tap water grown cells

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(4.2). Deionised water grown cells were far less refractile to the different treatments used for lysis, which implies that growth conditions play a significant role in determining cell wall strength.

The most significant contradictions to the results of section 4.2 were the greater efficiency of sarkosyl as the detergent in the general lysing procedure, as compared to SDS, and the finding that sarkosyl on its own gave better lysis than did use of the enzymatic method of lysis.

The method of Graham (1978), modified by the use of the salt solutions recommended by Kirby (1957) proved to be the best method of lysis, although even in this case lysis was only 25 %. This may be due to inefficient grinding of the cells.

A general conclusion which could be drawn from the results presented in sections 4.2 and 4.3, especially the cumulative lysis experiment results, plus the finding that plasmid DNA was present in a lysate prepared by the Graham method (section 4.6), was that the low yields of DNA were partly due to the refractility of the entire population, with leakage of a little DNA from each cell, rather than the presence of a small subpopulation of cells which were totally lysed with the rest being unaffected by the treatments used.

The effect of growth conditions on the ease of lysis was stressed when a breakdown in the deionising system led to water which was almost tap water with respect to ionic quality. Cells grown in this water were more refractile than those grown in deionised water and had features of both tap and deionised water grown cells (see 4.3.2).

A strain of *M. aeruginosa* isolated from Vaal Dam was also studied for lysis patterns (see 4.3.3). A significant finding was that DNA yields were higher than those found using freeze-dried cells of WR 70 which had been grown in tap water.

The low yields of DNA, together with the complete lack of evidence for plasmids in small scale lyses, led to the conclusion that nuclease activity in *M. aeruginosa* might not be as insignificantly low as was found in another unicellular cyanobacterium, *A. nidulans* (Norton and Roth, 1967). Relatively high nuclease levels were found (4.4) but the

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use of DEP or EDTA inhibited this activity. No evidence for sequence-specific endonucleolytic activity was found in a crude extract.

The presence of bacteria in the cultures used, due to difficulties still experienced in obtaining axenic cultures (e.g. Vance, 1966), gave rise to fears that any plasmids isolated could be of bacterial and not cyanobacterial origin. The contaminating bacteria were therefore isolated and characterised (4.5). A single species was found, which produced SDF and contained no plasmid DNA. The bacterium was not identified.

Mass lyses, using the methods described in sections 4.2 and 4.3, produced enough DNA for ethidium bromide-caesium chloride gradient analysis (4.6). No plasmid DNA was found in the contaminant, confirming the results of section 4.5, whilst plasmid DNA was present in WR 70. The DNA isolated from gradients was characterised as to mol % G+C and it was found that the plasmids had a mol % G+C (39,57) which was similar to that of strain WR 70 (39,25). The contaminant had a mol % G+C which was similar to that found for a second band in gradients (60,54 and 61,89 respectively). The second band was probably DNA from the bacterium.

Electron microscopic examination revealed the presence of two plasmids, whose molecular masses were calculated relative to that of plasmid RP4. The molecular masses were 2,05 Md and 5,02 Md . In the non-toxic strain, PCC 7005, a plasmid of 2 Md was found (Van Den Hondel *et al.*, 1979). It is tempting to speculate that the 5,02 Md plasmid is involved in the toxicity of strain WR 70. No plasmids were however found in the toxic strain NRC-1 (Lau, Sapienza and Doolittle, 1980) but this may not have been a *Microcystis* strain (Stanier *et al.*, 1971; Rippka *et al.*, 1979).

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CHAPTER 6

SUGGESTIONS FOR FUTURE RESEARCH

During the course of this study a number of lines for possible future research were thought of, some of which were obvious from the results obtained.

From a study of the literature available on cyanobacterial toxicity in general, and *Microcystis* toxicity in particular, it was obvious that there are a number of areas requiring urgent attention before further attempts can be made to study the genetics of toxin production in any species, especially *Microcystis*.

The first of these is a method for reliably plating out single cells of a cyanobacterium so that clonal studies can be undertaken. The results of Pretorius (1977) indicate that this is as yet not possible for *Microcystis*. Reliable genetic studies on subjects such as curing, etc., require single cell isolations.

The second, probably even more important area, is the development of a reliable assay for the toxin(s) which is also highly sensitive i.e. capable of using a colony from a plate as sufficient material. This is once again especially important for curing studies as the mouse test is not sensitive enough. Determination of the exact site and mode of action of the toxin(s) may assist in the development of such an assay. The importance of this assay cannot be too strongly emphasized.

The third is the isolation and characterisation, in as much detail as possible, of a reference strain of *Microcystis*. At the moment there is no such strain which has been characterised as to, for instance, mol % G+C, a vital characteristic for classification, which is relatively easy to determine accurately. Knowledge of the mol % G+C of a reference strain will allow workers to determine if they are definitely working with the same species. Stanier's group (Stanier *et al.*, 1971; Rippka *et al.*, 1979; Herdman *et al.*, 1979a; Herdman *et al.*, 1979b) have extensively characterised what may be a strain of *Microcystis* (PCC 7005). However PCC 7005 does not have gas vacuoles, is non-toxic and may not be *Microcystis* at all. In view of the contaminating

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cyanobacterium found in the culture of NRC-1 (Stanier *et al.*, 1971) this is not impossible.

Whilst on the subject of cultures the development of methods to get axenic cultures in a simple, routine way is another area of concern. Until such time as cultures are totally axenic a number of experimental results will always be in doubt.

From the results of the curing experiments a number of suggestions can be made. Firstly that the experiments be repeated with a number of other strains and with other agents as well. Larger culture volume should be used so that if the mouse test has to be used there is sufficient material for LD_{100} determinations. The stability of curing should be checked by growth in media in the absence of the curing agents.

Mutagenesis studies should be done if a plasmid is indeed found to be the determinant of toxicity in *Microcystis*, firstly to map the position of the responsible genes on the plasmid and secondly to determine whether or not the plasmid is purely regulatory with the toxin genes on the chromosome.

The possibility of lysogenic conversion to toxin production should receive attention. Even should this not be the case, isolation of a cyanophage acting on *Microcystis* only will be of great use in classification studies. The lack of a cyanophage for *Microcystis* would seem to indicate that lysogeny is the rule in all strains of *Microcystis*. This should receive attention by using agents such as mitomycin C for induction. Vance (1977) has reported preliminary results of such a study which sound interesting and it is unfortunate that this matter has received no further attention.

The lysis studies suggest two areas for further investigation. Firstly the chemical nature of the cell wall and secondly the effect of growth conditions on lysis. The huge difference in ease of lysis found between tap water and deionised water grown cells shows just how large an effect growth conditions have on lysis. Possibly a means can be found to produce cells which are easier to lyse.

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Precise physical studies, including the most recent approaches, e.g. fine structure melting studies (Azbel, 1979) on any plasmids isolated will be useful, especially if plasmid involvement in toxicity is proved.

The nucleases, especially sequence specific endonucleases, should be quantified and characterised. Isolation of nuclease deficient mutants may be of use in plasmid isolations.

Lastly studies on cell-free synthesis of the toxin may provide some interesting results. The number of different toxins found, even in a single strain (D.P. Botes, 1980, personal communication) suggests that a non-ribosomal method of synthesis, similar to that found in *Bacillus* spp. producing antibiotics (Katz and Demain, 1977) may be operative.

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CHAPTER 7

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APPENDIX: MEDIA AND BUFFERS

MEDIA

The modified medium of Volk and Phinney (1968) used in this study had the following composition:

$MgSO_4$	16,63 mg/ℓ
K_2HPO_4	5,06 mg/ℓ
$Ca_3(PO_4)_2$	21,03 mg/ℓ
$Ca(NO_3)_2.4H_2O$	235,69 mg/ℓ
Na_2CO_3	100,0 mg/ℓ
EDTA.Fe	1,2 mg/ℓ

A5 trace element solution (Stanier *et al.*, 1971) 1 ml/ℓ

BUFFERS

TE (Guerry, Le Blanc and Falkow, 1973)

0,01 M Tris pH 8
0,001 M EDTA

SSC (Marmur, 1961)

0,15 M NaCl
0,015 M trisodium citrate pH 7

Tris-acetate (Hayward and Smith, 1972)

0,04 M Tris pH 8
0,005 M Na acetate
0,001 M EDTA

Tris-borate (Koekman *et al.*, 1979)

0,089 M Tris pH 8,3
0,089 M Boric acid
0,0025 M EDTA

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