CAROTENOID BIOSYTHESIS IN *BREVIBACTERIUM LINENS*

by

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TEXT BOUND CLOSE TO THE SPINE IN THE ORIGINAL THESIS

Abstract

Medicine's increasing interest in the role of free radical damage in human morbidity has naturally focused upon the potential benefits of antioxidants such as the carotenoids. Since some of these compounds are also useful and safe food colourings, commercial interests have come to play an increasing part in their study.

Brevibacterium linens, which has been safely used in the ripening of cheeses for hundreds of years, was investigated as a possible commercial source of lycopene. Wild-type *B. linens* produces the orange coloured carotenoid 3,3'-dihydroxyisorenieratene, although inhibition of the lycopene cyclase gene with nicotine caused cultures to accumulate lycopene. By a process of random chemical mutagenesis using 1-methyl-3-nitro-1-nitrosoguanidine, cultures of *B. linens* were obtained which accumulated lycopene in the absence of nicotine. UV irradiation and ethyl methane sulphonic acid both failed to produce mutants with altered pigment phenotypes. Further mutagenesis showed that lycopene concentration per cell could be increased beyond the concentration of 3,3'-dihydroxyisorenieratene found in the wild-type. Lycopene accumulation was also accompanied by gross cellular morphological changes, as seen in TEM images, and a reduction in culture growth rate and cell mass yield compared with the wild-type strain. A number of *B. linens* strains were tested and the most commercially promising, in terms of lycopene production and cell mass yields, were selected for the analysis of media utilisation.

A number of growth media and conditions were tested in an attempt to enhance lycopene production and efficient medium utilisation of the selected strains. One carbohydrate by-product, Bundaberg Direct Consumption Raw Sugar (BRS), chosen because of its low cost, caused an increase in lycopene accumulation at the expense of cell mass, though it was not a suitable medium on its own. Deuterium labelled growth medium indicated a highly oxidative metabolism in the lycopene accumulating mutant tested, based upon the level of lycopene deuteration. Different organic and amino acids in the presence and absence of carbohydrates did not elicit significant improvements in culture yields, nor did concentrations of the vitamins thiamine and B_{12}.

In an attempt to find the most commercially viable medium, lycopene accumulating *B. linens* was grown on a Soya flour digest (NSP). Yeast extract was also tested and found, at a particular concentration, to promote lycopene production at the expense of cell mass. The minimum concentration of protein digest relative to BRS, below which cell mass yield declined, was identified, although this failed to affect lycopene accumulation. The overall maximum lycopene accumulation value obtained was 0.83% per cell dry mass for a *B. linens* lycopene accumulating mutant grown in a 3%(w/v) NSP plus 1.2%(w/v) yeast extract medium.

Growth temperatures of 33°C or above, as compared with 30°C, were detrimental to cell mass and lycopene concentrations (as was illumination), though at 27°C, cell and lycopene yields were highest. Culture pH was found to increase steadily over time, but attempts to limit pH were detrimental to cell mass yields. Aeration was found to improve cell mass yield and not diminish lycopene concentrations.

Attempts to amplify the lycopene cyclase genes of the mutant and wild-type strains of *B. linens* by PCR were unsuccessful, based upon the lack of homology of the sequences with the wild-type lycopene cyclase gene sequences from other organisms. A plasmid from a strain of *B. linens* was partially sequenced and may be a useful vector in future work involving genetic manipulations in *B. linens*.

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Abbreviations

AMP	adenosine monophosphate
BRS	Bundaberg direct consumption raw sugar
CrtB/*crt*B	phytoene synthase enzyme/gene
CrtE/*crt*E	GGDP synthase enzyme/gene
CrtI/*crt*I	phytoene dehydrogenase enzyme/gene
CrtY/*crt*Y	lycopene cyclase enzyme/gene
CrtZ/*crt*Z	β-carotene hydroxylase enzyme/gene
DHIR	3,3'-dihydroxyisorenieratene
DMADP	dimethylallyl diphosphate
DMSO	dimethyl sulphoxide
EMS	methane sulphonic acid ethyl ester
GDP	geranyl diphosphate
GGDP	geranylgeranyl diphosphate
IDP	isopentenyl diphosphate
LB	Luria Bertani medium
MNNG	1-methyl-3-nitro-1-nitrosoguanidine
MSG	monosodium glutamate
MVA	mevalonic acid
NMR	nucleic magnetic resonance
NSP	Oxoid neutralised soya peptone
PCR	polymerase chain reaction

PPDP	prephytoene diphosphate
SDS	sodium dodecyl sulphate
TCA	tricarboxylic acid cycle
TDP	thiamine diphosphate
TEM	transmission electron microscopy
TLC	thin layer chromatography
YE	yeast extract
YGA/YGB	yeast glucose broth/agar

Single letter amino acid codes

A	alanine	F	phenylalanine
E	glutamic acid	L	leucine
S	serine	R	arginine
Q	glutamine	K	lysine
T	threonine	D	aspartic acid
G	glycine	M	methionine
W	tryptophan	N	asparagine
H	histidine	Y	tyrosine
C	cysteine	I	isoleucine
P	proline	V	valine

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To Gill, Isabelle and ...

'There is eternal simplicity to a solution once it has been discovered!'

Aleksandr I. Solzhenitsyn

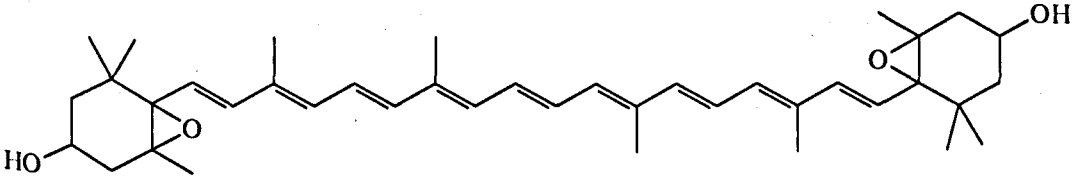
The Gulag Archipelago 1918-1956

CHAPTER 1

INTRODUCTION

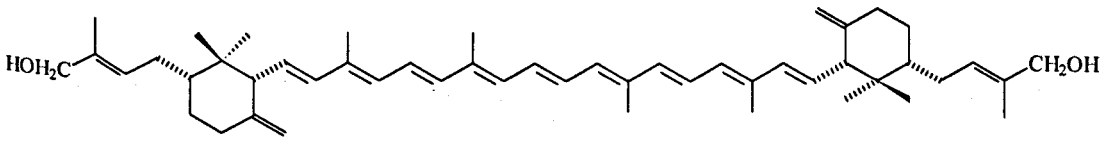
1.1 Characteristics of carotenoids

Carotenoids are natural pigments (biochromes) which absorb light in the wavelength range 380-550nm. More than 600 of these isoprenoid pigments have been identified. When carotenoids contain an oxygen function they are referred to as xanthophylls which may contain hydroxy- (*e.g.* 3,3'-dihydroxyisorenieratene (see Introduction to *Brevibacterium linens*: The use of *Brevibacterium linens* for the commercial objective of lycopene production), methoxy-, epoxy- (*e.g.* violaxanthin (see below)), oxo-, aldehyde- or carboxylic acid moieties which, in appropriate cases may be esterified or glycosylated (*e.g.* zeaxanthin diglucoside (Figure 1.6(b))).



Violaxanthin

Both xanthophylls and the hydrocarbon carotenes are noted for their range of coloration, usually red, orange or yellow (when seven or more conjugated double bonds are present) though some biosynthetic intermediates are colourless and may be fluorescent. In addition to the normal C_{40} tetraterpene compounds, examples with C_{30} (found in *Staphylococcus*), C_{45} and C_{50} skeletons also occur, for example sarcinaxanthin, found in *Micrococcus luteus.*



Sarcinaxanthin, the final pigment in the carotenoid biosynthesis pathway of *Micrococcus luteus*

Several important factors affect the characteristics of a carotenoid molecule and therefore its light absorption characteristics (and thereby its colour) its ability to act as an antioxidant and its possible physiological role. With increased chromophore length, there is increased π-electron delocalization, so that excitation is more easily achieved with lower light energies (longer wavelengths). So for example, the three conjugated double bonds present in phytoene lead to UV-vis absorption peaks at 275, 285 and 296nm compared with 440, 470 and 502nm for lycopene which contains eleven conjugated double bonds. It is possible to find carotenoids containing from three up to fifteen conjugated double bonds. Features such as cyclization and *cis*-isomerization alter the carotenoid molecule and hence the absorption spectrum.

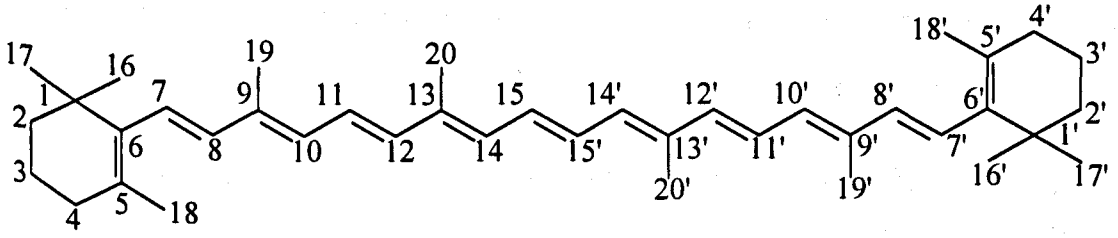
1.2 Natural distribution of carotenoids

The most significant sources of carotenoids in terms of quantity are microscopic aquatic algae such as diatoms in which carotenoids are found both inside and outside the chloroplast. In all, approximately one hundred million tons of carotenoids are synthesised in living organisms each year (Klaui, 1982). They are well known as plant pigments in chromoplasts, accounting for fruit and flower colours, but

also occur in egg yolks, skin and feathers of birds, in fish, amphibians, reptiles, insects and the reproductive organs of animals. Crustaceans such as *Homarus gammarus* contain carotenoproteins such as α-crustacyanin. In the context of cellular location, the very high hydrophobicity of carotenoids imposes limits upon the environments in which they can exist, though they can be made more water soluble when glycosylated or complexed with proteins.

1.3 Nomenclature

The numbering of the carbon atoms of carotenoid molecules reflects their symmetrical carbon skeleton, for example with β-carotene (β,β-carotene) (Figure 1.3). Commonly one end or both ends of the molecule are cyclized. A variety of Greek letter designations describe the end groups (Figure 1.3).



β-carotene (β,β-carotene)

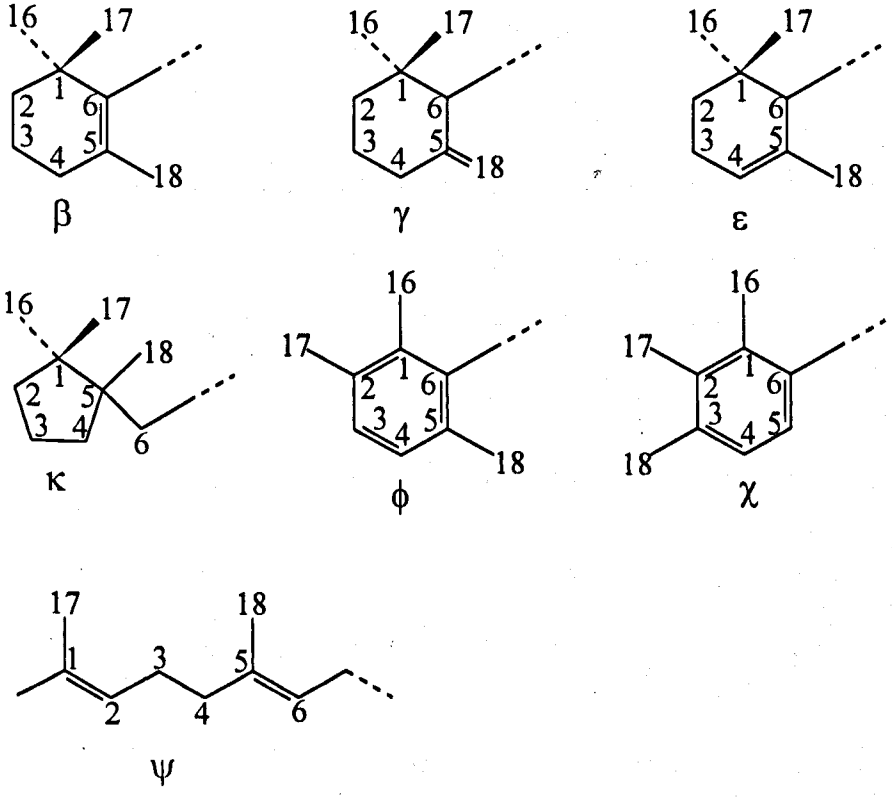


Figure 1.3 Examples of the numbering system employed for carotenoids.

1.4 Functions and actions of carotenoids

In many cases, the coloration afforded by carotenoids must surely give a significant selective advantage for the organism that makes them. Also, as protective agents against the damaging side effects of otherwise essential reactions such as photosynthesis and as accessory pigments in the efficient harvesting of light, carotenoids play very important roles. Their actions in animal systems, especially in human health, are also important.

1.4.1 Free radicals

The importance of free radicals in human disease has gained a growing interest, particularly in the West where an increasingly aged population becomes more susceptible to the morbid effects of degenerative conditions, which proportionately have become more significant as individuals survive for longer periods (Cutler, 1991).

Where an atom or molecule contains any number of unpaired electrons, the resulting species is said to be a free radical (Halliwell and Gutteridge, 1989). Free radicals take a number of forms; they are not only capable of causing damage to biomolecules, for example through lipid peroxidation, protein deactivation and DNA damage (Sies *et al* 1992; Burton, 1989) and thus promoting processes that lead to cell damage and death (Krinsky, 1979), but may also drive the formation of other radical species and so cause damage indirectly.

Within animals, most free radicals are generated through the body's own physiological processes. To a lesser extent, they result from the effects of external physical events including ionising radiations, UV irradiation, rarely by ultrasound (Riesz *et al.*, 1985), certain drugs including paracetomol (Wendel *et al.*, 1979) and probably best known of all, cigarette smoke (Church and Pryor 1985).

Oxygen is highly soluble in a hydrophobic medium (Windrem and Plachy, 1980) and carotenoid molecules are found in precisely such an environment. Ground state molecular oxygen ($^3O_2$), singlet state oxygen O_2^* ($^1O_2$) and the superoxide anion radical (O_2^-) are all variously implicated in degradative reactions involving unsaturated fatty acids, purines and aromatic amino acids (Farmilo and Wilkinson, 1973) and, in the case of the superoxide anion radical, in the formation of hydrogen peroxide.

Physiological processes may themselves be the source of free radicals. The liver may form radicals as a result of the detoxification of certain substances and, in smokers, nitric oxide (NO•) and the nitrogen dioxide radical (NO_2•) may be implicated in the indirect pathogenesis of cigarette smoke through the formation of oxidants (Pryor *et al.*, 1986). The respiratory burst which takes place during phagocytosis is of clear benefit but this too can lead to DNA lesions, protein or lipid damage. Oxygen free radical formation is also promoted *via* electron leakage, within the vicinity of the mitochondrial electron transport chain (Chance *et al.*, 1979).

Free radical damage is implicated in the pathologies of not only cancer but also systemic lupus erythematosis, rheumatoid arthritis, artherosclerosis, erythropoietic protoporphyria and essential hypertension. Singlet-state oxygen is believed to play a significant role in the pathology of erythropoietic protoporphyria (Mathews-Roth *et al.*, 1970) and improvements have been noted in patients administered supplements of dietary β-carotene (Mathews-Roth 1986).

The elimination of free radicals, known as quenching, has led to a considerable volume of research into the potential of carotenoids as antioxidant molecules, though there is often debate regarding their action. The arguments concerning the protective value of β-carotene are a case in point, where evidence has been produced which conflicts with generally accepted hypotheses (see the Carotene and Retinol Efficiency Trial, mentioned below), putting into question the entire concept of the value of certain carotenoids as protective antioxidant molecules.

1.4.2 Carotenoids in micro-organisms

The Gram positive bacterium *Micrococcus luteus* is known to be more resistant to singlet oxygen damage than its carotenoid-devoid mutants or other non-carotenoid pigmented Gram positive bacteria (Dahl *et al.*, 1989), so the presence of carotenoid pigments would seem to provide a defence against photosensitised damage. In particular, those carotenoids with nine or more conjugated double bonds quench singlet state oxygen most effectively (Mathews-Roth *et al.*, 1974). In considering the protection offered by particular carotenoid molecules or their isomers

however, it is suggested that total cellular carotenoid quantity is in fact more important than the quantities of individual carotenoids (Mathews-Roth and Krinsky, 1970).

Carotenoids are also thought to have an important role in maintaining the dynamic properties of membranes. In naturally occurring membranes for example, it has been shown that carotenoid producing strains of *Staphylococcus aureus* are able to mitigate the increase in membrane fluidity due to oleic acid compared with non-carotenoid producing strains (Chamberlain, *et al*, 1991).

1.4.3 Photoprotection

Carotenoids offer protection against near UV irradiation (wavelength range 320-400nm) but not against far UV (wavelength range 200-300nm). When carotenoids are present, the photosensitising molecules activated by NUV (near-UV) which would usually attack the membrane are seen to be rendered less detrimental (Tuveson *et al*, 1988).

The radical quenching properties of carotenoids have generated interest in their possible application in the prevention of the malignant transformation of DNA by sunlight. Inclusion of β-carotene and canthaxanthin in the diets of mice for instance, has been shown to reduce the incidence of UV-induced skin tumours (Mathews-Roth, 1982). In micro-organisms work by Konicek *et al* (1988) indicates the extraordinarily high resistance of some carotenoid-producing micro-organisms to

doses of UV light which are many times greater than those lethal to *E. coli*. Where cell membrane proteins are the target of the photosensitizer toluidine blue, the carotenoids of *Micrococcus luteus* are seen to offer protection. In contrast, no such protection is available against the DNA damage instigated by 8-methoxypsoralen which is not a photosensitizer (Mathews, 1963). *E. coli* expressing the carotenogenesis genes of *E. herbicola* were found to be resistant to near-UV irradiation (and phototoxic molecules (Tuveson *et al.*,1988)) when accumulating neurosporene, β-carotene and zeaxanthin. In contrast, lycopene and ζ-carotene, offered no such protection (Sandmann *et al.*, 1998).

1.4.4 Light harvesting in plants

Carotenoids can absorb light from wavelengths not utilised by chlorophyll. Once excited, carotenoids acting as accessory light-harvesting pigments, rapidly transfer energy to chlorophyll as their excitation level decays (Codgell and Frank, 1987). In plants, survival is made impossible in the absence of carotenoids because singlet oxygen is generated by photoexcited chlorophyll as it transfers energy to molecular oxygen in photosystem II (PSII). Carotenoids protect against the damage caused by singlet oxygen by quenching excited chlorophyll before singlet oxygen is generated or by quenching singlet oxygen itself or reacting with it in preference to other parts of the photosynthetic apparatus.

1.4.5 Carotenoids in human health

The focus of much of the current research undertaken on carotenoids is concerned with their action in humans when provided in the diet. Notable epidemiological studies such as the Carotene and Retinol Efficiency Trial (CARET) (see below), have in some cases demonstrated links between the consumption of carotenoid supplements or carotenoid rich foods and a concomitant decline in the probability associated with contracting certain disorders such as heart disease and some cancers. Certain other diseases are known to be directly associated with a deficiency of carotenoids; these include cataracts and age-related macular degeneration (Jacques *et al* 1988 and Bendich 1994). There are many other physiological processes known to involve harmful reactive species which merit the study of their interactions with carotenoid molecules; these include autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus and normal cellular events such as respiration (involving the release of electrons from the mitochondrial electron transport chain) and the oxidative burst elicited in the defensive response of immune system polymorph cells.

Carotenoids are absorbed from food are carried in the blood, largely by low density lipoproteins, though their absorption is at first dependent upon their level of bioavailability. The factors which influence bioavailability and bioconversion (where the carotenoid is to be converted into vitamin A) relate to the age and physiological state of the individual consuming the food, the manner in which the carotenoid is complexed within the food matrix, the way the food is prepared and

the type of meal in which it is consumed. Whilst extremes of heat and cooking time destroy carotenoids, food preparation has the potential to increase carotenoid bioavailability by promoting their release from plant tissue.

Carotenoids are well known for their role as vitamin A sources, affecting night vision, cell growth, reproduction, immunity and epithelial cell integrity (Basu and Dickerson, 1996). Diseases associated with a deficiency of vitamin A include night blindness, conjunctival dryness and corneal damage, also the skin and mucosa can become keratinized causing cellular water loss and changes in cell morphology. Vitamin A deficiencies are also associated with respiratory disease and diarrhoea. Indeed, in a workshop on the Bioavailability & Bioconversion of Carotenoids (Davidson, 1995), participants concluded that in populations at-risk of nutrient deficiencies (including vitamin A) in developing countries, the consumption of carotenoid-rich fruits and vegetables should be encouraged.

In epidemiological terms, associations have been noted between groups of the population and their carotenoid intake. Forman *et al* (1996) found that carotenoid intake was lower among current smokers compared with non-smokers, the less educated compared with the college educated, younger adults (age 18-39) compared with older adults, those who regularly eat meals at restaurants compared with those who eat at home, and women who take oral contraceptives compared with those that do not.

Controversy has sometimes accompanied the use of carotenes in human health. In the Carotene and Retinol Efficacy Trial carried out in the US, an apparent link was discovered which suggested that β-carotene may actually increase the risk of lung cancer among long term smokers and asbestos workers. In a second study, there appeared to be no good or bad effect on cancer or heart disease arising from β-carotene intake. However, a most important caveat which relates to the bioavailability and bioconversion of carotenoids, is that neither of the studies used natural source β-carotene or natural source carotenoid complexes. Both studies contrast with work by Ziegler (1989; 1993) in which Serum β-carotene levels were associated with reduced cancer risk.

Until very recently, whilst levels of carotenoids consumed have perhaps been considered inadequate, foods have never posed any significant proven threat to the levels which are already present in the body, though both smoking and drinking may deplete carotenoids and other antioxidants. With the introduction of the sucrose polyester 'Olestra', designed as a non-fattening fat substitute, data have become available which show a decline in carotenoid levels when Olestra is consumed. It has been estimated that moderate Olestra consumption could lead to 2,400 to 9,800 additional cases of prostate cancer each year in the US and that a 10 percent drop in serum carotenoids could cause 32,000 extra deaths in the United States per year (Fackelmann, 1996). It is interesting to note the expected impact of low serum carotenoid levels on the incidence of certain diseases.

Lycopene, which is located mainly in the prostate gland, liver, testes and adrenal glands, is not thought to be stored for long periods in the body since levels decrease in people on a low-lycopene diet, though they increase dramatically within one day of eating a high-lycopene content meal. In a number of studies beneficial links have demonstrated between the consumption of foods containing lycopene and certain cancers and heart disorders. In a study of the dietary habits and health of 47,894 men, Giovannucci (1995) found that those who ate 10 or more servings of tomato foods weekly appeared to be 45 percent less likely to develop prostate cancer. A 45-55% reduction in the likelihood of contracting stomach cancer has also been associated with the consumption of seven or more tomato servings per week. In the murine model, a 60% reduction in lung cancer appeared to be the result of dietary lycopene. In other forms of cancer, lycopene was found to be a more potent inhibitor of endometrial, lung and mammary malignant cell growth than either α- or β-carotene *in vitro*, inhibiting cancer cell growth in a dose- dependent manner when present in micromolar concentrations. Sharoni and Levy (1994) also found evidence to suggest that lycopene intervenes in the signal transduction mechanism in endometrial cancer cell growth. As well as more effectively suppressing mammary tumour formation when administered to rats (in comparison with α- or β-carotene), lycopene was also seen to inhibit the growth of human skin fibroblasts *in vitro.*

1.5 Commercial exploitation

Quite apart from their serious therapeutic applications, carotenoids are also used as colouring agents in the food industry on account of their low or absent toxicity and high colour. In aquaculture, canthaxanthin, the main pigment of *Rhodococcus ruber,* is combined with astaxanthin in trout and salmon feeds to achieve the aesthetically pleasing flesh colour which is absent in farmed salmonids. Palm oil is used as a carotene source to obtain yellow to orange food shades and capsanthin and capsorubin both give orange pigmentation from paprika. β-Carotene is used both to colour and fortify a variety of fruit drinks and margarines.

1.6 Biosynthesis

In the classically recognised isoprenoid pathway for the origin of carotenoid pigments isopentenyl diphoshate (IDP) is derived from mevalonic acid (MVA). After isomerisation of IDP, the resulting dimethylallyl diphosphate (DMADP) is combined with a further molecule of IDP to form geranyl diphoshate (GDP). Geranylgeranyl diphosphate (GGDP) results after the addition of two more IDP molecules. GGDP is therefore a committed C_{20} precursor of carotenoids. The first C_{40} carotene phytoene, is formed when two GGDP molecules condense to produce PPDP (prephytoene diphosphate) which is then converted into phytoene either as the 15-*cis* or the all-*trans* isomer. With 3 conjugated double bonds, phytoene has a λ_{max} value of 286nm. A series of desaturations follow after which cyclization and related reactions at the C-1,2 double bond and final modification complete the biosynthetic pathway. The isoprenoid pathway to GGDP is shown in Figure 1.6.

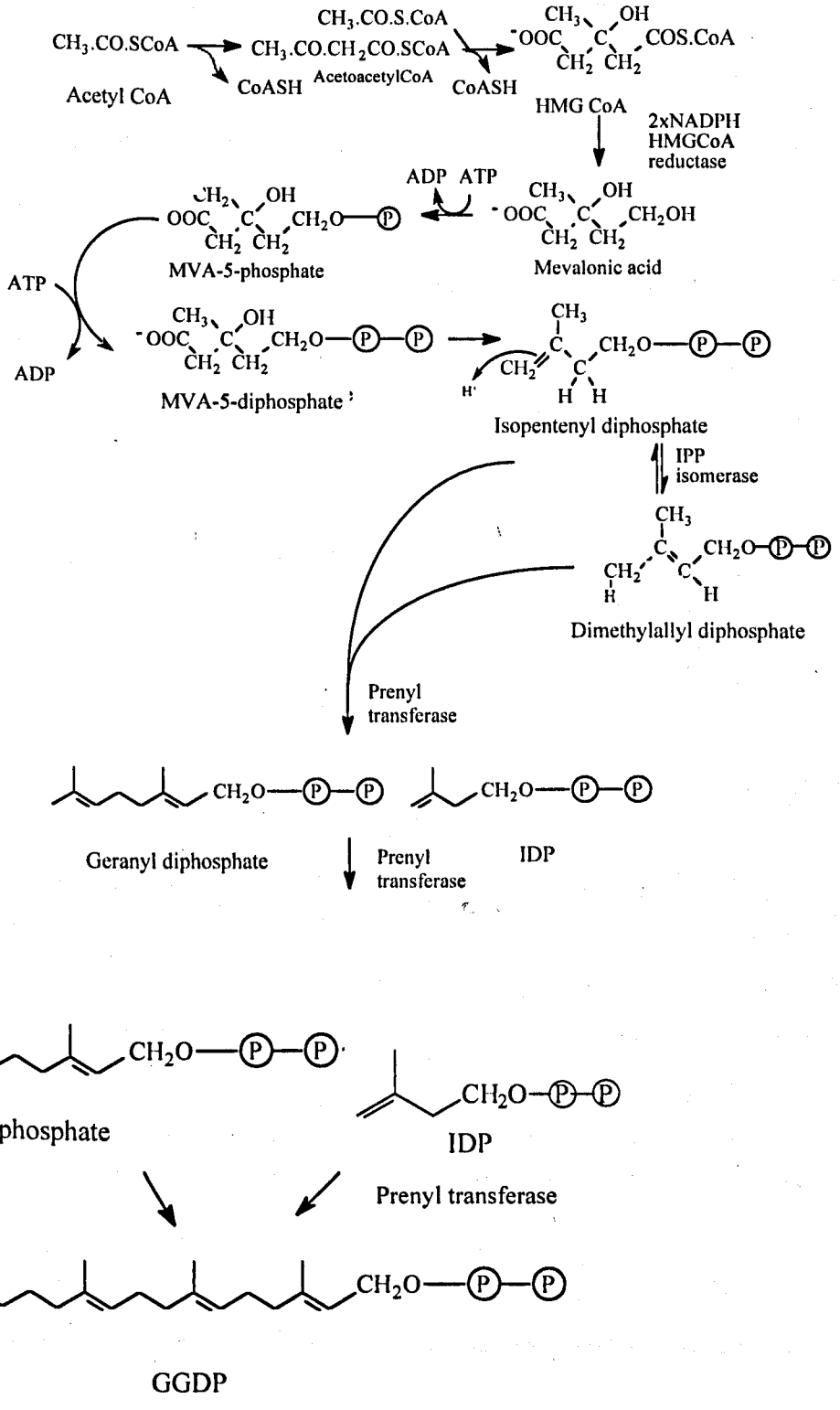


Figure 1.6 The classical isoprenoid pathway to geranylgeranyl diphosphate (GGDP), *via* mevalonic acid.

Different organisms go on to build a variety of pigment molecules *e.g.* zeaxanthin diglucoside, the main pigment found in the Gram negative, yellow non-phototrophic, phytopathogenic bacterium *Erwinia herbicola*, which is found in soil, water and in plants (Starr, 1981; Billing and Baker, 1963; Hundle, *et al.*, 1991). Following the desaturation of phytoene to lycopene, cyclization, hydroxylation and glycosidation then yield the final carotenoid (see Figure 1.6(b)).

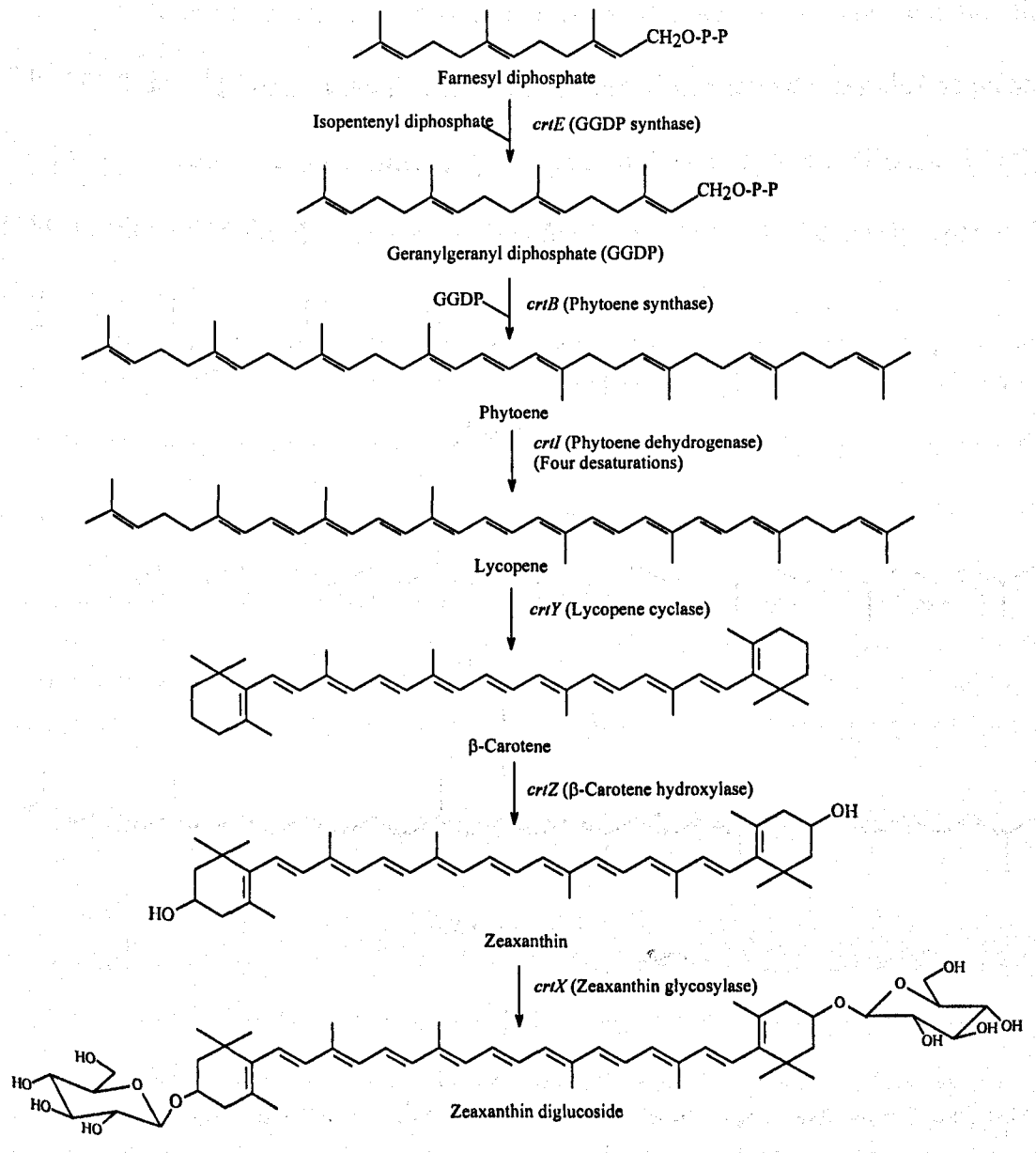


Figure 1.6(b) The carotenoid biosynthesis pathway as it occurs in *Erwinia* sp. (Hundle *et al.*, 1991). Italicised codes indicate genes, and the enzymes for which they code are named in parentheses.

Desaturation reactions introduce further double bonds and extend the conjugated double bond system to give the coloured carotenoids. By this sequence lycopene is made *via* phytofluene, ζ-carotene and neurosporene (Figure 1.6(c)). Isomerisation of 15-*cis* phytoene is required for the synthesis of all-*trans* lycopene.

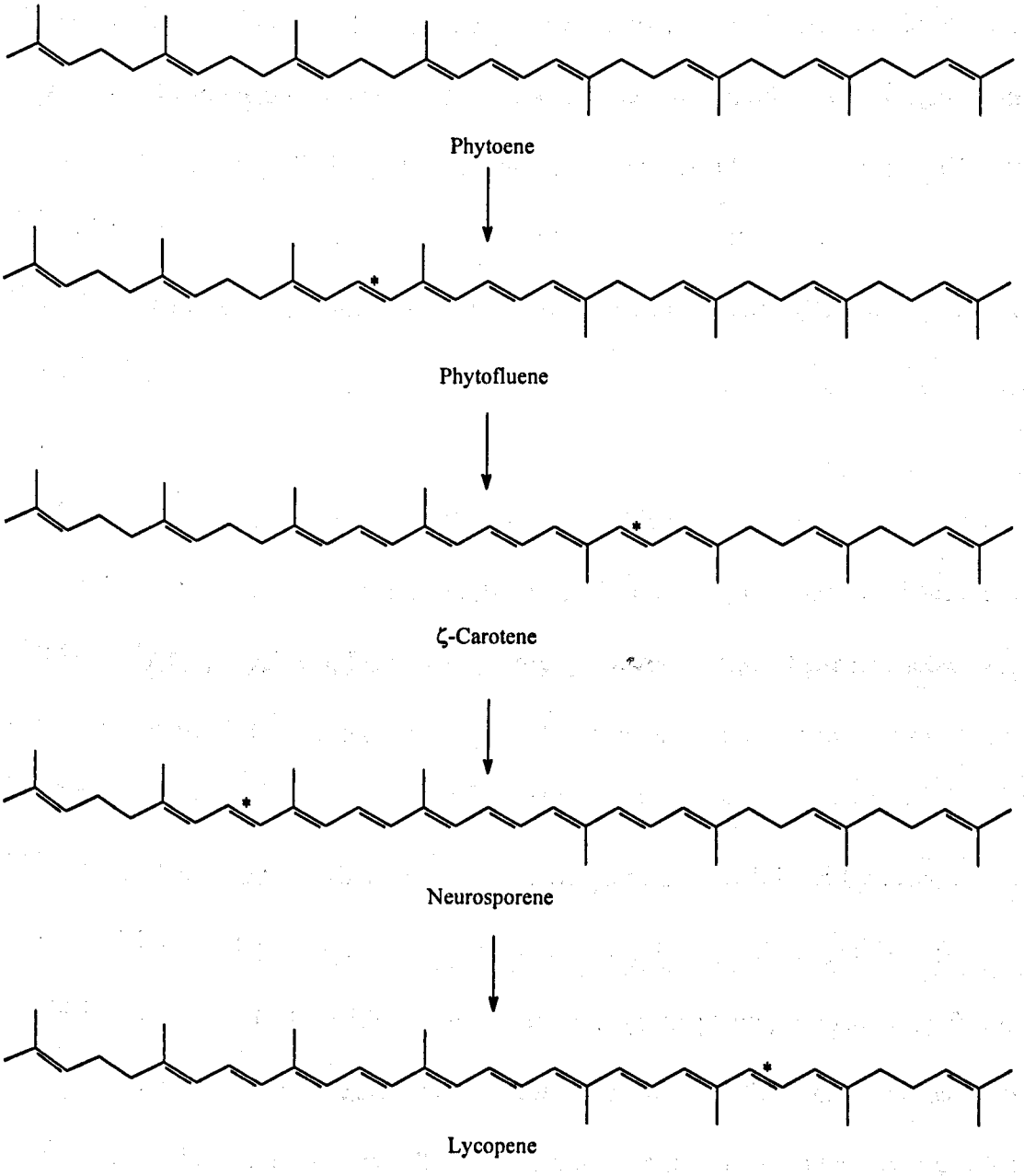


Figure 1.6(c) The sequence of desaturations from phytoene to lycopene. (* indicates the insertion of a double bond).

After two dehydrogenations forming ζ-carotene, lycopene is formed from ζ-carotene *via* a further double dehydrogenation. In plants, two enzymes are involved in catalysing the conversion of phytoene to lycopene, encoded by the genes *Pds* and *Zds*. The bacterial enzyme responsible for the conversion of phytoene to lycopene, phytoene desaturase, is encoded by the gene *crt*I. In plants, ζ-carotene desaturase catalyses the formation of lycopene from ζ-carotene *via* a double dehydrogenation with the substrate supplied by the dehydrogenation of phytoene to pytofluene, catalysed by the enzyme phytoene desaturase (PDS) which may also be responsible for the isomerisation of phytoene in the 15-*cis*- form to all-*trans* (Sandmann, 1994; Fraser, 1992).

In plants, lycopene constitutes the point at which the carotenogenic pathway diverges, to give the two ring structures β- and ε-. In bacteria, lycopene cyclisation is catalysed only by β-cyclases, leading to β-carotene and subsequent β-ring molecules. Cyclisation can result in a variety of compounds, originating either from neurosporene or lycopene. Proton attack at the C-2 and C-2' positions of the acyclic precursor carotenoid yields an intermediate carbocation which is then stabilised by proton loss from the C-1 or C-4 positions. Proton loss from the C-6 position yields a β-ring. Similar loss from the C-4 position provides the ε-ring. In plants, the two ring forms can be found on the same carotenoid molecule, *e.g.* lutein. Whilst carotenoids with either one β- and one ε-ring or two β rings are common, carotenoids with two ε rings are rare. When the β- and ε-cyclase genes of *Arabidopsis* are introduced into lycopene accumulating strains of *E.coli*, lycopene is converted into β-carotene by the β-cyclase enzyme, but only a monocyclic molecule, δ-carotene, is produced through the action

of the ε-cyclase enzyme (Cunningham *et al.*, 1996). The β-cyclases of *Erwinia uredovora* and *Capsicum annuum* are not restricted to the cyclization of lycopene, but they have been shown to be capable of cycling the 7,8-dihydro-ψ-end group i*n vitro* in place of the normal cyclization of the ψ-end group of lycopene to a β-group (Takaichi *et al* 1996).

The formation of xanthophylls by the introduction of various oxygen functions, occurs as the final stages of the biosynthetic sequence. Thus, for example, oxygenation of the C-4 position yields canthaxanthin from β-carotene (Lotan and Hirschberg, 1995). Other ketocarotenoids may be derived from β-carotene, namely echinenone, canthaxanthin, adonirubin and astaxanthin (Hirschberg, 1998). The most commonly occurring xanthophylls are those with hydroxy groups at C-3 and/or C3', *i.e.* lutein, formed by hydroxylation of α-carotene and zeaxanthin, produced by hydroxylation of the C-3 and C-3' carbons of β-carotene. The formation of zeaxanthin from β-carotene requires molecular oxygen and involves the monohydroxy β-cryptoxanthin as an intermediate (Britton, 1988).

Work by Rohmer, *et al*, (1996) has revealed an alternative route for formation of the isoprene unit as IDP. Experiments involving the use of substrates labelled with ^{13}C, showed that the acetate-mevalonic acid route is replaced by a glyceraldehyde-3-phosphate/pyruvate pathway. Thus in the green alga *Scenedesmus,* β-carotene and lutein were seen to be formed by this alternative pathway (Schwender *et al.*, 1996) which also operates in many bacteria (Figure 1.6(d)).

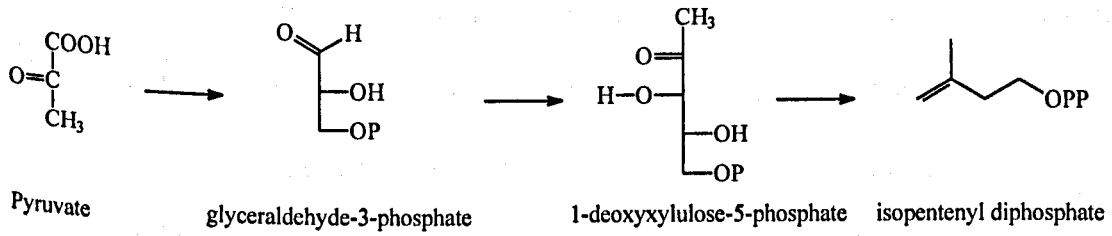


Figure 1.6(d) A scheme for the non-mevalonate pathway proposed by Rohmer *et al.*, (1996). It is proposed that an initial condensation step takes place between a pyruvate derived C_2 unit and a triose phosphate. A 1-deoxyxylulose-5-phosphate intermediate is formed by combining with the C_2 unit from the triose phosphate

1.6.1 The enzymes and their regulation

In plants and algae, the first reaction specific to carotenogenesis is the tail-to-tail dimerisation of two GGDP molecules forming prephytoene diphosphate. In the cyanobacterium *Synechoccus* PCC7942 and other organisms, the enzyme catalysing this reaction, phytoene synthase, serves to catalyse both the steps from GGDP to (15-*cis*)-phytoene (Sandmann, 1994). This reaction is dependent upon the availability of the divalent manganese cation (Dogbo *et al,* 1988). The phytoene synthase complex is known to be loosely associated with envelope membranes in *Cyanophora paradoxa* (Lütke-Brinkhaus *et al.*,1982) and although the *Synechococcus* phytoene synthase is thought to be a single polypeptide, often the entire carotenoid biosynthesis enzyme system is believed to occur as a complex, as in *Erwinia herbicola* for example.

Phytoene desaturases (CrtI in bacteria and Pds in plants) are known to demonstrate functional diversity, thus the phytoene desaturase enzyme of *R. capsulatus* for example, yields mainly *trans*-neurosporene but the CrtI enzyme of *E. uredovora* produces *trans*-lycopene and two *cis*-isomers of ζ-carotene. The

Neurospora enzyme also desaturates to lycopene (Linden *et al.,* 1991). Transformed *E. coli* have been used to isolate the *Synechoccus* phytoene desaturase enzyme, which is known, in its plants, to be associated with the thylakoid membrane (desaturating to ζ-carotene). It is also known to be sensitive to feedback regulation by carotenes (Fraser, Linden and Sandmann, 1993).

The plant β-cyclase enzyme lycopene cyclase, which shows significant homology with the ε-cyclase, has been isolated from *Capsicum* chromoplasts (Camara and Dogbo, 1986) and the cyanobacterial lycopene cyclase has been expressed in *E. coli* (Cunningham *et al* 1993). In higher plants and some classes of algae more than one cyclase enzyme is present, *i.e.* a β-cyclase and an ε-cyclase. The proportions of β,β- and β,ε-carotenoids in *Arabidopsis* for example, are strictly controlled by these two different enzymes (Cunningham *et al.*, 1996). At least *in vitro* circumstances, a cyclase enzyme has a requirement for NADPH (Hornero-Mendez and Britton, 1996), contrasting with the phytoene dehydrogenase enzyme (see below).

In many instances, there is conservation between equivalent enzymes in carotenogenic organisms. Amongst the carotenogenic bacteria for example, there is significant amino acid sequence homology in the predicted data, *e.g.* for the bifunctional 34KDa phytoene synthase enzyme (CrtB), which in two reactions yields phytoene from GGDP (Hirschberg, 1998; Armstrong *et al*, 1990), for the 33KDa GGDP synthase (CrtE) (which shows significant homology amongst its plant, archaeal and eubacterial equivalents (Armstrong *et al* 1993, Adiwigala *et al* 1996; Wiedemann *et al* 1993), and for the 54KDa phytoene desaturase (phytoene

dehydrogenase) CrtI, which dehydrogenates phytoene four times to yield lycopene (Schmidt *et al*, 1978; Lang *et al*, 1994; Armstrong *et al.*, 1990), in contrast with photosynthetic eukaryotes which utilize two separate enzymes for the conversion of phytoene into lycopene (Hirschberg, 1998; Pecker *et al*; 1992; Albrecht *et al* 1995; Linden, *et al* 1994). The mechanism of phytoene dehydrogenase is thought to involve nucleotide coenzymes. The hydrophobic N-terminal region of the enzyme has an FAD or NAD(P) binding domain (Armstrong *et al.*, 1993; Armstrong *et al*, 1989; Gari *et al*, 1992; Lang *et al* 1995).

Traditionally, carotenoid biosynthesis enzymes are very difficult to isolate and purify. The *crtI* gene from *E. uredovora* was cloned and overexpressed in *E. coli* and was made active after the removal of urea (Fraser *et al.*, 1992); an FAD requirement was observed and NAD(P) was found to be inhibitory. The CrtI enzyme is also reported to require ATP (Fraser *et al*, 1992; Lang *et al*, 1994). The enzymes CrtE (Weidemann *et al.*, 1993) and lycopene cyclase (CrtY) (Schnurr *et al.*, 1996) also contain a dinucleotide binding motif in common with the phytoene desaturases and ζ-carotene desaturases (Hirschberg, 1998).

The xanthophyll violaxanthin has as its precursor zeaxanthin, which is epoxidised at the 5,6 and 5',6' positions in a reaction catalysed by the enzyme zeaxanthin epoxidase which, in common with the CrtI enzyme, displays domains for ADP and FAD binding (Schwartz *et al* 1997). Zeaxanthin is derived from β-carotene *via* β-cryptoxanthin, catalysed by the enzyme β-carotene hydroxylase (Haycock C.,

1996). Another enzyme responsible for the conversion of zeaxanthin, is the 45KDa zeaxanthin glycosylase CrtX, which requires UDP-activated substrates.

The enzymes responsible for the formation of lycopene, β-carotene and xanthophylls are thought of as being located in an intrabilayer position (Beyer P. *et al.*,1982; Schmidt A. *et al.*, 1989) so that the nonpolar product of phytoene synthase may enter the lipid bilayer for further modification. This provides advantages in the metabolic channelling of the intermediates (Michalowski *et al.*, 1991). In agreement with this hypothesis, the activity of zeaxanthin glucosylase (CrtX) has been observed in the cytosol and in membrane fractions but, within membranes, its activity is five-fold higher. This disagrees with the work of Haycock (1996) with transformed *E. coli* who found a putative CrtX protein that was restricted to the cytoplasmic fraction. The location of CrtX may be dependent upon two hydrophobic regions which possibly serve to anchor the enzyme in the cell membrane (Kyte and Doolittle, 1982).

In contrast with CrtX, β-carotene hydroxylase (CrtZ) activity is not found in membrane fractions though, as with other enzymes from disrupted cells, it may be dependent upon the presence of membrane-based redox reactions which are disturbed by cell fractionation. When expressed in *E. coli* maxi-cells, carotenoid biosynthesis proteins from *Erwinia herbicola* were found in a variety of locations (Haycock, 1996). Protein bands corresponding to CrtI (phytoene dehydrogenase) were evident in periplasmic, inner membrane and cytoplasmic fractions, in partial agreement with Lang (1994) where overexpression of *Rhodobacter sphaeroides* CrtI in *E. coli* produced a mainly cytoplasmic enzyme association, contrasting with the membrane

bound phytoene desaturase found by Schmidt *et al* (1989). A band corresponding to the molecular weight of CrtB (phytoene synthase) appeared to associate with the outer membrane and cytoplasm (Haycock, 1996). A possible CrtE (GGDP synthase) protein coincided with outer membrane, periplasm and cytoplasm fractions. The 43KDa lycopene cyclase enzyme appeared to favour associations with the cytoplasm and inner membrane, though it has been found to be active in cell-free lysates.

1.6.2 Regulation of biosynthesis

Carotenoid biosynthesis is controlled for example, by the rate limiting IDP synthase and FDP synthase enzymes in *Micrococcus luteus* (Takatsuji *et al.*, 1983; Ruiz-Vázquez *et al.*, 1993) and *via* outside influences such as the level of illumination, as in the Gram negative non-photosynthetic *Myxococcus xanthus* (Babalobre *et al*, 1987; Martínez-Laborda *et al.*, 1990) and in *Rhodobacter capsulatus* (Armstrong, 1989) where the shift from dark (chemoheterotrophic) to light (anaerobic photosynthetic) induces the expression of at least six carotenogenesis genes. Carotenogenesis in *Mycobacterium* is also photoinduced and is probably regulated at the transcriptional level. It is thought that the *crtB* gene product may constitute a rate-limiting enzyme in the carotenoid biosynthesis pathway since when present on a multicopy plasmid in *Thermus thermophilus*, carotenoid content is seen to rise (Hoshino *et al*, 1994) (see Section 5.3). There is evidence of feedback inhibition in certain carotenoid systems, such as *Phycomyces blakesleeanus*, where neurosporene, lycopene, β-zeacarotene and γ-carotene all inhibit phytoene desaturation (Giuliano *et al* 1993; Corona *et al* 1996). Whatever the regulatory influence, carotenogenesis is

normally controlled at a pre-translational level, though a few organisms, such as the daffodil (*Narcissus pseudonarcissus*) for example, have both active (membrane bound) and inactive (soluble fraction) forms of their carotenoid biosynthesis enzymes (Schledz, 1996; Al-Babili, 1996).

In *Rhodobacter sphaeroides* where lowered oxygen levels induce photosynthesis component production, the photopigment suppression gene (*pps*) suppresses bacteriochlorophyll and carotenoid synthesis at the transcriptional level under aerobic conditions (Penfold and Pemberton, 1991).

Whilst the levels of xanthophylls in leaves are affected by light intensity, fruit and flower carotenogenesis is developmentally regulated (Gillaspy *et al* 1993), for example in the tomato fruit, where a 500-fold increase in lycopene concentration coincides with fruit ripening. Such an increase is known to be the effect of developmental control which takes place at a transcriptional level (Corona *et al* 1996). Though the development of the chloroplast is light regulated, the expression of the genes responsible for chloroplast carotenoid production is not (Hirschberg, 1998). Thus plants grown in the dark contain in place of chloroplasts, etioplasts, which lack chlorophyll but contain xanthophylls (Britton, 1988). In contrast with green plants, algae such as *Scenedesmus* and *Chlorella* both require light to activate the production of carotenoids (Sandmann, 1994).

Light induction of carotenogenesis is not restricted to plants and bacteria, it is also found in fungi such as *Phycomyces blakesleeanus* and *Neurospora crassa.* In *N.*

crassa blue light controls the expression of the genes *al-1,* which encodes a phytoene desaturase enzyme; *al-2* encoding phytoene synthase and *al-3,* which encodes the geranylgeranyl diphosphate synthase (Nelson *et al.*, 1989; Baima *et al.*, 1991; Baima *et al*, 1992; Carattoli *et al*, 1991; Schmidhauser *et al.*, 1990; Li and Schmidhauser, 1995). β-Carotene accumulation is known to be photoinduced in the fungus *Mucor rouxii* (Mosqueda-Cano and Gutierrez-Corona, 1995) and the red yeast *Phaffia rhodozyma* also gives enhanced pigmentation when exposed to constant illumination (Meyer and Du Preez, 1994).

1.6.3 The genes

The carotenoid biosynthesis genes and their arrangements have been characterised in a number of organisms. In the gram negative, purple, non-sulphur, facultative, photosynthetic *Rhodobacter capsulatus* which inhabits muddy lake bottoms and sewage lagoons, nine *crt* (carotenoid biosynthesis) genes are to be found in a 46kb photosynthesis gene cluster. Seven genes *crtA, B, C, D, F, I* and *K* within the cluster, form a subcluster of 11kb and are arranged in four operons (Armstrong, 1994; Marrs, 1981; Armstrong, 1989, 1990; Gari *et al* 1992 and Lang *et al* 1995). The ninth gene *crtJ* is separated from the others by approximately 12kb (Zsebo and Hearst, 1984). It appears that the *crtA* gene, responsible for the oxidation of spheroidene to spheroidenone, undergoes stimulated transcription in response to oxygen and thereby protects the photosynthetic system from photooxidative damage by scavenging otherwise harmful oxidation (Zhu *et al.*, 1986).

In *Erwinia herbicola,* which produces β-cryptoxanthin and zeaxanthin mono- and di-glucosides as its major carotenoids (Starr, 1981; Perry *et al.*, 1986)), the carotenoid biosynthesis genes were found within a 12.4kb chromosomal fragment and identified by functional complementation (Perry *et al*, 1986 and Misawa *et al*, 1990). When elevated growth temperatures were applied to certain cultures of *E. herbicola,* pigment devoid strains which were incapable of reversion were found to carry their carotenogenesis genes on plasmids (Chatterjee and Gibbins, 1971; Hoshino *et al.*, 1993) as with the thermophilic bacterium *Thermus thermophilus* (Tabata *et al.*, 1994) where the genes for carotenogenesis are found in a cluster on a 250 kb plasmid.

In the Gram-negative bacterium *Flavobacterium* strain R1534, which is a natural producer of zeaxanthin, a 5.1 kb segment containing the carotenoid biosynthesis genes has been sequenced. This cluster consists of five genes arranged in at least two operons, the proteins encoded by which show significant homology to the *crtE* (GGDP synthase), *crtB* (phytoene synthase), *crtY* (lycopene β-cyclase), *crtI* (phytoene desaturase) and *crtZ* (β-carotene hydroxylase) gene products of other carotenogenic organisms (Pasamontes *et al.*, 1997).

Where carotenoid biosynthesis enzyme encoding genes are arranged in clusters, interspecies similarities between clusters are evident. In *E. uredovora* for example, there are no open reading frames between *crtE* and *crtZ* (Misawa *et al.*, 1990) In *M. xanthus, M. fulvus* and *E. herbicola* in contrast, one or more open reading frames are located within the functional region (Figures 1.6 (e) and (f)) (Kleinig, 1975; Ruiz-Vazquez, 1993), the whole of which (in the case of *Myxococcus*) appears to be under the control of a light induced promoter. A proposed mechanism for the photoinduction of carotenogenesis in *Myxococcus xanthus*, is based on control by the *carR* gene product (Figure 1.6(g)) which apparently suppresses carotenogenesis in the dark (McGowan *et al.*, 1993; Hodgson, 1993), hence carotenogenesis becomes constitutive when mutations are present in the *carR* gene. *carQ* mutations in contrast, affect pigmentation so that no *crtE* product is produced, which is again irrespective of the level of illumination. The *crtI* gene responsible for the dehydrogenation of phytoene, is governed by the level of illumination as well, but is also dependent upon stationary phase or carbon deficiency (Fontes, 1993).

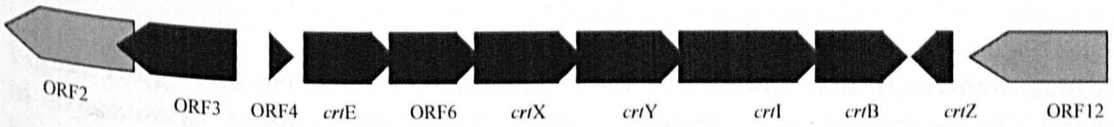


Figure 1.6(e) The carotenoid gene cluster from *Erwinia herbicola* (Armstrong, 1994)

Figure 1.6(f) Carotenoid biosynthesis gene cluster of *M. xanthus* (Haycock, 1996, adapted from Botella *et al.*, 1995 and Armstrong, 1994)

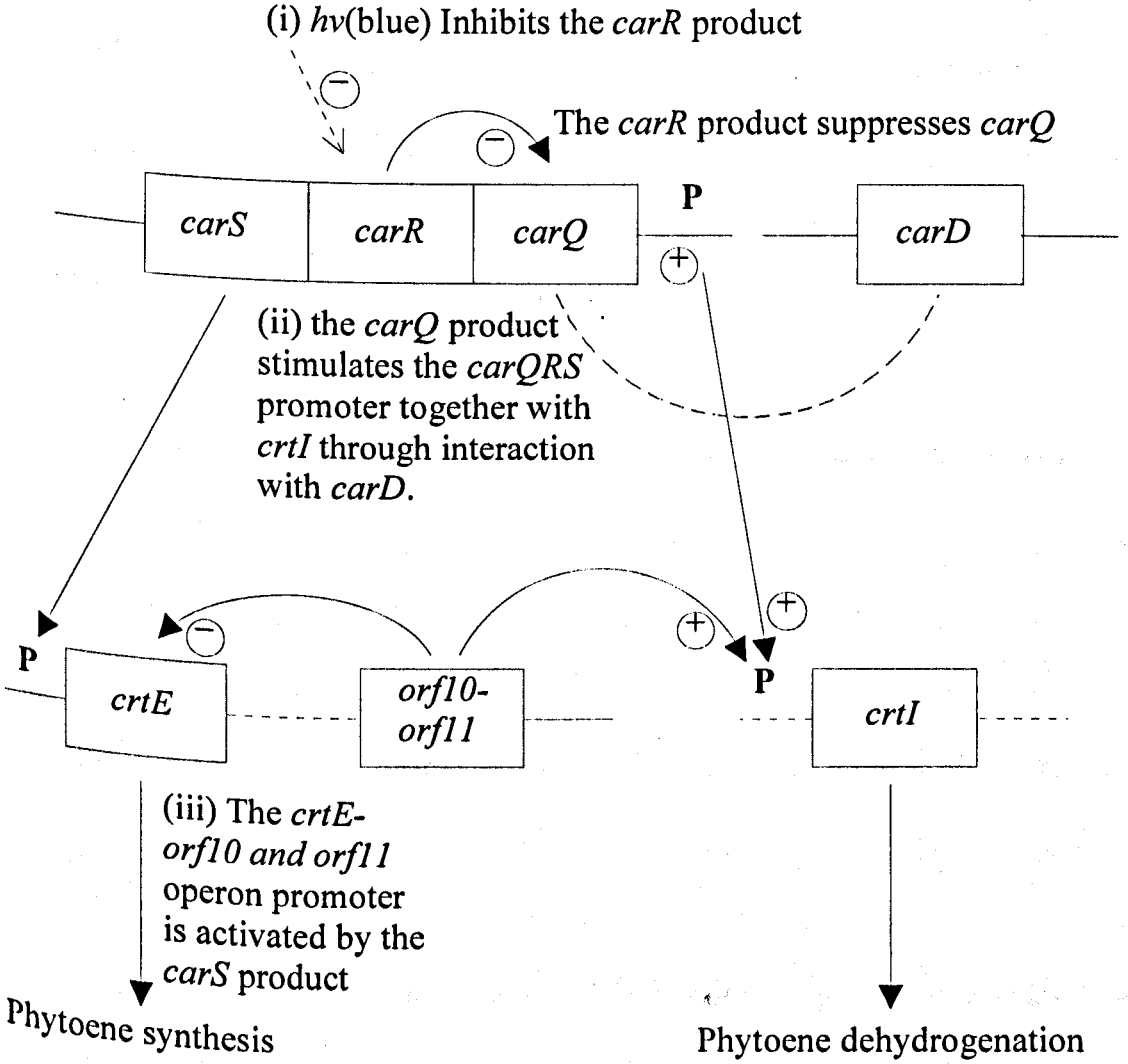


Figure 1.6(g) The proposed mechanism of the photoinduction of carotenogenesis in *Myxococcus xanthus* (Haycock, 1996, adapted from Nicolas *et al.*, 1994). Symbols of polarity indicate suppressive or enhancive effects. There is a possibility that singlet oxygen may act as an initiator *via carR*. In darkness, *carQ* appears to be restricted to a membrane location through the action of *carR*. Illumination derived *carR* suppression permits the escape of *carQ* so that it is able to promote carotenogenesis.

There may be an unexpectedly small number of carotenogenic genes despite the number of carotenoids isolated so far. In *Agrobacterium aurantiacum* for example, the enzymes CrtZ (β-carotene hydroxylase) and CrtW (β-carotene C-4 oxygenase)

have substrates with large differences in polarity, so that β-rings and 4-keto-β-rings are both utilized by CrtZ and β-rings and 3-hydroxy-β-rings by CrtW (Misawa *et al.*, 1991; Yokoyama and Miki, 1995). In *A. aurantiacum* the organisation of the carotenoid gene cluster is similar to that in *Erwinia spp.* for the genes *crtY, crtI* and *crtZ*, but the order of transcription is different.

In cyanobacteria the phytoene desaturase from *Synechocystis* (encoded by the genes *crtP* or *Pds*) has been located (Chamowitz *et al* 1991) along with lycopene cyclase (Cunningham *et al* 1993; Cunningham *et al* 1994) using functional complementation techniques based upon herbicide resistances related to the two genes. When the cyanobacterial gene for phytoene synthase was located (Chamowitz *et al* 1992), homology was detected with the fruit-ripening associated tomato gene pTOM5 which was subsequently confirmed as encoding for a phytoene synthase (Bartley, 1992). Whilst there is conservation between the phytoene desaturase genes of both plants and bacteria, there is a greater level of homology among the equivalent plant carotenogenesis genes (Hirschberg, 1998), thus it is feasible that a shared origin of evolution exists between plant carotenogenic genes which is separate to that of bacteria. Similarities of this kind are reflected amongst the lycopene cyclases of cyanobacteria and plants, whilst in their bacterial counterparts, homologies are limited to short regions specific to dinucleotide and possibly substrate binding. (Cunningham, *et al* 1996; Cunningham, *et al* 1994; Pecker, *et al* 1996). Lycopene cyclase genes for bacteria (Misawa *et al* 1990; To *et al* 1994; Hundle *et al* 1994) *Synechococcus* (Cunningham *et al* 1993 and Cunningham *et al* 1994), tomato (Pecker *et al* 1996), pepper (Hugueney *et al* 1995), daffodil (Al-Bablili *et al* 1996) and *Arabidopsis*

(Cunningham *et al* 1996 and Scolnik *et al* 1995) have all been cloned. Despite the homologies observed across a variety of organisms, it is important to note that eukaryote carotenoid genes are not clustered as they are in prokaryotes so, for example, in eukaryotes, phytoene desaturase and phytoene synthase are the only carotenoid genes belonging to the same operon.

1.7 Genetic manipulation in carotenogenic systems

In view of the potential cost benefits and the possibility of yielding novel compounds, much recent carotenoid research has been directed towards the genetic manipulation of carotenogenic organisms or of host organisms which are not naturally carotenogenic. The carotenoid biosynthesis genes of *Rhodobacter sphaeroides* have been expressed in the non-photosynthetic, phylogenetically related *Paracoccus denitrificans, Agrobacterium tumefaciens, Agrobacterium radiobacter* and *Azotomonas insolita* (Pemberton and Harding, 1987). As with the majority of microbial molecular genetics, specific enzymes able to catalyse the biosynthesis of carotenoids have frequently been expressed in *E. coli*. Even carotenoid biosynthesis enzymes from different phyla have been shown to interact productively in the same host (Hirschberg, 1998; Lotan and Hirschberg, 1995; Cunningham *et al* 1993; Chamowitz *et al* 1992; Linden *et al* 1991; Kajiwara *et al* 1995; Misawa *et al* 1994; Martinez-Ferez *et al* 1994; Raisig *et al* 1996).

By combining carotenoid biosynthesis genes in another carotenoid pigmented organism, novel compounds can be elicited, as with the expression of the *crtI* gene

from *E. herbicola* in *R. sphaeroides* for example (Hunter *et al.*, 1994). In *E. coli* the genes responsible for carotenoid biosynthesis in *Erwinia herbicola* have been placed under the control of the T7 promoter (Haycock, 1996). In the related *E. uredovora*, the carotenoid biosynthesis genes *crtB, crtE, crtI* and *crtY* expressed in the ethanol producer *Zymomonas mobilis* through conjugal transfer, yield β-carotene (Misawa, Yamano and Ikenaga, 1991), but when expressed in *Agrobacterium tumefaciens*, the final pigment yielded is zeaxanthin diglucoside (Nakagawa and Misawa, 1991).

Changes in carotenoid phenotype and improvements in carotenoid yield have been achieved very effectively by the cruder methods of chemical mutation and through media optimisation. N-methyl-N'-nitro-N-nitrosoguanidine is a commonly used and potent mutagen which has given chromatic variants in the filamentous fungus *Blakeslea trispora* (Mehta *et al*, 1995) and increasing carotenoid content in the fungus *Phycomyces blakesleeanus* (Cerdá Olmeda, 1985).

Whilst many mutations carried out on carotenogenic organisms have been achieved by treatment with chemicals or radiation, other methods including the use of transposon mutagenesis have been applied (Vertès *et al*, 1994). Interestingly, certain targeted deletions of small regions of carotenogenic genes may promote improved productivity. This is the case with geranylgeranyl diphosphate synthase where the deletion of fourteen amino acids at the C-terminal end and thirteen at the N-terminal end (replaced by four novel amino acids) resulted in increased geranylgeranyl diphosphate synthase activity (Chamowitz *et al*, 1993).

1.8 Aims

The following experimental work has been targeted towards the production of lycopene using a largely empirical, biotechnological approach. The results below seek to prove that *Brevibacterium linens* forms lycopene in its carotenoid biosynthetic pathway and that random mutagenesis can be used to maintain the production of lycopene in preference to other carotenoid biosynthesis pathway intermediates.

In a process of further random mutagenesis of lycopene accumulating mutants, the manipulation of media through the trial of growth substrate combinations and the effects of factors external to the medium composition, such as light and temperature, an attempt is made to enhance the biosynthesis and accumulation of lycopene.

Based upon the assumption that the cause of lycopene accumulation can be attributed to a mutation carried by the lycopene cyclase gene, molecular biology techniques are utilised in an attempt to arrive at the sequences of both the mutated and wild type lycopene cyclase genes. As a prelude to potential future genetic manipulations of *B. linens*, the partial sequence of an endogenous *B. linens* plasmid is revealed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacteria

The bacterial strains used in this work were as follows:

Brevibacterium linens NCIMB 8546 (National Collection of Industrial and Marine Bacteria Aberdeen, Scotland)

Brevibacterium linens CECT 75 Spanish Type Culture Collection, Spain (containing 7.75kb plasmid)

Brevibacterium linens Christian Hansen (Chr. Hansen (UK) Ltd., Reading, Berks) BL1 and BL2

Brevibacterium linens W (Wiesby) Visby Labarotorium Tønder ApS, Denmark

E. coli XL1-Blue MRF': Δ(*mcrA*) 183 Δ(*mcrCB-hsdSMR-mrr*) 173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn10 (Tet^{r})] (Stratagene, CA, USA)

2.2 Reagents

Special reagents were obtained as follows:

MNNG 1-Methyl-3-nitro-1-nitrosoguanidine (Sigma Chemical Co., St. Louis, MO, USA) (Chemical formula: $CH_3N(NO)C(=NH)NHNO_2$; RMM: 147.09)

Egg white lysozyme (Pharmacia Biotech, Herts., UK)

Grade I aluminium oxide (Woelm Pharma, Germany).

Dimethyl sulphoxide (Sigma Chemical Co., St. Louis, MO, USA)

Pure, DNAse free, 18MΩ water (Sigma Chemical Co., St. Louis, USA)

Restriction enzyme buffer (Promega Ltd., Southampton, UK)

Acetylated 10mgml^{-1} BSA (Bovine serum albumin) (Promega Ltd., Southampton, UK)

dATP, dCTP, dGTP and dTTP (Promega, Ltd., Southampton, UK)

Taq DNA Polymerase Buffer (Promega, Ltd., Southampton, UK)

Taq DNA Polymerase (Promega Ltd., Southampton, UK)

$MgCl_2$ (Promega, Ltd., Southampton, UK)

Mineral oil (Sigma Chemical Co., St. Louis, USA)

Sodium caseinate (Eastman /Fisher Scientific, UK).

2.3 Antibiotics

Tetracycline (Sigma Chemical Co., St. Louis, MO, USA) was incorporated into LB medium at a concentration of 12.5μgml^{-1}.

Ampicillin was dissolved in water to a final concentration of 35-50μgml^{-1} and then filter sterilized before adding to solid LB medium at 55°C or liquid LB medium.

2.4 Buffer compositions

The compositions of buffers as used in this work are as follows:

TE buffer (pH8.0): 10mM Tris Cl and 1mM EDTA

Lysozyme buffer (pH 8.0): 25mM Tris, 10mM EDTA, 10.3% w/v sucrose and lysozyme 1mgml^{-1}

Lysis reagent: 1% SDS plus 0.1M NaOH

3M Sodium acetate (pH 4.8)

Citrate buffer (pH4.0): 18ml 0.2M NaOAc to 82ml 0.2M HOAc

2.5 Plasmid vectors

Vectors used in this work were as follows:

pBlue script KS+ plasmid (Stratagene CA, USA) size = 2.96kb, encodes ampicillin resistance

pGEM®-T Easy (Promega, Corp., WI, USA) size = 3.02kb encodes ampicillin resistance

2.6 DNA marker ladders

Agarose gels were calibrated using the Kb DNA Ladder (Stratagene, CA, USA). Size marker length and quantity are given per 500ng total aliquot:

(bp)	quantity (ng)
12,000	50
10,000	50
9,000	50
8,000	50
7,000	50
6,000	40
5,000	42
4,000	42
3,000	43
2,000	40
1,500	10
1,000	8
750	8
500	7
250	10

2.7 Equipment

Special equipment was obtained as follows:

Transilluminator model UVP TMP-36E (UVP Ltd., Cambridge, UK).

Philips UV/VIS scanning spectrophotometer (model PU8750, Philips, England).

Thermal cycler block PHC-3 (Techne, Cambridge, UK)

Ultrawave sonicator bath

2.8 Growth media

2.8.1 YGB/A (Yeast glucose broth/agar) (AFRC, 1990)

The standard YGB growth medium contained the following:

	g dm^{-3}
Nutrient broth no. 2* (Oxoid, Unipath, Hampshire, England)	25
glucose	5
yeast extract (Gibco, Paisley, Scotland)	3
(agar (Gibco, Paisley, Scotland)	15

The medium was sterilised autoclaving at 121°C. *The composition of Nutrient Broth no. 2 is given in the Appendix.

2.8.2 Semi-Defined Medium (Tanaka *et al.*, 1971)

A semi-defined *B. linens* growth medium consisted of the following:

	g dm^{-3}
Fumaric acid	50
Ammonium dihydrogen phosphate	2.5
Malt extract	2.0
$VitaminB_{12}$	$2.0x10^{-6}$
$NaHPO_4.12H_2O$	3.0
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.01
$FeSO_4.7H_2O$	$5.0x10^{-3}$
$MnSO_{4\cdot n}H_2O$	$5.0x10^{-3}$

When added, molasses was present at a concentration of 40 gdm^{-3}. This medium was sterilised by filtration.

2.8.3 Medium for assessment of carbohydrate utilisation (Yamada and Komagata, 1972)

A medium for the assessment of carbohydrate utilisation consisted of the following:

	g dm^{-3}
Tryptone	5.0
Sugar/sugar alcohol	5.0
Yeast extract	0.1
K_2HPO_4	1.0
NaCl	5.0

The medium was adjusted to pH7.0 and sterilised by filtration.

2.8.4 Test medium for assimilation of organic or amino acid (Yamada and Komagata, 1972)

A test medium for the assimilation of organic and amino acids consisted of the following:

Organic or amino acid as sodium salt	5.0g (~0.03mol)
Glucose	0.2g
Yeast extract	0.1g
Tryptone	0.1g
K_2HPO_4	1.0g
NaCl	5.0g
Water	$1dm^3$

This medium was adjusted to pH7.0 and sterilised by filtration.

2.8.5 Luria-Bertani medium (Maniatis *et al*, 1982)

The composition of the Luria-Bertani (LB) medium is as follows:

	g dm^{-3}
Bacto-tryptone	10
Yeast Extract	5
NaCl	10

The pH was adjusted to 7.5 with NaOH and the solution was autoclaved at 121°C.

2.9 Growth conditions

2.9.1 Standard growth conditions for all *B. linens* strains

A 10% (v/v) mid log phase inoculum was used to inoculate 50ml of culture medium in baffled 250ml glass shake flasks with foam stoppers. Cultures were grown in darkness at 30°C, with orbital agitation at approximately 100 revolutions per minute. Cultures were harvested at maturity (corresponding with stationary phase plus an additional period in which maximum pigmentation (estimated by eye) was allowed to develop).

Standard medium consisted of YGB (see below), though inocula for other media were first washed free of YGB medium and then resuspended in the medium to be inoculated.

2.9.2 pH controlled fermentations

A 2.5 litre fermenter vessel containing one litre of medium agitated at a rate of 250rpm, aerated at a rate of one volume per minute was pH controlled using an LH (no longer trading) pH controller set to the appropriate maximum. pH was maintained by addition of 7M hydrochloric acid.

2.9.3 Adjustment of pH at inoculation

50ml aliquots of YGB were mixed with concentrated hydrochloric acid or concentrated sodium hydroxide prior to inoculation until the desired pH values were achieved.

2.9.4 Selection for *B. linens* strains able to grow on carbohydrate media

A single colony of *B.linens* mutant Wkiii was diluted in 250ml YGB medium. 250µl aliquots were spread onto 140mm diameter plates containing 2.5%(w/v) neutralised soya protein, 0.5%(w/v) Bundaberg raw sugar plus 0.1%(w/v) yeast extract in 1.5%(w/v) agar. After 3.5 days of incubation at 30°C, the largest colonies were selected and suspended as above and used to inoculate a medium containing

2.0%(w/v) neutralised soya protein, 1.0%(w/v) Bundaberg raw sugar plus 0.1%(w/v) yeast extract in 1.5%(w/v) agar. The process of incubation, selection, suspension and inoculation was repeated on media in which the protein concentration was progressively reduced and carbohydrate concentration progressively increased, thus neutralised soya protein concentration was progressively reduced by 0.5%(w/v) whilst Bundaberg raw sugar concentration was increased by the same amount to a final medium composition of 2.5%(w/v) Bundaberg raw sugar, 0.5%(w/v) neutralised soya protein, plus 0.1%(w/v) yeast extract in 1.5%(w/v) agar.

2.9.5 Cold shock treatment

B. linens was grown in YGB medium under standard conditions until 23.5, 53.5 or 89.0 hours after inoculation. At these times, cultures were refrigerated for two hours at 4°C in darkness.

2.9.6 Effects of illumination on pigmentation of *B. linens*

YGA plates bearing *B. linens* lawns were covered with aluminium foil and exposed at a distance of 20cm from a bank of 40W fluorescent lamps. The foil of one plate was cut to allow illumination of the colonies. Plates were incubated at 30°C for seven days.

Alternatively, the same illumination intensity was applied to liquid cultures in 50ml volumes contained in 250ml shake flasks incubated at 30°C, with agitation at a rate of 100rpm.

2.10 Use of nicotine ([-]-1-Methyl-2-[3-pyridyl]-pyrrolidine) as an inhibitor of cyclisation

Solutions of YGA were prepared and autoclaved. After the media had cooled to below 55°C nicotine was added such that a range of nicotine concentrations were available. Molten YGA containing nicotine was poured into petri dishes. When set, plates were inoculated with wild type *B. linens* under sterile conditions. Plates were incubated at 30°C for seven days.

2.11 Mutation techniques

2.11.1 Mutation using ultra violet radiation at 254nm

Mid log phase cultures of *B.linens* NCIMB 8546 were grown in YGB medium to an absorbance of approximately 2 at 600nm. Cells were diluted 10,000 times in YGB. Aliquots of 100µl were spread onto YGA plates. Plated cells were immediately exposed to ultraviolet radiation at 254nm at a range of intensities from 100 to 8000 joules m^{-2} over time periods ranging from one second to twenty four minutes. Irradiation was provided by a UV cross linker (Stratagene Cloning Systems, La Jolla, California/Stratagene Limited, Cambridge, England). After irradiation, cultures were

incubated at 30°C for 5-7 days in darkness.

2.11.2 Mutation with methane sulphonic acid ethyl ester (EMS)

Methane sulphonic acid ethyl ester (Sigma Chemical Co., Dorset) was added to 1ml of mid-log phase *B. linens* NCIMB 8546 culture (approximately at an absorbance of 2 at 600nm) at a concentration of 480μM (Bishop, 1971) or 0.14M (Levin, 1971). After 12h incubation in an orbital incubator at a rate of approximately 100rpm, at 30°C, cells were harvested and washed three times with YGB medium. Cells were diluted five-fold and resuspended in 1ml YGB, and were spread onto YGA plates and incubated at 30°C for five to seven days.

2.11.3 Mutation with 1-methyl-3-nitro-1-nitrosoguanidine (MNNG)

24μl of a filter-sterilised (Millex-GV_{13} filter (Millipore, Bedford, MA, USA)) solution of MNNG in DMSO (dimethyl sulfoxide) (410μgμl^{-1}) was added to 10ml of early log phase (A_{600nm} ≈1) cell culture. After 15 min incubation at 30°C in darkness with orbital agitation at a rate of approximately 100rpm, the MNNG-containing cultures were diluted one hundred fold in YGB medium. Diluted cultures were used to inoculate 140mm diameter YGA plates by pouring to excess and draining. This was followed by five to seven days incubation at 30°C in darkness.

2.11.4 Estimation of kill rate in cultures exposed to mutagenic treatment

Before mutagenesis, 10µl of cell culture was diluted 10,000, 100,000 and 1,000,000 fold in sterile YGB medium. Aliquots of each dilution were plated onto YGA and incubated at 30°C for seven days. Colony numbers for untreated cells were compared with those for treated cells.

2.12 Confirmation of isolated colonies as *B. linens*

Once isolated, suspected mutant strains were confirmed as *B. linens* by Gram staining and examination of microscopic morphology. The identity of all strains was also confirmed by means of the api CORYNE identification system (bioMérieux sa, Lyon, France), which consists of a pretreated microtube card containing dehydrated substrates for the demonstration of sugar fermentation or enzyme activity. Reactions were performed according to the manufacturer's protocol.

2.13 Lyophilization of bacterial strains

Freeze drying medium:

Sterile horse serum (Oxoid, Unipath, Hampshire, England) (or glycerol)	30ml
Glucose	3g
Oxoid Nutrient Broth No.2	130mg
Water	10ml

The freeze-drying medium was made by first dissolving the glucose and nutrient broth in the water. After the addition of the horse serum, the mixture was filter-sterilised through a 0.47μm filter. Aliquots (0.2ml) were introduced into sterile glass ampoules. Sterile swabs were used to inoculate aliquots very heavily to produce thick suspensions.

2.14 Qualitative carotenoid extraction

A quantity of cells grown to stationary phase on 90mm diameter petri dishes on YGA medium were transferred from the agar surface to a glass vial. A mixture of acetone/methanol (8:2 (v/v)) (approximately four volumes) was added to the collected cells and the mixture was subjected to sonication in an Ultrawave sonicator bath for fifteen minutes. Cell fragments were allowed to settle until the solvent turbidity was minimal, and the collected supernatant was filtered through a plug of glass wool in a pipette to remove finer debris. After drying under a stream of nitrogen, the residue was redissolved in approximately 1ml of diethyl ether. Spectra over the wavelength range 350-550nm were obtained by means of a Philips UV/VIS scanning spectrophotometer (model PU8750, Philips, England).

2.15 Quantitative carotenoid extraction

After the required incubation, cells were harvested by centrifugation at 22,000 x g for two minutes and twice washed with water. After removal of excess water, cells were frozen and lyophilised in preweighed tubes until no further moisture could be removed.

After the dry cell mass was determined by weighing, the cells were resuspended in one third the original cell culture volume of lysozyme buffer (25mM Tris, 10mM EDTA, 10.3% w/v sucrose, pH 8.0 (Santamaria *et al.*,1984)). Cell suspensions were then sonicated in an Ultrawave sonicator bath for a minimum of 45 minutes and egg white lysozyme (Pharmacia Biotech, Herts., UK) at a concentration of $1 mg ml^{-1}$ was added before overnight incubation at 37°C.

The lysozyme treated cells were then repeatedly extracted with a mixture of acetone/methanol (of 8:2 (v/v), approximately one third the original cell culture volume). After each addition of solvent, cells were subjected to further sonication for fifteen minutes. Extracted cells were again collected by centrifugation at 4000 x g for thirty seconds and supernatant, containing dissolved carotenoid, was added to 40ml diethyl ether in a 250ml separating funnel. Extractions with acetone/methanol were repeated, with sonication, until no further pigment could be recovered from cell debris.

The pigment extract was transferred to diethyl ether in a separating funnel and the ethereal solution washed with water or saturated sodium chloride solution. The diethyl ether, containing the total carotenoid, was filtered through anhydrous sodium sulphate. The solution was concentrated by rotary evaporation, and made up to a known volume of diethyl ether. Quantification analysis was performed by spectrophotometry, by means of the absorbance measurement at 472nm. The $A^{1\%}_{1cm}$ value for lycopene is 34,000, so an absorbance of 0.34 corresponds to a lycopene concentration of $1\mu g\mu l^{-1}$. Dried carotenoids were stored under nitrogen at -20°C.

2.16 Thin layer chromatography

TLC plates (thickness 0.75mm) were prepared from a slurry of 50g silica gel G in 100ml distilled water. Plates were dried for 5-10 minutes and then placed at 100-120°C until ready for use. Mobile phases used are given in the relevant sections of Results and Discussion.

2.17 Mass spectrometry

2.17.1 Purification of solvents

Solvents to be used for purification of samples for mass spectrometry were purified by slow filtration through 10-20g activated alumina.

2.17.2 Purifying lycopene for MS/NMR

The lycopene sample was dissolved in purified petrol and chromatographed on a small column of alumina activity grade III. The lycopene was eluted in a small volume of further petrol (containing <2%(v/v) diethylether). The major lycopene containing fraction was collected and evaporated under nitrogen. No plastics were used.

2.17.3 Mass spectrometer

A VG Quattro quadruple mass spectrometer operated in the positive ion mode was used to obtain lycopene mass spectra of purified carotenoids. The ion source temperature employed was 240°C, with emission current at 200μA and electron energy at 70eV.

2.18 Nuclear magnetic resonance spectroscopy

Spectra of lycopene in $CDCl_3$ were recorded at 400mhz in a Bruker 400 instrument at Unilever Research Limited, Colworth House, as kindly arranged by Miss L. Gambelli.

2.19 Transmission electron microscopy (TEM)

Colonies were scraped from solid medium and collected by centrifugation in 3% glutaraldehyde in 0.1M cacodylate buffer (pH7.4) fixative. Cells were incubated at room temperature overnight and then washed in 0.1M cacodylate buffer (pH7.4) for three 5 min periods. A second fixation step used 1% osmium tetroxide in 0.1M cacodylate buffer (pH7.4), with incubation at room temperature for one hour. The 0.1M cacodylate buffer washes were repeated as before. Samples were dehydrated in 70% ethanol for one hour, 90% ethanol for one hour with two changes and 100% ethanol for one hour with three changes. Dehydrated cells were embedded by incubation in propylene oxide for 30 minutes, followed by two hours in 1:1 propylene oxide:resin (Epon-Araldite), then an overnight incubation in 1:2 propylene oxide:resin. Samples were finally embedded in resin over twelve hours with two changes. The samples were placed in BEEM capsules, which were topped up with resin and polymerised at 80°C for 48 hours.

Blocks were sectioned with a Reichert Ultracut E ultramicrotome set to 120nm section thickness. Sections were picked onto 200 mesh hexagonal thin bar copper grids and stained in 2% uranyl acetate for 20 minutes followed by Reynold's citrate for five minutes. Sections were observed by use of a Philips CM10 Transmission electron microscope at 80kV. Images were recorded on Kodak 4489 film.

2.20 Molecular biology techniques

2.20.1 Extraction of genomic DNA

One ml of late log phase culture with an absorbance at 600nm of approximately 2, was collected by centrifugation at 12,000 x g for ten minutes in a microcentrifuge and separated from the supernatant. After the addition of 400µl lysis buffer (25mM Tris, 10mM EDTA, 10.3% w/v sucrose, pH 8.0) containing lysozyme at a concentration of $1mgml^{-1}$, cells were incubated at 37°C for three hours. After collecting cells by centrifugation for 30mins at 12,000 x g, the pellet was combined with 340µl of Nucleon reagent 'B' according to the kit protocol. Nucleon kits I and II (Scotlab, Scotland) protocols were employed from this point.

2.20.2 Extraction of plasmid DNA

A Qiagen Plasmid Maxiprep kit (Qiagen Ltd., Surrey) was employed according to the kit protocol after an additional lysozyme step was incorporated into the first (P1) buffer stage. Modified buffer composition was as follows: 50 mM Tris-HCl, pH8.0; 10mM EDTA; 25% (w/v) sucrose; 100 mg/ml RNAse A; 100 mg/ml lysozyme. The cell collected by centrifugation were incubated in the modified buffer P1 for 20 minutes at 37°C (Wilson, 1996).

2.20.3 Restriction digestion of plasmid DNA

DNA was cut with restriction enzymes according to the method described below. Restriction enzymes used are given in the Results and Discussion section.

0.2-1.5μg substrate DNA was dissolved in pure, DNAse free water and combined with reaction components as listed below;

Pure, DNAse free water to make a final volume of 20μl (Sigma Chemical Co., St. Louis, USA)

2μl 10X concentration restriction enzyme buffer (Promega Ltd., Southampton, UK)

0.2μl acetylated 10mgml^{-1} BSA (Promega Ltd., Southampton, UK)

1μl substrate DNA solution

After mixing by pipetting, 0.5μl of restriction enzyme at a concentration of ten units per μl was introduced. Reactions were incubated for 3-4 h at 37°C.

2.20.4 Polymerase chain reaction

The composition of PCR reactions was as follows:

Approximately 1.2pg target DNA

50pmol each primer (Perkin Elmer Limited, Cheshire)

10nmol each of dATP, dCTP, dGTP and dTTP (Promega, Ltd., Southampton, UK)

Taq DNA Polymerase Buffer diluted from 10 X concentration to 1 X final concentration (Promega, Ltd., Southampton, UK)

125nmol $MgCl_2$ (Promega, Ltd., Southampton, UK)

DNAse free pure 18MΩ water (Sigma Chemical Co., St. Louis, USA) to a final volume of 50µl

1 unit Taq DNA Polymerase (Promega Ltd., Southampton, UK)

One drop of mineral oil (Sigma Chemical Co., St. Louis, USA) was overlaid onto each reaction.

Reactions not including target DNA were established as negative controls.

2.20.5 Polymerase chain reaction thermal cycle profile

Appropriate strains of *B. linens* were collected by centrifugation and their DNA was isolated according to the Nucleon protocol. Purified genomic DNA was used as template in PCR reactions with primers. PCR reactions took place in a Techne thermal cycler, model PHC-3 (Techne, Cambridge). The temperature of the thermal cycler block was allowed to reach 94°C. This temperature was held until all tubes containing reaction components were loaded into the block. Cycle interruption was then removed allowing one minute at 94°C. This step was only applied to samples once and constituted an initial denaturation.

The cycle was as follows:

Denaturation @ 94°C for 1 minute

Primer annealing @ 55°C for 1 minute

Elongation @ 72°C for 2.5 minutes

After 35 cycles, reactions were subjected to a final elongation period of three minutes followed by a block cooling stage to bring the temperature of the block towards 5°C.

2.20.6 Agarose gel electrophoresis

Agarose gels were prepared in TAE buffer (Maniatis, *et al*, 1982) and electrophoresis conducted in the same buffer at 100V.

2.20.7 Purification of DNA from agarose gel slices containing the products of previous PCRs

Slices of LMP agarose (Sigma Chemical Co., St. Louis, USA) corresponding to the molecular weight desired were weighed. 0.04 volumes of Agarase buffer (750mM Bis-Tris plus 250mM EDTA) (Boehringer Mannheim, East Sussex, UK) at 25X concentration was added to gel slices where 1g is equivalent to 1ml). After adding buffer, gel slices were melted at 65°C for 15 minutes. After cooling to 45°C in a water bath, agarase enzyme was added. 1 unit of agarase is required to digest 100mg agarose. Mixed buffer, gel and agarase were incubated at 45°C for one hour.

2.20.8 PCR of DNA released by agarase from agarose gels containing the products of previous PCRs

Three bands were extracted with agarase, (no attempt was made to assess DNA concentration). For each band two tubes were prepared, one to hold 0.5µl extract, the other to hold 1µl. A total of six tubes were thus used plus one negative control tube containing no target DNA. PCR components were combined as shown in the standard PCR protocol (above).

2.20.9 Production of competent cells by means of a rapid $CaCl_2$ transformation procedure

20ml of LB (Luria-Bertani) medium containing tetracycline to a final concentration of 12.5μgml^{-1} was inoculated with 1ml of overnight *E. coli* XL1-Blue MRF' culture and incubated for three hours at 37°C with agitation at 150rpm in shake flasks.

The culture was chilled on ice for 20 minutes, and the cells were collected by centrifugation for 5mins at 2500 x g at 4°C. Cells were again collected by centrifugation for 10mins following resuspension in 10ml ice cold 0.1M $CaCl_2$ solution. Cells, now considered competent, were finally resuspend in 1ml of 0.1M $CaCl_2$ before chilling on ice for one hour or more.

2.20.10 Transformation of competent cells

For each transformation, 100µl competent cell aliquots were transferred to 1.5ml eppendorf tubes prechilled on ice. A quantity of transforming plasmid DNA (2-20ng) was gently mixed with the competent cells. As a control, one tube of cells contained no added DNA. Tubes were again chilled on ice for 30-40 minutes.

Competent cells were heat shocked at 42°C for 2 minutes prior to chilling on ice for 2 minutes. Finally, transformed cells were diluted in 900µl N.B. (Nutrient

broth CM1 at a concentration of 15gdm^{-3} Oxoid, Unipath, Hampshire, England) and incubated at 37°C for 1 hour at 225rpm.

100µl transformed cell aliquots were spread onto plasmid selective agar containing ampicillin, Xgal and IPTG at a concentration of 35-50µgml^{-1} and incubated at 37°C overnight. Untransformed cells were also plated as a negative control. White colonies were picked and checked for the presence of recombinant plasmids.

2.20.11 Preparation of Xgal and IPTG plates

A stock solution of Xgal was dissolved in dimethyl formamide to a concentration of 50mgml^{-1} and stored at 4°C. IPTG was dissolved in water to a concentration of 0.1M. LB plates also containing tetracycline were prepared with a final concentration of filter-sterilised 0.1mM IPTG and 40µgml^{-1} X-Gal.

2.20.12 Ligation of plasmid vector and insert DNA

Ligations were carried out using the according to protocol (Doyle *et al.*, 1996) with an appropriate vector:insert ratio. Between 50-200ng vector DNA was used plus 1u T4DNA ligase and 1µl 10X ligase buffer to a final volume of 10µl in nuclease-free water. All ligations were conducted on protruding end DNA molecules and incubations were at 16°C over night or at 22°C for three hours. If the vector to be used contained two compatible ends after restriction digestion, it was first treated with calf intestinal alkaline phosphatase (CIAP) (Doyle *et al.*, 1996). 10µl of CIAP 10X

reaction buffer was mixed with 0.01u CIAP enzyme per pmol DNA ends, plus the DNA to be phosphorylated, to a final volume of 100µl. Reactions were incubated for 15 minutes at 37°C, then another 0.01u CIAP per pmol ends was added. Incubation then continued for another 30 minutes.

2.20.13 Nucleotide sequencing

Sequences were obtained by means of an ABI (Warrington, Chester) 377 DNA sequencer and ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit. Reactions were conducted according to kit protocols with 300-500ng target DNA and 10ng primer per reaction. The thermal cycle profile employed was as follows: denaturation, 96°C for 30 seconds; primer annealing 50°C for 15 seconds and extension at 60°C for four minutes. Reactions products were precipitated with ethanol and run on a 4% gel for seven hours.

2.20.14 Nucleotide and amino acid sequence comparisons

Nucleotide and amino acid sequence comparisons were carried out by means of the GAP algorithm of the Wisconsin Genetic Computer Group's Sequence Analysis Software Package (1994). Deduced amino acid sequences were derived by use of the TRANSLATE algorithm. The BESTFIT algorithm was used to assist in the alignment of overlapping sequence. The algorithms PRETTY and CONSENSUS were used for multiple sequence alignments and to obtain consensus sequences.

CHAPTER 3

OBTAINING LYCOPENE ACCUMULATING *BREVIBACTERIUM LINENS*

3.1 Introduction to *Brevibacterium linens*: The use of *Brevibacterium linens* for the commercial objective of lycopene production

As discussed in the general introduction (Chapter 1), this work is targeted at the commercially viable production of lycopene. The lycopene to be manufactured would almost certainly enter the human food chain, possibly as a food colouring or as a pharmaceutical. With this in mind, certain limitations are imposed upon the organism which is used to produce it; in particular, there is a need to avoid organisms which are not proven to be harmless to humans. After the consideration of a number of a number of carotenogenic organisms, the bacterium *Brevibacterium linens* was chosen.

B. linens has the advantage that it is a well known food organism used in the ripening of certain cheeses (see below). At the same time *B. linens* is known to produce carotenoids and it is assumed to follow the well known pathway to C_{40} carotenoids as discussed in the introduction, with the formation of the aromatic rings of 3,3'-dihydroxyisorenieratene (see below) comprising the last steps. Hence GGDP is expected to be converted into phytoene, phytoene into lycopene, lycopene into β-carotene and β-carotene into β-isorenieratene. These assumptions are supported by the work of Haycock (1996) who obtained data to support the presence of 3,3'-dihydroxyisorenieratene, monohydroxyisorenieratene, monohydroxy-β-isorenieratene, β-carotene and *cis*-β-carotene. It is therefore assumed that mutations in the phytoene desaturase gene (*crt*I) (assuming it is present), would lead to mutants accumulating phytoene and that a similarly dysfunctional lycopene cyclase enzyme would lead to mutants with a lycopene accumulating phenotype.

By exposing cells of a carotenoid-producing organism to an inhibitor which acts upon the conversion of an intermediate in the carotenoid biosynthesis pathway, it becomes possible to elucidate some of the steps which compose the pathway. Furthermore, this information can be used to determine the value of a mutation strategy aimed at the formation of phenotypes which accumulate intermediates in the carotenoid biosynthesis pathway. Nicotine has been seen to block the cyclization of carotenoids in fungi and plants as well as in photosynthetic and nonphotosynthetic bacteria including *Brevibacterium* sp. KY4313 (Hseih *et al.,* 1974). Since lycopene is normally accumulated in the presence of nicotine, these inhibition experiments indicate that lycopene is the first carotenoid molecule to undergo cyclization. In *Flavobacterium sp.* R1519, nicotine has been shown to inhibit lycopene cyclization and therefore to block production of zeaxanthin (the wild-type final carotenoid pigment). At low nicotine concentrations however, the monocyclic compound rubixanthin was present in place of lycopene and underwent conversion into β-cryptoxanthin and zeaxanthin (McDermott *et al.,* 1974). Similarly, the phytoene desaturase inhibitor diphenylamine, first seen to block the formation of bacterial pigments (Kharasch *et al.,* 1936), promotes the accumulation of phytoene in treated cells. It is expected that diphenylamine treatment would lead to the accumulation of phytoene in the strains of *B. linens* tested here. If so, the creation of phytoene-accumulating mutants would also be of commercial potential, since the compound may have application in topical prophylaxis aimed at the prevention of the exposure of skin to UV radiation. It would appear that compounds sharing similar structures (including diphenylamine) often block carotenogenesis in certain organisms and it has been suggested that these molecules bear similarities to a region of the carotenoid

molecule which is subject to dehydrogenation and therefore may act as competitive inhibitors (Rilling, 1965).

Brevibacterium linens derives its name from its 'small rodlet' morphology and its spreading or smearing growth pattern. It is a member of the Gram-positive Coryneform bacteria, a group of mostly soil-based organisms, some of which are used industrially for the production of amino acids, in particular glutamic acid and lysine (Vidaver, 1982; Kinoshita, 1959). *B. linens*, in contrast (Mulder, 1966), was described by Wolff (1909) as a major cheese-ripening bacterium and was given its present name by Breed in 1953. *B. linens* develops after the yeast present in the cheese has exhausted all available lactate and the pH has risen above 6 (*B. linens* has been reported as unable to grow on rind below pH 5.85 (Kelly and Marquardt, 1939)). The cheeses ripened (and to some degree coloured (Albert *et al.*, 1944)) by *B. linens* (also known as red smear cheese) are Livarot, Limburger, Brick, Camambert, Munster, ComtÇ and Roquefort (Mulder *et al.*, 1966; Bernard, *et al*, 1993). As well as a possible role in the removal of unpleasant taste components of cheeses, *B. linens* is known to convert L-methionine into methanethiol (CH_3SH) (Pitcher and Noble, 1978). It is known that methanethiol is an important constituent in the aroma of cheddar cheese, thus *B. linens* may impart flavour or aroma characteristics to the surface-ripened cheeses.

Because an attempt is being made to produce lycopene from mutants of *B. linens* with a commercial objective, the efficient conversion of growth substrates is important, to provide both cell mass and pigment. *B. linens* is a chemoorganotroph

which grows under strictly aerobic conditions and its metabolism is respiratory and never fermentative (no acids are formed in peptone media in the presence of glucose) (Keddie and Jones, 1981). Optimum growth temperatures vary from 20-30°C depending upon the strain and good growth is shown on peptone-yeast extract medium at neutral pH. *B. linens*, in common with all Brevibacteria, is halotolerant or halophilic; indeed up to seven strains have been grown in 15%(w/v) sodium chloride. *B. linens* is also non-motile and displays a rod-coccus cycle on complex media so that old colonies of 3-7 days are mainly coccoid of 0.6-1.0μm diameter, though length may vary. Colonies, if young (24-48 hours) are opaque, 0.5-1mm in diameter, convex, smooth and shiny. Older colonies are larger, of 2-4mm in diameter after 4-7 days of incubation.

Though Gram-positive, some strains or old colonies readily decolourize, so that stains can be equivocal. Capsule formation is non-characteristic but slime may occur (Colwell, 1969) which will not wash off in SDS, acidic or alkaline rinses. Though no true mycelium exists among *B.linens*, primary branching may be observable. The species is catalase-positive, not acid-fast and does not produce endospores. Two strains of *B. linens* have been reported to produce bacteriocins (linecins) which inhibit *B. linens* growth (Kato *et al.*, 1984). The production of these is conferred by plasmids (Sakakibara and Tomizawa 1974). Other strains known to contain plasmids include *B. linens* CECT 75. The function of the 7.75 kb plasmid in CECT 75, known as pBL 100, is not known, although plasmids found in pathogenic coryneforms often confer virulence as well as resistance to antibiotics (Kono *et al*, 1983). Alternatively, pBL100 may have a fatty acid breakdown or bacteriocin-producing capability (Kato *et*

al 1984; Veldkamp, 1970). In this work, an attempt will be made to elucidate the sequence of this plasmid with a view to its potential as an endogenous vector in the genetic manipulation of *B. linens*.

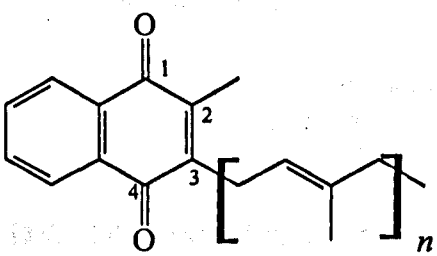
In taxonomical terms, *B. linens* is grouped closely with the genus *Arthrobacter*, based upon a variety of biochemical tests including acid production from sugars, utilisation of organic acids as sole carbon sources and DNA base composition (Bousfield, 1972). Fairly close comparison has been found with *Nocardia* which, like *Arthrobacter*, is generally isolated from soil. The similarity with *Arthrobacter* falters however, when amino acid composition and morphology are compared (Yamada and Komagata, 1972). Although *B. linens* is normally associated with cheese, it has also been found on the surface of sea fish (Mulder, 1966) though, based upon DNA homology studies, only a small proportion of the *B. linens*-like bacteria found on sea fish were actually related to *B. linens* (Keddie and Jones, 1981).

The cell wall composition of *B. linens* strains appears unique amongst the coryneforms since it contains glycerol teichoic acids in the wall polysaccharides (Keddie and Jones, 1981; Fiedler *et al.*, 1981). As with all bacterial cell walls, phosphate is present and the basic pattern of glycerol, glucose, glucosamine and/or galactosamine is followed. No strains of *B. linens* contain mycolic acids (Collins *et al.*, 1979), but some strains may contain galactose, arabinose, ribitol teichoic or mannitol teichoic acids (Fiedler *et al.*, 1981; Keddie and Jones, 1981). Peptidoglycans are of group A (*i.e.* there is direct cross-linkage between positions 3 and 4 of the

peptide subunits) and *meso*-diaminopimelic acid is present though cell-wall arabinose is absent.

The major fatty acids in *B. linens* are 12-methyltetradecanoic acid (*anteiso*-C_{15}) and methylhexadecanoic acid (*anteiso*-C_{17}) (Suzuki and Komagata, 1983). As well as *anteiso*- and *iso*-methyl-branched fatty acids being present, small amounts of unbranched saturated fatty acids are also found. The polar lipids diphosphatidylglycerol and phosphatidylglycerol (Collins *et al.*, 1980) also occur together with phosphatidylinositol (Komura *et al.*, 1975).

The only isoprenoid quinones detected in Brevibacteria are menaquinones (see below) and of which those with eight isoprene units in a dihydrogenated form (MK-8[H_2]) constitute the major components as with the genus *Rhodococcus* (Collins and Goodfellow 1979). MK-7[H_2] menaquinones are also present in *B. linens* though in small quantities relative to MK-8[H_2].



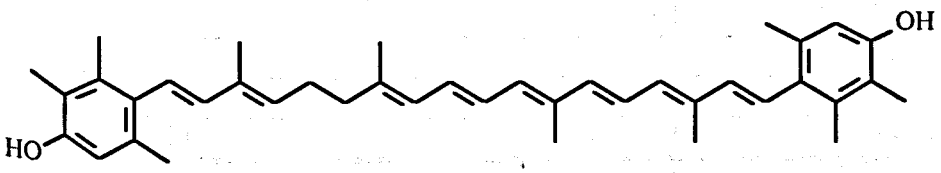
Menaquinone-n

Brevibacterium linens is known to assimilate a variety of organic acids, namely acetic, pyruvic, L-lactic, D-lactic, malic, succinic, fumaric, α-ketoglutaric, citric, formic, propionic, butyric, oxalic, malonic, glutaric, adipic, pimelic, glycolic, glyoxilic, gluconic, hippuric and uric acids (decomposed to urea) (Yamada and Komagata, 1972a; Bousfield 1972), though some strains, such as B3 for example, are unable to utilise acetate. Sugar and sugar alcohols utilised include glucose and glycerol, fructose and galactose (Bousfield, 1978) though sucrose and lactose are not utilised by all strains (Mulder *et al*, 1966). Alanine and tyrosine have also been listed as carbon sources (Bousfield, 1972), together with arginine, serine and lysine. All Brevibacteria are proteolytic so that gelatin, milk and casein are hydrolysed by most strains (Colwell *et al.*, 1969; Bousfield 1972). More recently an extracellular alkaline serine protease has been isolated from *B. linens* and found to have a pH optimum between 7.0 and 8.5 (Juhasz and Skarka (1990)).

The genome of *B. linens*, which is 3105kb in length, has been resolved into eight fragments with the restriction enzyme *DraI* and fifteen fragments with *AseI* (Correia *et al*, 1994). The DNA base GC percent values of 60-67% are fairly high in *B. linens* compared with the other coryneform bacteria which are considered heterogeneous based upon DNA-DNA hybridisation studies (Fiedler *et al.*, 1981).

Brevibacterium linens was originally thought to be the exclusive bacterial producer of the phenolic carotenoid 3,3'-dihydroxyisorenieratene (see below), until the same compound was discovered in a strain of *Rhodococcus ruber* (Haycock, 1996). The location of this pigment was analysed in 78 *B. linens* strains and it was

found to be associated with membrane fractions, and not in other cell components including the cell wall (Jones, Watkins and Erickson, 1973). The majority of strains are reported to have light-dependent pigment production which may be affected by oxygen levels but strain B4 is only orange (the colour of 3,3'-dihydroxyisorenieratene) if 4% sodium chloride is present in the medium (Mulder, 1966); this is not the case in other *B. linens* strains.



3,3'-dihydroxyisorenieratene

3.1.1 The use of nicotine, a cyclase inhibitor, to establish the presence of lycopene in the carotenoid biosynthesis pathway of *B. linens*

As discussed in the introduction, carotene ring formation is known to be inhibited by nicotine, so that the cyclase enzyme substrate lycopene would be expected to accumulate in the presence of sufficient non-lethal concentrations of nicotine.

Results of incubation of *B. linens* NCIMB 8546 for seven days at 30°C in the presence of nicotine without exposure to light, on solid YGA medium (see Materials and Methods Section 2.9.1) are shown in Table 3.1 and Plate 3.1. The approximate concentrations of nicotine used were between 0.1 and 4.0mM as shown in Table 3.1. Figure 3.1 shows the mass spectrum of lycopene extracted from cells of B. linens

NCIMB 8546 grown in the presence of nicotine (see Materials and Methods Sections 2.10, 2.14 and 2.17). Figure 3.1(b) shows the UV-vis spectrum of lycopene extracted from nicotine treated cells.

Approximate concentration of nicotine (mM)	Colony colour	Level of inhibition
0.1	Orange	Not observable
0.2	Peach	Medium
0.5	Peach	Medium
1.0	Peach	Medium
2.0	Deep Pink	High
4.0	Pink	High

Table 3.1 The effects of the cyclase inhibitor nicotine upon the pigmentation of *B. linens* strain NCIMB 8546, grown on solid YGA medium.

Plate 3.1 The effect of the cyclase inhibitor nicotine on *B. linens* strain NCIMB 8546. Cells were grown on YGA medium containing nicotine at the following concentrations; 0.1mM, 0.2mM, 1.0mM (beginning from bottom of left column); 2.0mM, 4.0mM (beginning from bottom of right column)

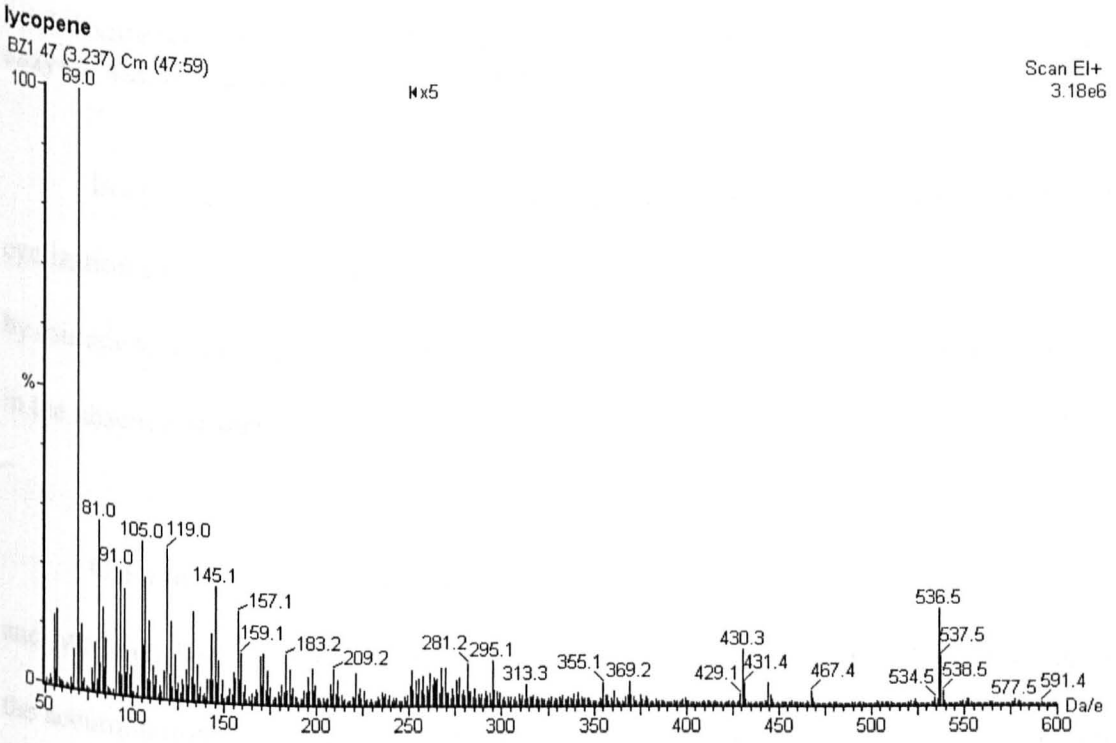


Figure 3.1 Mass spectrum of lycopene extracted from *B. linens* NCIMB8546 treated with nicotine

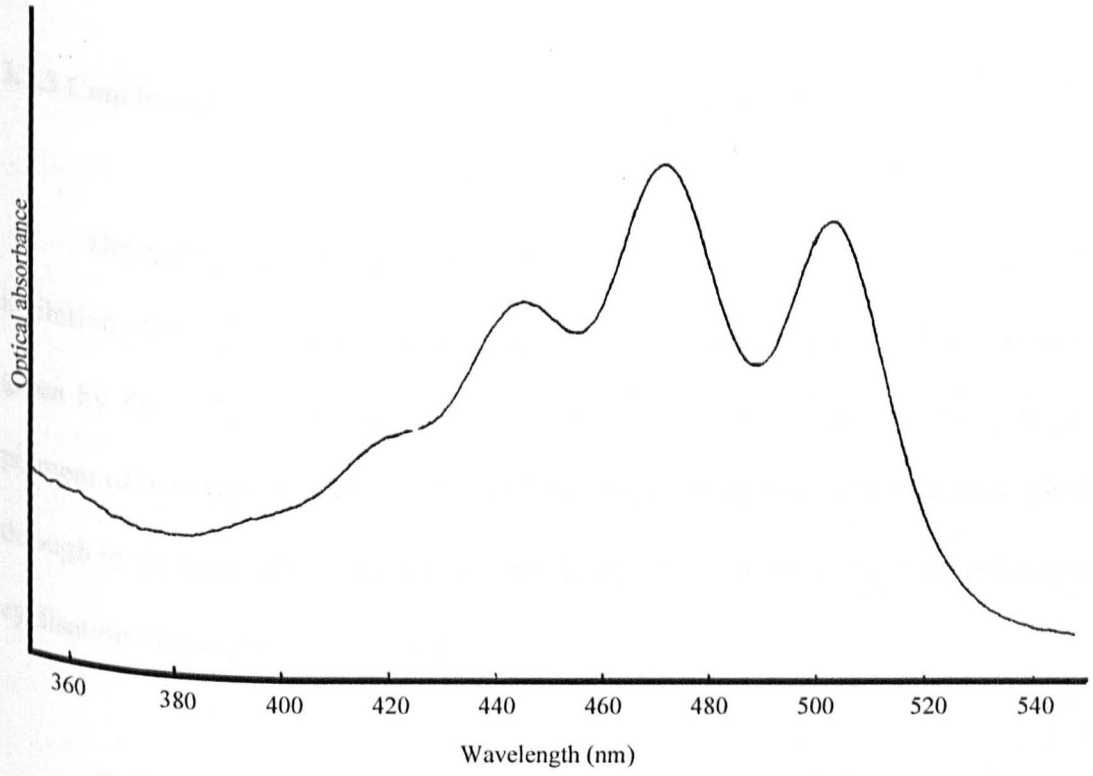


Figure 3.1(b) UV-vis spectrum of lycopene extracted from *B. linens* NCIMB8546 treated with nicotine

3.1.2 Evidence for the role of nicotine as an inhibitor of the lycopene cyclase enzyme and not as a mutagen of the lycopene cyclase gene

In order to establish that nicotine was in fact acting as an inhibitor of lycopene cyclization rather than exerting any permanent effect upon the cells exposed, namely by mutation, cells accumulating lycopene in the presence of nicotine were then grown in the absence of nicotine.

Cells of *B. linens* NCIMB 8546 grown in liquid YGB medium (see Materials and Methods Section 2.9.1) containing concentrations of nicotine sufficient to cause the accumulation of lycopene were diluted in sterile YGB medium prior to plating out onto YGA medium containing no nicotine. In spite of the accumulation of lycopene in liquid culture, cells grew normally on nicotine-free medium.

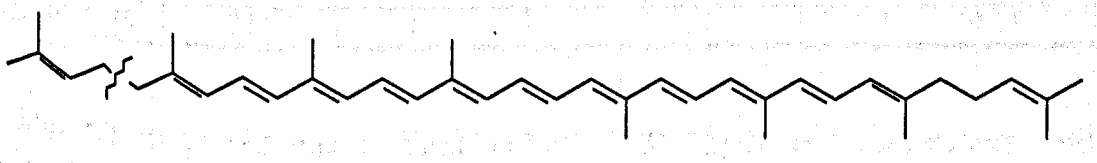
3.1.3 Conclusions

Depending upon the concentrations of nicotine used, low, intermediate or full inhibition of the lycopene cyclization appeared to be taking place. Evidence of this is given by the variation in hue from the orange of 3,3'-dihydroxyisorenieratene, the pigment of wild type *B. linens* NCIMB 8546 which can be seen at low nicotine levels, through to the pink of the lycopene-accumulating strain in which nicotine inhibits the cyclisation of lycopene (see Plate 3.1).

The pink to red appearance of nicotine-treated cells and also of the mutant cells (see Section 3.4 below) of B. linens NCIMB 8546 suggests the presence of

lycopene. This was confirmed by UV-vis spectroscopy (see Figure 3.1(b)) and mass spectrometry of the isolated pigment (see Figure 3.1). The UV-vis spectrum is consistent with that for reference samples of lycopene with absorption maxima corresponding to 446, 472 and 503 nm. The mass spectrum for lycopene is shown in Figure 3.1 and is identical to that of an authentic crystalline sample of lycopene. The molecular ion M^+ 536 corresponds to $C_{40}H_{56}$, the ion at *m/z* 467 denotes the M-69 fragment which confirms the lycopene end group below. The fragment ion at *m/z* 430 (M-106), is characteristic of acyclic carotenoids and denotes the loss of xylene from the molecule.

The above data provide support for the common carotenoid biosynthesis pathway in *B. linens*, discussed in the introduction with lycopene as a precursor to the cyclic carotenoids which follow. The result of the inhibition of the cyclase enzyme shows that the blocking of the cyclase reaction through mutation of the cyclase gene is a valid strategy with which to create lycopene-accumulating strains of *B. linens*.



The characteristic M-69 fragment of lycopene with the separated end group

3.2 Quantitative analysis of 3,3'-dihydroxyisorenieratene in wild-type strains of *B. linens*

The wild-type strains of *B. linens* examined in this work do not appear, when inspected by eye, to accumulate the same concentrations of 3,3'-dihydroxyisorenieratene. Since the concentration of pigment in non-mutated cells is likely to reflect the overall production of carotenoids, these values are of importance with regard to the potential of subsequent mutant strains for lycopene production.

Wild-type cells of the *B. linens* strains BL1 (Christian Hansen), CECT75, NCIMB 8546 and Wiesby were extracted quantitatively and the carotenoid contents determined spectrophotometrically. The results are shown in Table 3.2.

B. linens strain	Dry cell mass (mg)	A472 in 20ml diethyl ether*	Total 3,3'-dihydroxyisorenieratene (DHIR) (μg)	Percentage DHIR per cell dry mass
BL1	195	0.85	194	0.10
CECT75	221	1.74*	316	0.14
Wiesby	99	1.28	233	0.24
NCIMB 8546	230	1.63	296	0.13

Table 3.2 Dry cell mass, total culture 3,3'-dihydroxyisorenieratene and percentage 3,3'-dihydroxyisorenieratene per dry cell mass values for *B. linens* wild-type strains in 50ml culture volumes. (*Values with the * suffix were obtained from 3,3'-dihydroxyisorenieratene dissolved in 25ml diethyl ether).

The concentrations of 3,3'-dihydroxyisorenieratene in wild-type *B. linens* suggests that certain strains may offer greater possibilities in terms of pigment production than others. This assumes that subsequent mutants will yield lycopene

concentrations which are reflective of their 3,3'-dihydroxyisorenieratene concentrations. Based upon pigment concentration, mutants derived from Wiesby *B. linens* are likely to yield the highest concentrations of lycopene, though strain CECT75 yields the greatest mass of total culture pigment.

3.3. Evidence of the presence of compounds supporting the proposed carotenoid biosynthetic pathway in wild-type *B. linens*

Thin layer chromatography was used to separate and purify the carotenoids present in wild-type *B. linens* from Wiesby, and the carotenoids were analysed by UV/vis spectroscopy and mass spectrometry.

The final carotenoid biosynthesis pathway pigment in wild type *B. linens* is 3,3'-dihydroxyisorenieratene which accounts for the orange colouration of cells (Plate 3.3). The carotenoid mixture extracted from wild type *B. linens* (including phenolic 3,3'-dihydroxyisorenieratene) displays a UV-vis spectrum in diethyl ether as shown in Figure 3.3. By TLC separation (see Materials and Methods Section 2.16), three pigments bands were initially resolved from the wild type extract (approximate RF values 0.5, 0.8 and 0.9). Further resolution of the uppermost pigment band yielded six further bands (approximate RF values between 0.5 and 0.8).

The most significant fractions from thin layer chromatograms were purified and analysed by mass spectrometry. The data suggested the presence of 3,3'-dihydroxyisorenieratene, through the presence of fragments at *m/z* 149, indicative of

aromatic end groups, an intense peak at *m/z* 468 (M-92) which is typical of the presence of a dicyclic compound and the molecular ion *m/z* 560. In a further fraction, the monohydroxylated ϕ,ϕ-caroten-3-ol$^+$ is indicated by a molecular ion at *m/z* 544. A fragment at *m/z* 133 is characteristsic of the unhydroxylated aromatic end group. The molecular ion at *m/z* 528 is consistent with isorenieratene, that at *m/z* 536 of β-carotene and that at *m/z* 532 of β-isorenieratene. These data lend support to the existence of a biosynthetic pathway in which the aromatic end groups are formed from β-rings one at a time, followed by the addition of one hydroxyl and then two hydroxyl functions. The UV-vis spectrum of ϕ,ϕ-caroten-3-ol is shown in Figure 3.3(b).

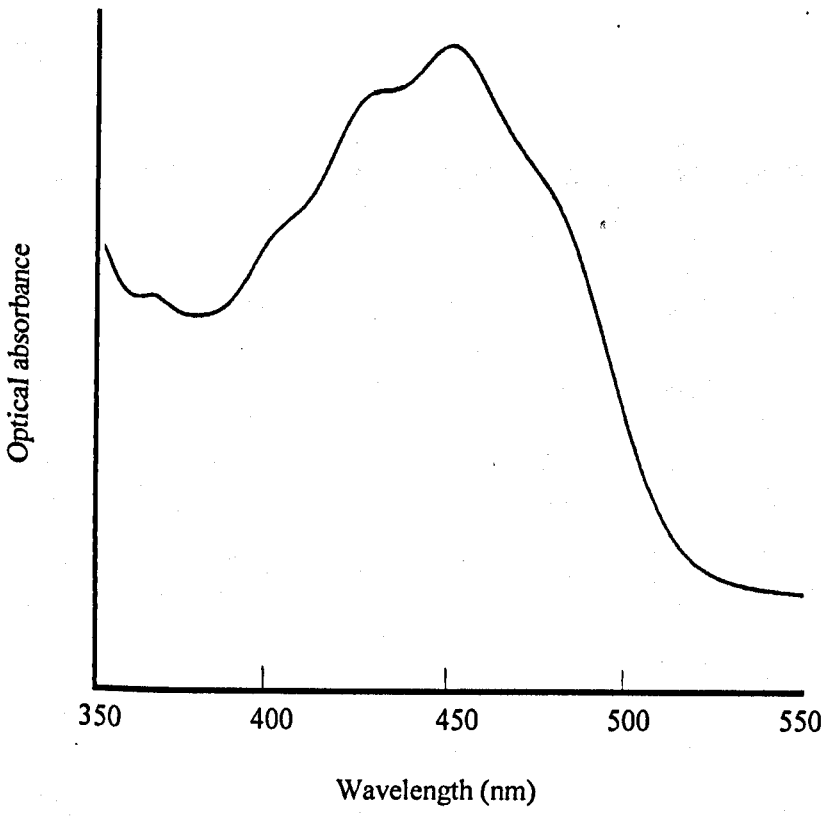


Figure 3.3 The UV-vis spectrum of *B. linens* NCIMB 8546 pigment extract including 3,3'-dihydroxyisorenieratene

Plate 3.3 *B. linens* lycopene accumulating mutant 'Pink' (*Pink*) and NCIMB 8546 wild-type (*Orange*) grown on YGA medium


Pink
Orange

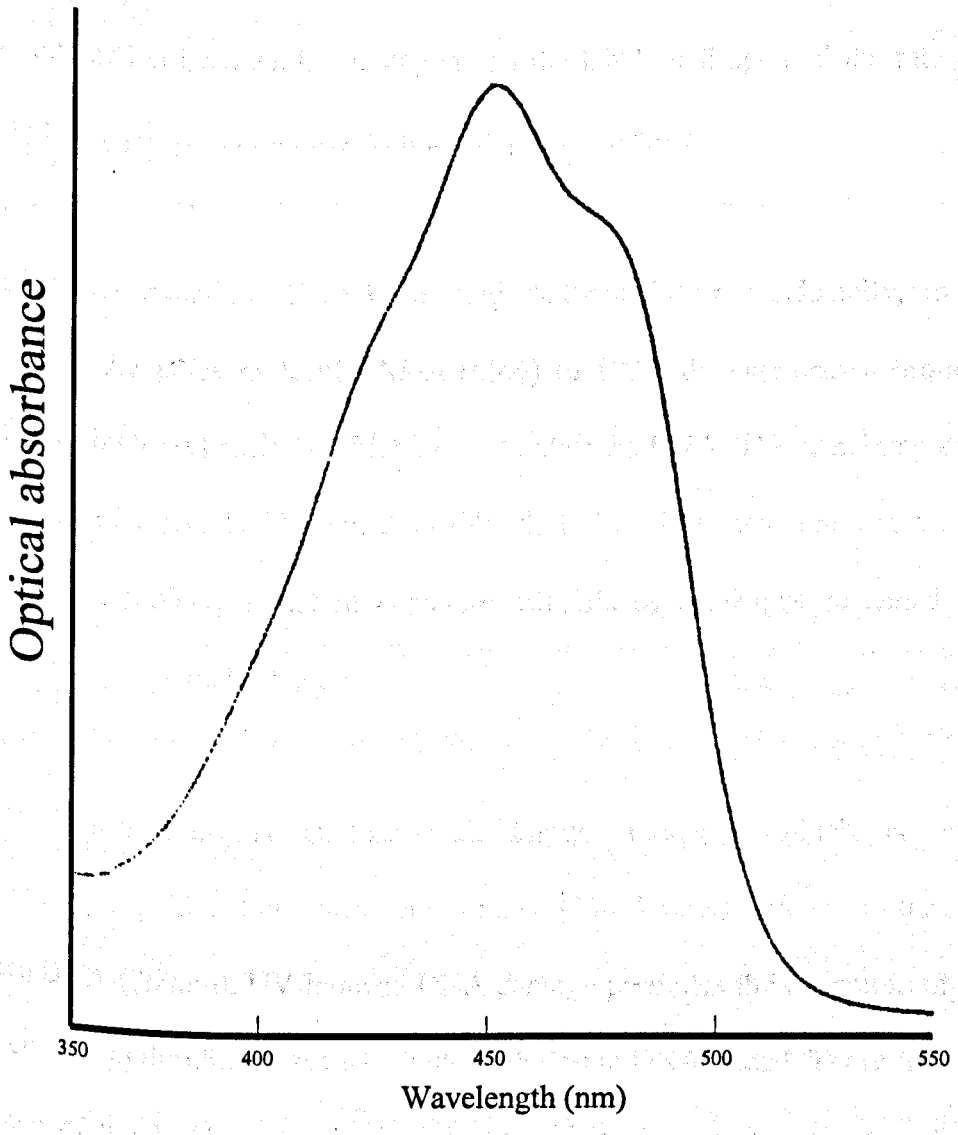


Figure 3.3(b) The UV-vis spectrum of ϕ,ϕ-caroten-3-ol extracted from *B. linens* NCIMB 8546

3.4 Mutation to obtain lycopene accumulating strains

Chemical mutation and mutation using UV light and ionising radiations have long been used as a crude, undirected but nevertheless effective mechanism by which new strains of micro-organisms are generated. In industrial environments mutant organisms have been developed with increased yields, either of the whole cell or some desirable product. Sometimes the end product itself is altered through mutation.

Different mutagens affect cellular DNA in different ways and, alongside the direct or indirect interaction between the mutagen and the DNA, cell age and the rate of cell replication relative to the rate of repair are important factors.

Ionising radiations have been used deliberately or accidentally, to cause mutations in the DNA molecule. Most (65%) of DNA damage arises through the formation of hydroxyl radicals which go on to alter the DNA. The remaining damage is the result of direct DNA ionisation (Ward, 1988). The most important damage resulting from ionising radiation exposure, consists of breakages sustained in the deoxyribose-phosphate backbone.

UV irradiation is another well known mutagen, which is actually pathologically significant at a wavelength range (300-400nm) different to that which damages DNA (260nm). UV-induced DNA damage promotes the formation of cross-links between pyrimidine bases which share the same DNA strand. These may affect the shape of the DNA molecule, interfering with replication processes or forming repair-resistant lesions (Chan *et al.*, 1985).

Alkylating agents such as methane-sulphonic acid ethyl ester (EMS) (which is known for causing point mutations and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) ((which must first undergo metabolic conversion) see below) attack nucleophilic regions of the DNA molecule, primarily at the N^7-guanine, N^3-adenine, O^6-guanine and O^4-thymine sites (Roberts, 1978; Singer and Kusmierek, 1982). Upon alkylation, the *N*-glycosylic bond is destabilised, leading to the formation of abasic sites within

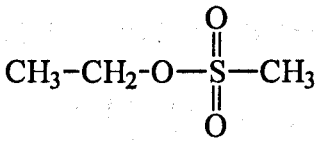
DNA (Loeb and Preston, 1986). DNA is not, however, universally susceptible to alkylation and, in particular, guanine-rich regions are notably sensitive (Richardson and Richardson, 1990), the O^6 and N^7 positions of guanine being readily accessible to MNNG within the DNA double helix (Dizdaroglu *et al.,* 1991). Depurination also takes place as a result of the alkylation of guanine residues, since the bond between the purine nitrogen and the deoxyribose molecule can be broken. Should this take place when the replication fork reaches the damaged region before repair has been made, an adenine is normally inserted opposite the apurinic strand, resulting in a GC to TA transversion in a subsequent round of replication (Myrnes and Krokan, 1986).

Carotenogenic organisms lend themselves to mutation analysis because screening is elementary and rapid owing to the pigmentation changes associated with altered carotenoid phenotype. At a concentration of around $1gdm^{-3}$, MNNG is effective in inducing pigmentation changes in *Brevibacterium flavum* and *Brevibacterium* sp. M27. Tetracycline and streptomycin resistant strains were also produced by the same methods in *Brevibacterium sp.*M27 and *B. flavum* thus indicating the randomness of the mutagen (Konicková-Radochová *et al,* 1988; Konicek *et al* 1988). In yeasts too, carotenoid mutations have been noted as with *Phaffia rhodozyma* whereby UV or EMS treatment yields phytoene- and β-carotene-accumulating as well as non-carotenoid containing phenotypes where the wild-type pigment is astaxanthin. Mutants accumulating higher than normal concentrations of astaxanthin have also been isolated (Girard, *et al* 1994).

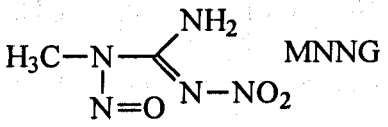
Any mutations induced, even if they are to affect pigmentation exclusively, must not be significantly detrimental to the physiology of the cell. Geranylgeranyl diphosphate (GGDP) for example, is required for the synthesis of the phytyl or geranylgeranyl side chain of plant and bacterial chlorophylls, so no carotenoid pathway mutations would be expected before GGDP in the carotenoid biosynthesis pathway of photosynthetic organisms (Goodwin, 1980).

In this work, an attempt has been made to mutate wild type strains of *B. linens* so that lycopene becomes the exclusive end product of carotenoid biosynthesis. Experiments conducted with the cyclase inhibitor nicotine (see Section 3.1.1) suggest that deleterious mutations in the gene encoding the lycopene cyclase enzyme should lead to mutants which accumulate lycopene. In contrast with the requirement for GGDP (discussed above), cyclase inhibition experiments suggest that the absence of carotenoids beyond lycopene is non-lethal.

Although not used here, more advanced mutation techniques have been applied to other Corynebacteria such as transposon mutagenesis whereby a mobilisable element of *Corynebacterium glutamicum* DNA (insertion sequence IS*1831*) for example, inserts itself into the genome of *Brevibacterium flavum* MJ233C (Vertès *et al.*,1994).

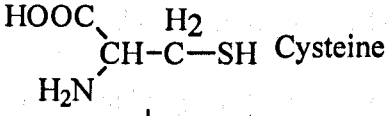


Methane-sulphonic acid ethyl ester

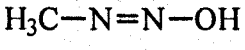


MNNG

+



Cysteine



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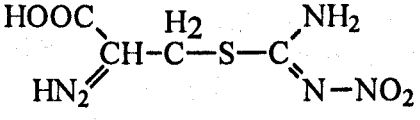
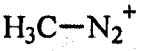
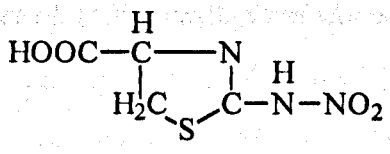






Metabolic activation of MNNG

3.4.1 Mutation using ultra violet radiation at a wavelength of 254nm

Mid log phase cultures of *B. linens* NCIMB 8546 diluted 10,000 times in YGB spread onto YGA plates in aliquots of 100µl were exposed to ultraviolet radiation at a wavelength of 254nm at a range of intensities from one hundred to eight thousand joules per square metre (see Materials and Methods Section 2.11.1) over time periods ranging from one second to 24 minutes (see Table 3.4). In all cases, progeny cell phenotype appeared identical to that of the wild type. Colony numbers subsequent to exposure are given in Table 3.4 (after incubation to maturity in darkness at 30°C). These results, coupled with the high resistance of *B. linens* to levels of UV radiation many times those lethal in *E. coli* imply a very efficient DNA protecting mechanism which, based upon the known quenching properties of some carotenoids against photooxidative damage, suggests that the carotenoid pigmentation in *B. linens* provides a protective effect against light damage both to cell functions and DNA. This has been the case with *E. coli* expressing *Erwinia herbicola* carotenogenic genes which were found to provide significant protection against photosensitising molecules and near ultraviolet light (Tuveson *et al*, 1988).

Carotenoids are frequently associated with cellular membranes so it is perhaps not surprising that they may have an influence on cell membrane structure and integrity and thus the capability of the cell to endure harmful radiation. In experiments conducted by Chamberlain *et al.* (1991) with *Staphylococcus aureus* 18Z, the killing effect of oleic acid, which increases membrane fluidity, was mitigated in the presence of membrane carotenoids.

Intensity (Joules m^{-2})	Dosage period (minutes)	Total colonies
100	0.5	6
100	1	0
100	2	0
100	3	23
100	6	50
100	12	45
100	24	0
500	0.5	20
500	1	13
500	2	3
500	3	9
500	6	3
500	12	0
500	24	0
2000	0.2	38
4000	0.2	22
8000	0.2	9

Table 3.4 The effect of exposure to ultra violet light (254nm) upon survival in 100µl aliquots of *B. linens* NCIMB 8546. All colonies post irradiation displayed colour phenotype identical with wild-type cells.

3.4.2 Mutation using Methane-sulphonic acid ethyl ester (EMS)

EMS was added to 10ml of mid log phase cells of *B. linens* NCIMB 8546 in YGB medium at a concentration of 0.14M (Levin, 1971) (see Materials and Methods Section 2.11.2). After agitated incubation for 3.5 hours at 30°C, cell suspensions were diluted five-fold and aliquots of 100µl were plated onto YGA medium.

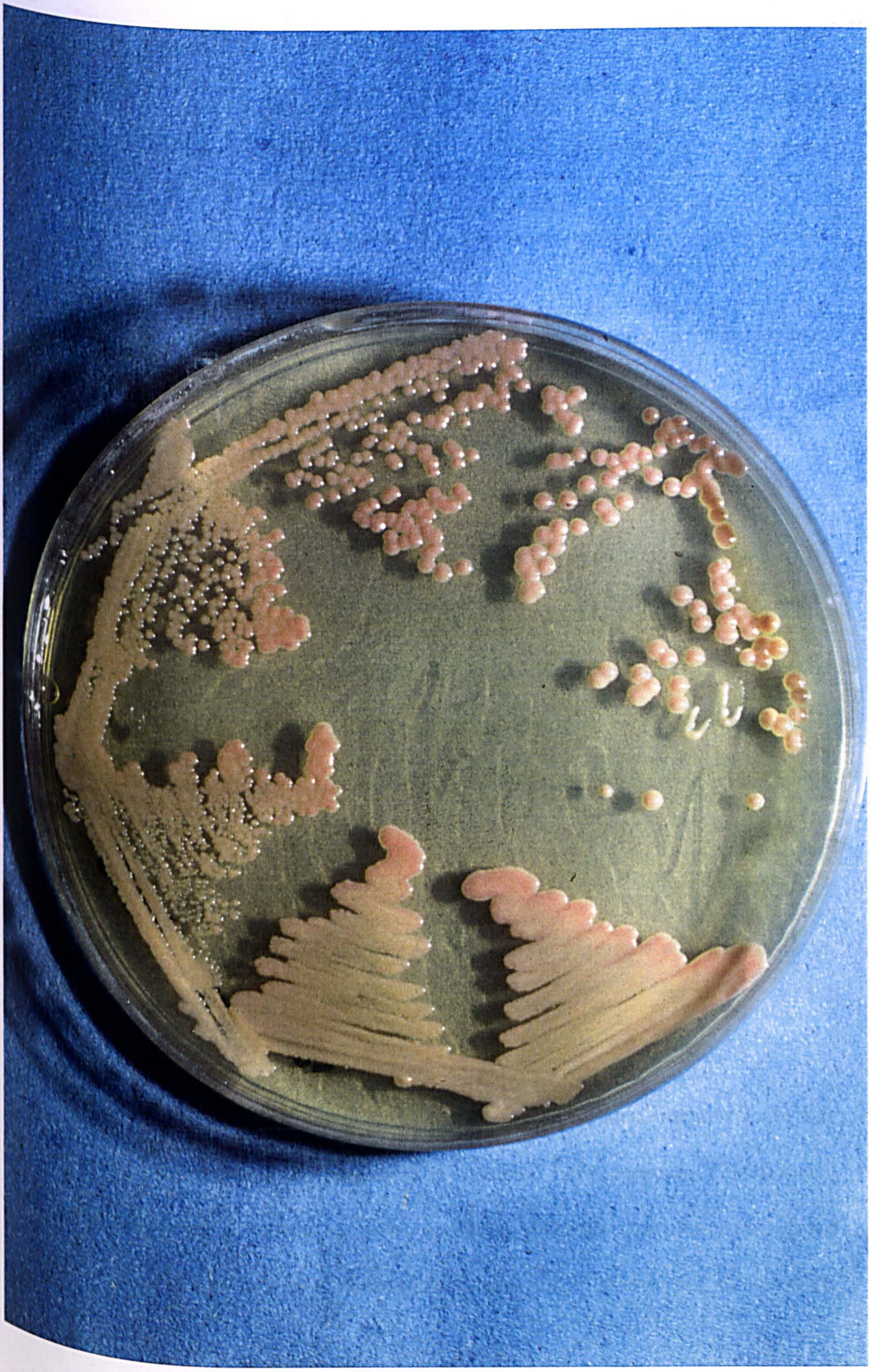
Despite a kill rate in the order of 99.8% (see Materials and Methods Section 2.11.4), colonies resulting from cells treated with EMS displayed pigment phenotypes identical with those for untreated wild type *B. linens* NCIMB 8546.

EMS is known to be a powerful alkylating agent, so it is difficult to envisage any mechanism by which the genome of *B. linens* is rendered less sensitive to its effects. It is perhaps feasible to suggest that the presence of cellular carotenoids may promote a membrane structure which is in some way less permeable to the compound than in the case of other, more easily mutated organisms.

3.4.3 Mutation by 1-methyl-3-nitro-1-nitrosoguanidine (MNNG)

Of the mutagenic treatments employed, only 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) yielded strains of *B. linens* which displayed mutant pigmentation phenotypes (see Materials and Methods Section 2.11.3). *B. linens* strain NCIMB 8546 yielded occasional pink strains which responded to further rounds of mutation, resulting in increased lycopene yield. Strains were consistently found to be of stable phenotype. Cell and lycopene yield values for the mutant designated 'Pink' under standard growth conditions (see Materials and Methods Section 2.9.1) are in the order of 200mg dry cell mass, which yields approximately 0.1% lycopene per cell dry mass (Plate 3.4) (exact values are given in Table 3.4(b)). This dry cell mass value contrasts with that seen in the wild-type strain which is in the order of 230mg (see Table 3.2), representing a decline in dry cell mass of approximately 31% based upon the peak 'Pink' strain value. The kill rate of MNNG was estimated at approximately 99.98%. The contrast between *B. linens* NCIMB 8546 wild-type and strain 'Pink' is clearly shown in Plate 3.3.

Plate 3.4 *B. linens* lycopene accumulating mutant 'Pink' grown on YGA medium



Dry cell mass (mg)	A_{472} in 50ml diethyl ether	Total lycopene (μg)	Percentage lycopene per cell dry mass
199	1.80	264	0.13
177	1.70	250	0.14
160	1.49	219	0.14

Table 3.4(b) Dry cell mass, lycopene mass and percentage lycopene per cell dry weight values for *B. linens* NCIMB 8546 mutant strain 'Pink' in 50ml culture volumes.

3.4.4 Characterisation of lycopene accumulated in mutants

The confirmation of the presence of lycopene in the mutants was obtained by UV-vis spectrophotometry, (see Figure 3.1(b)), TLC against an authentic crystalline sample and mass spectrometry (see Figure 3.1). The identification of lycopene in the most promising strain (Wkiii, see Section 3.6) was confirmed by NMR spectroscopy; the NMR spectrum of the isolated lycopene was identical to that of an authentic synthetic sample.

3.5. Mutant phenotypes which appear to accumulate pigments other than, or in addition to, lycopene and 3,3'-dihydroxyisorenieratene

Whilst lycopene-accumulating mutations could be isolated, mutants displaying low or undetectable levels of pigmentation appeared more common. Occasionally colony colours such as yellow or brown appeared, although more rarely than those with a pink or red phenotype. It is conceivable that yellow phenotypes still produce

the final product 3,3'-dihydroxyisorenieratene although at a reduced concentration when compared with wild type *B. linens* (a so-called 'leaky' mutation).

Incubation of *B. linens* NCIMB 8546 with 1-methyl-3-nitro-1-nitrosoguanidine also resulted in a dull-brown mutant (Plate 3.5). The extracted carotenoid had a UV/visual spectrum consistent with that for 3,3'-dihydroxyisorenieratene (Figure 3.3), though total carotenoid extraction using lysozyme (see Materials and Methods Section 2.15) appeared impossible in the brown mutant whilst being achievable in wild type *B. linens*, though the brown pigmentation is not likely to be due to the presence of carotenoids. Brown pigmentation appeared to become enhanced during storage which may indicate the effect of oxidation on pigmentation. Gradual colour changes have also been seen in *E.coli* possessing carotenogenic genes from *E. herbicola*. Here very faint pigmentation, only evident after several days of storage, is present in cells of *E. coli* carrying a mutated *crt* E (GGDP synthase) gene (Hundle *et al*, 1994).

A further pink pigmented mutant created from a Wiesby dairy strain of *B. linens* was found to release a brown pigment, soluble in solid YGA medium and eventually diffusing to uniform density (Plate 3.5(b)). It is perhaps the case that this pigment is similar to that released by *B. casei* when incubated on milk agar.

UV-vis spectra were also obtained from a weakly coloured mutant, M22, that was derived from *B. linens* NCIMB8546 (Plate 3.5(c)) in an attempt to isolate a phytoene accumulating mutant. Spectra from solvent extracts were consistent with the

Plate 3.5 *B. linens* brown pigmented mutant (see text) grown on YGA medium

Plate 3.5(b) A *B. linens* mutant that releases a brown pigment, alongside a non-pigment releasing lycopene accumulating strain. The culture was grown on YGA medium

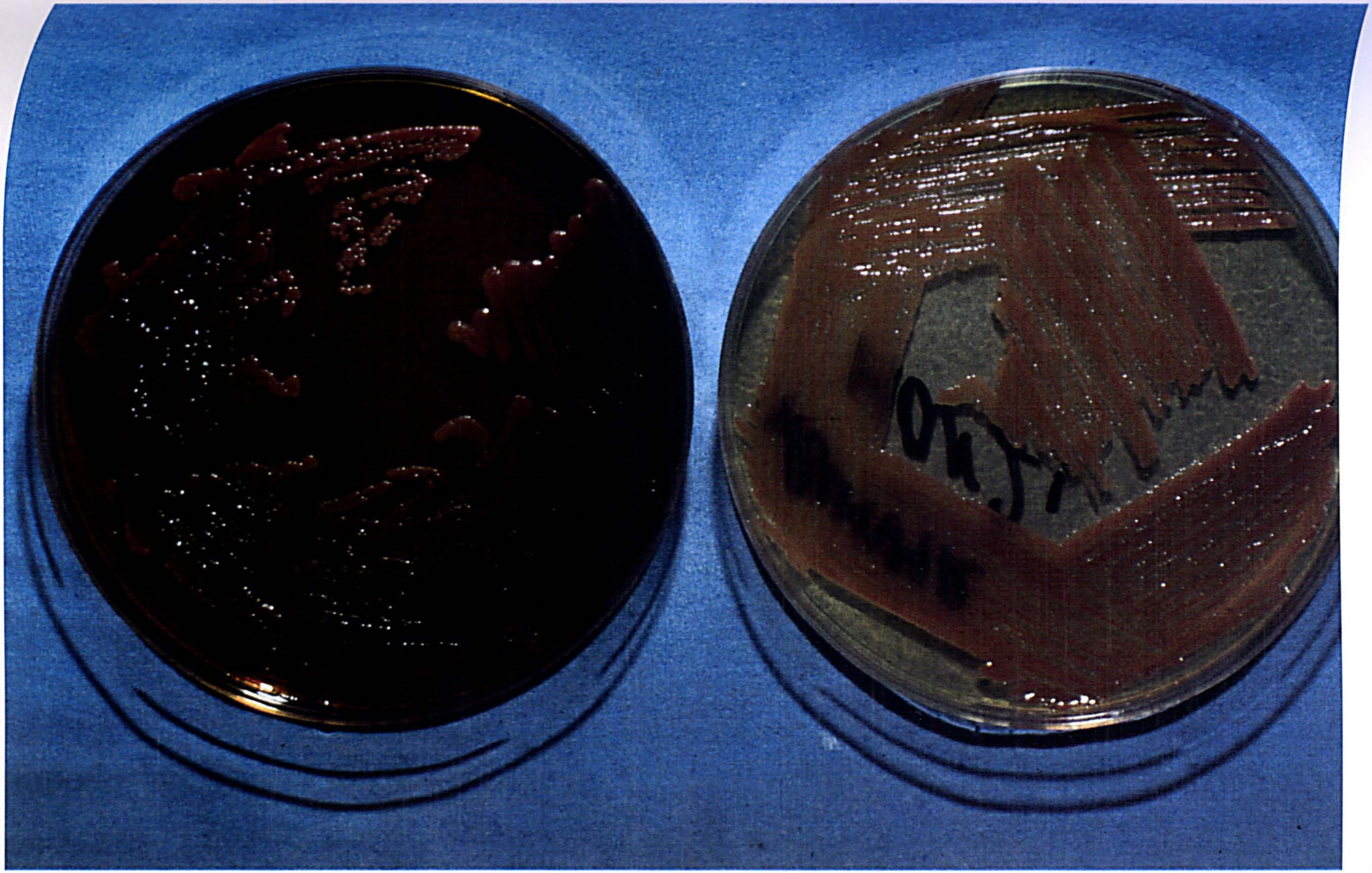


Plate 3.5(c) *B. linens* mutant M22, which is a possible phyotene accumulating strain. Grown on YGA medium

possible presence of phytoene phytofluene and ζ-carotene, structures for which are given in the introduction, Figure 1.6(c).

3.6 Mutation strategies to enhance the accumulation of lycopene in *B. linens* strains

Mutants selected for lycopene accumulation were subjected to further mutation by MNNG in an attempt to enhance pigment yield through alterations in the regulation of biosynthesis. Two selected strains designated 'RR' and 'MR' yielded values in the ranges 0.12-0.14% lycopene per cell dry mass and 0.20-0.27% lycopene per cell dry mass respectively when grown under standard conditions (Table 3.6). Dry cell mass yield appeared to follow an opposing pattern to the level of pigmentation such that mean values were seen to fall by 46% (for strain 'RR') and 62% (for strain 'MR') when compared with strain 'Pink'.

Mutant designation	Dry cell mass (mg)	A_{472} in 25ml diethyl ether	Total lycopene (μg)	Percentage lycopene per cell dry mass
RR	108	1.78	131	0.12
RR	95	1.77	130	0.14
RR	86	1.66	122	0.14
MR	58	2.15	158	0.27
MR	67	1.85	136	0.20
MR	80	2.29	168	0.21
MR	64	2.21	163	0.25

Table 3.6 Dry cell mass, lycopene mass and percentage lycopene per cell dry weight values for *B. linens* NCIMB 8546 mutant strains 'RR' and 'MR in 50ml culture volumes.

In light of the slow growth rate, low cell mass yield and lycopene yield values found in *B. linens* NCIMB 8546 derived mutants, further strains of *B. linens* were examined visually and by growth rate (see Section 3.8.1) in view of their mutation to obtain more efficient media conversion to pigment and cell mass.

Strains BL1 and BL2 of *B. linens* were acquired from the dairy suppliers Christian Hansen (Chr. Hansen (UK) Ltd., Berkshire) (Plate 3.6) and were subjected to mutagenic treatment with MNNG. Since the levels of pigmentation and cell mass yield during the initial mutagenesis were relatively low (means 0.02% and 0.06% lycopene per dry cell mass respectively, compared with mean 0.14% for strain 'Pink'), mutant colonies were not subjected to further mutation. Lycopene accumulation values for mutants BL1 and BL2 grown under standard conditions (derived from the wild-type strains BL1 and BL2 respectively) are given in Table 3.6(b). Plates 3.6(b) and 3.6(c) show the appearance of the two mutants.

Mutant designation	Dry cell mass (mg)	A_{472} in 20ml diethyl ether	Total lycopene (μg)	Percentage lycopene per cell dry mass
BL1	167	0.50	29	0.02
BL1	173	0.23	14	0.01
BL1	171	0.66	39	0.02
BL2	167	2.03	119	0.07
BL2	165	1.75	103	0.06
BL2	226	2.05	121	0.05

Table 3.6(b) Dry cell mass, lycopene mass and percentage lycopene per cell dry mass values for *B. linens* mutant strains 'BL1' and 'BL2' in 50ml culture volumes.

A sample of *B. linens* strain 'W' supplied by Wiesby (Visby Labarotorium Tønder ApS, Denmark) was subjected to mutagenic treatment using MNNG. After selection for the most highly pigmented presumed lycopene accumulating mutants a range of strains were investigated after growth under standard conditions. Selection by eye was proven to be sufficient to detect mutants capable of accumulating higher levels of lycopene than previously encountered. In particular, strain WBLM1Mk yielded a mean percentage lycopene yield of 0.24% in contrast with a mean of 0.23% for mutant strain 'MR'. Although a mean dry cell mass of 31mg did not compare favourably with that of MR at 67mg. Whilst these figures suggest that 'MR' strains merit further investigation, their slow growth rate in contrast with Wiesby based strains (see Section 3.8.1) militates against this. Lycopene accumulation and dry cell mass values for Wiesby derived mutants are displayed in Table 3.6(c). Plates 3.6(d) and 3.6(e) show wild type *B. linens* from Wiesby and contrast *B. linens* Wiesby mutant WBLM1Mk with strain 'Pink'. Further WBLM1M derived strains are shown in Plates 3.6(f) and 3.6(g).

Plate 3.6 *B. linens* wild-type strain BL2 from Christian Hansen, grown on YGA medium

Plate 3.6(b) *B. linens* mutant strain BL1M (and others) originating from the wild-type strain from Christian Hansen after mutagenic treatment with 1-methyl-3-nitro-1-nitrosoguanidine. Cells were grown on YGA medium

Plate 3.6(c) *B. linens* mutant strain BL2M originating from the wild-type strain BL2 from Christian Hansen after mutagenic treatment with 1-methyl-3-nitro-1-nitrosoguanidine. Cells were grown on YGA medium

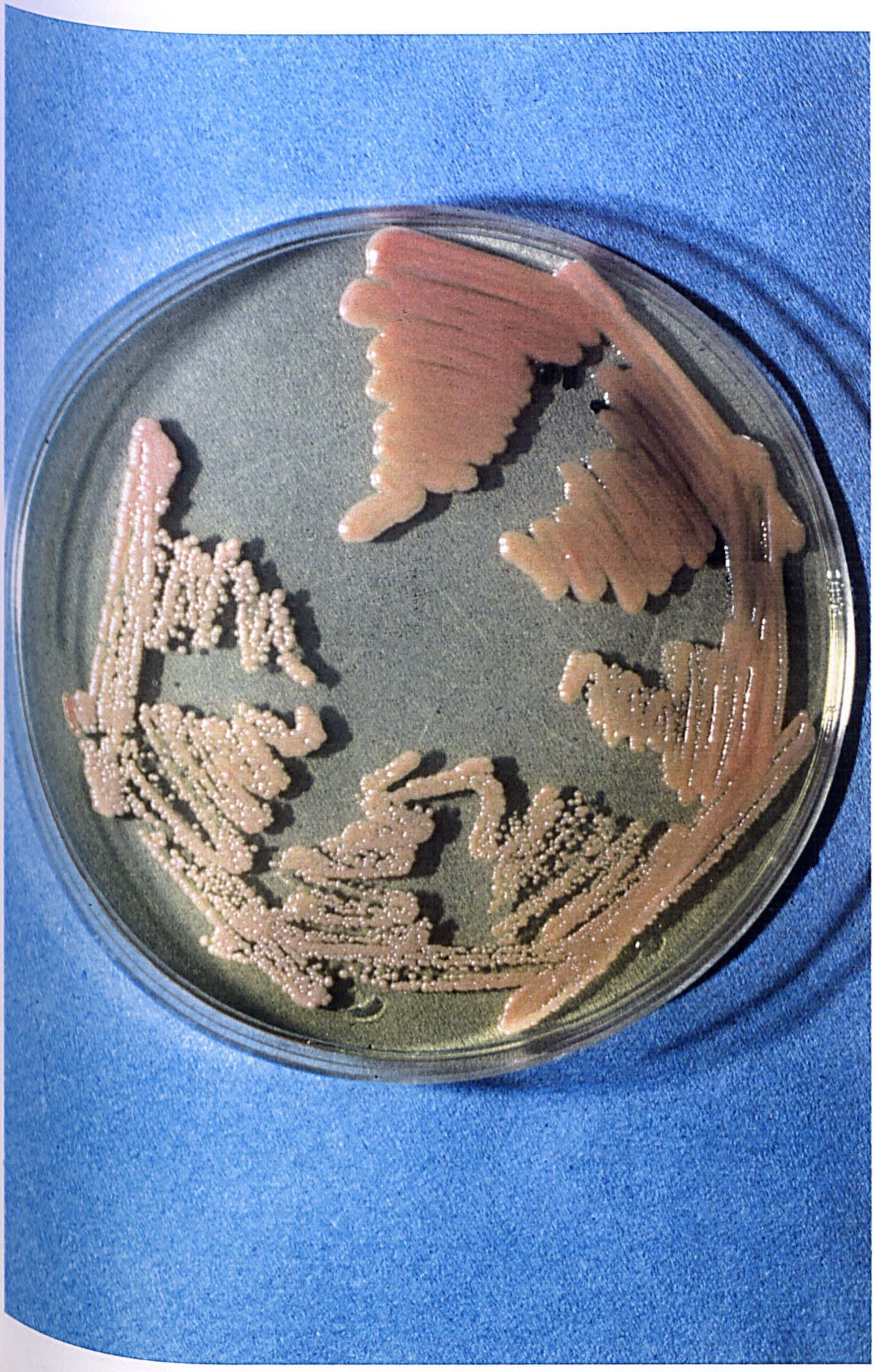


Plate 3.6(d) *B. linens* wild-type strain from Wiesby (Visby Labarotorium) grown on YGA medium

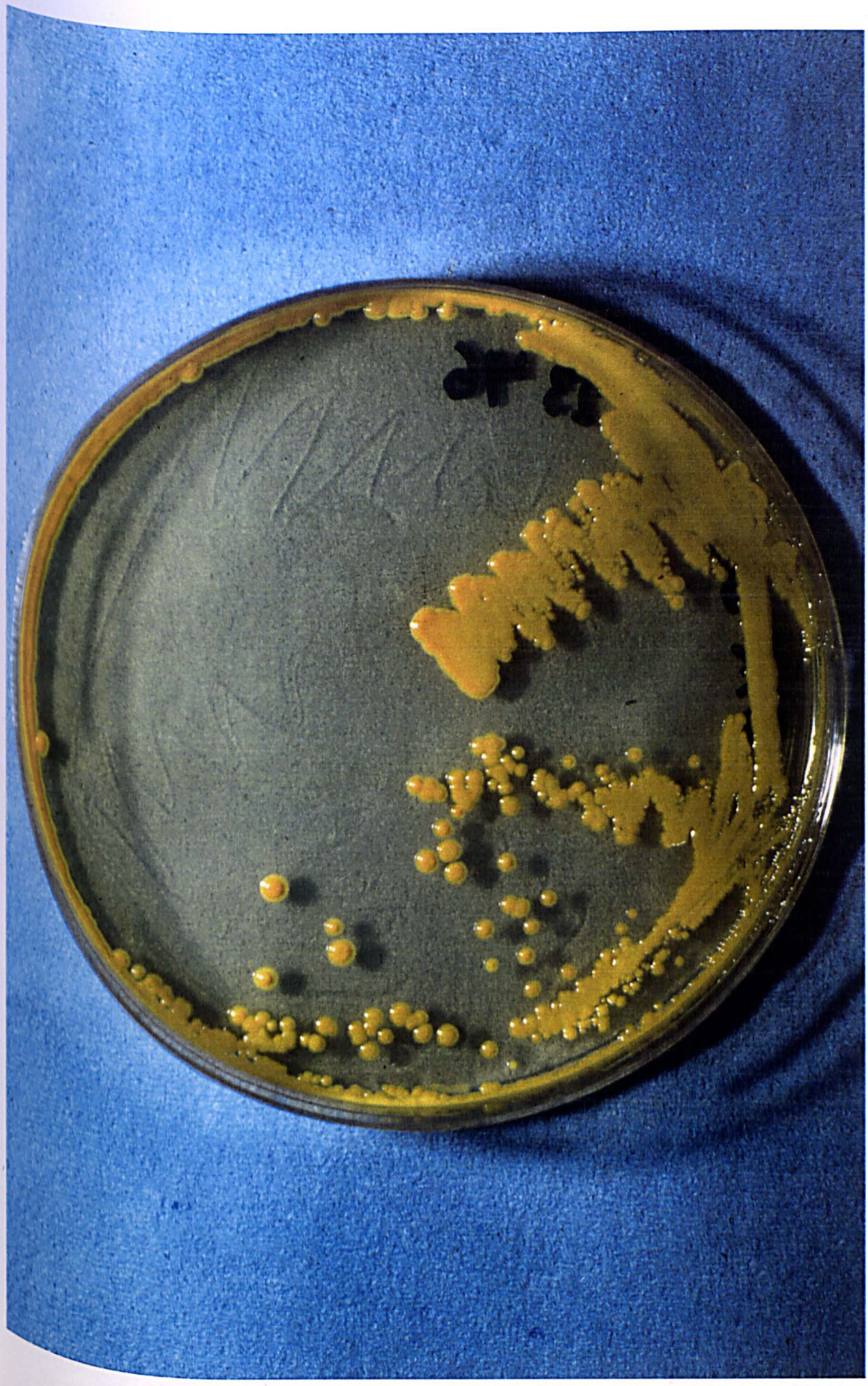


Plate 3.6(e) Shows the comparison between *B. linens* lycopene accumulating mutant strains 'Pink' (bottom) and Wkiii (top) grown on YGA medium

Plate 3.6(f) Mutant strains derived from mutant WBLM1M after further mutation with 1-methyl-3-nitro-1-nitrosoguanidine

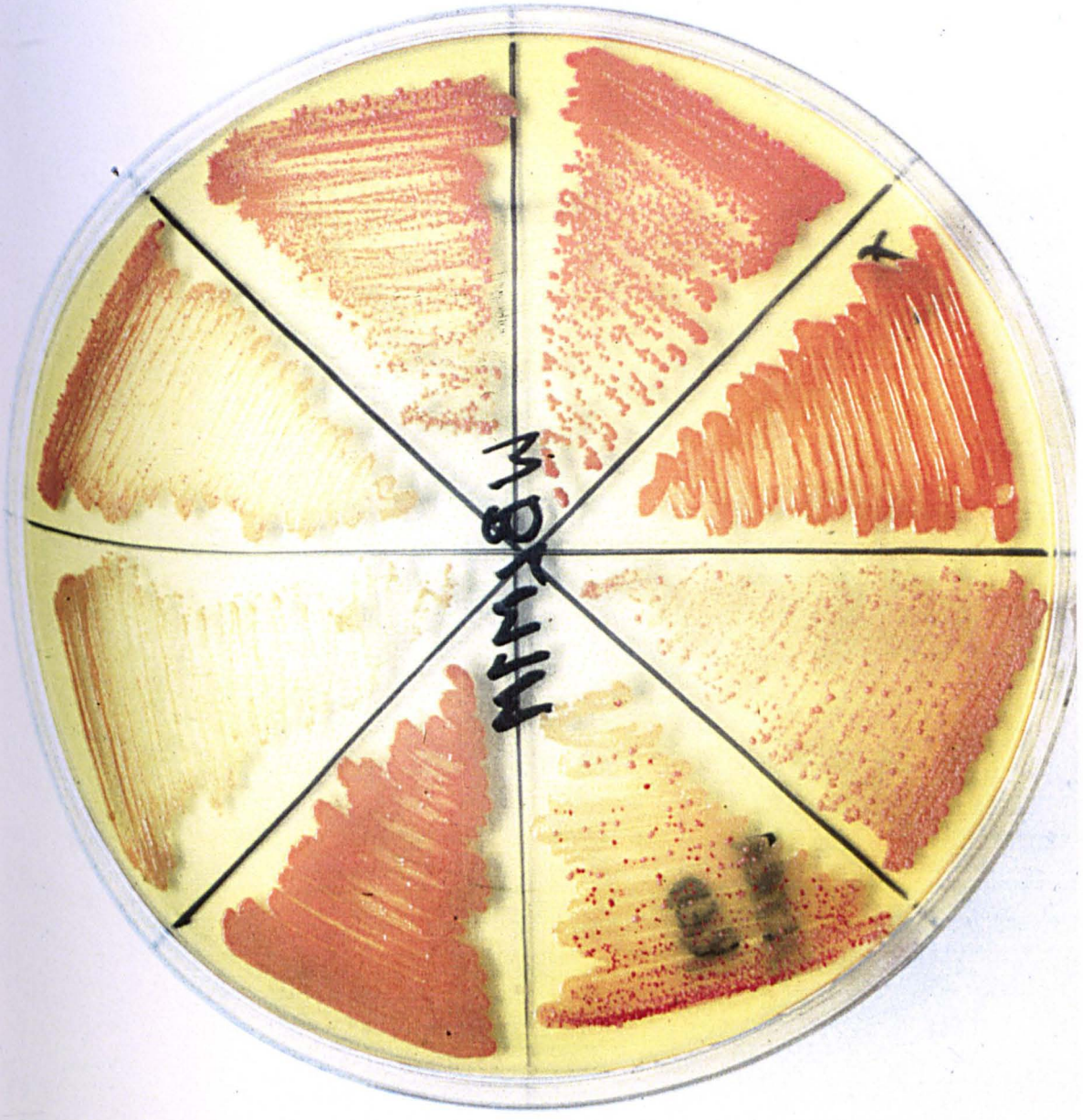

3 DAYS

Plate 3.6(g) A mixture of strains derived from mutant WBLM1M after further mutation using 1-methyl-3-nitro-1-nitrosoguanidine. Cells were grown on YGA medium

Mutant designation	Dry cell mass (mg)	A_{472} in 10ml diethyl ether*	Total lycopene (μg)	Percentage lycopene per cell dry mass
WBLM1Md	74	1.04*	77	0.10
WBLM1Md	72	2.25	66	0.09
WBLM1Md	74	1.28*	94	0.13
WBLM1Mj	10	0.57	17	0.17
WBLM1Mj	13	0.74	22	0.17
WBLM1Mj	11	0.57	17	0.15
WBLM1Mk	25	2.53	74	0.30
WBLM1Mk	42	2.35	69	0.16
WBLM1Mk	27	2.25	66	0.25
WBLM1Mg	46	2.89	85	0.18
WBLM1Mg	46	1.78	52	0.11
WBLM1Mg	62	2.24	66	0.11

Table 3.6(c) Dry cell mass, lycopene mass and percentage lycopene per cell dry weight values for *B. linens* mutant strains WBLM1Md, WBLM1Mj, WBLM1Mk and WBLM1Mg in 50ml culture volumes. (*A_{472} for values with * suffix are taken from pigment dissolved in 25ml diethyl ether).

Strain WBLM1Mk was subjected to further MNNG treatment yielding mutants designated Wki-Wkiv, some of which displayed promising increases in cell yield and lycopene accumulation in comparison with strain 'MR'. Table 3.6(d) shows values for these strains. Lycopene values as a percentage of cell dry mass reach a mean of 0.57% for strain Wkiv, which represents a yield increase of 148% when compared with strain 'MR'. With a mean dry cell mass value for strain Wki of 197mg, biomass yields were seen to increase dramatically as well (by 194% when comparing strain 'MR' with strain Wki).

Mutant designation	Dry cell mass (mg)	A_{472} in 100ml diethyl ether*	Total lycopene (μg)	Percentage lycopene per cell dry mass
Wki	188	1.99	585	0.31
Wki	179	1.84	541	0.30
Wki	223	2.43	715	0.32
Wkii	139	1.88	553	0.40
Wkii	116	1.43	421	0.36
Wkii	123	1.44	424	0.34
Wkiii	148	2.02	594	0.40
Wkiii	174	2.34	688	0.40
Wkiii	150	1.98	582	0.39
Wkiv	12	1.13*	67	0.55
Wkiv	11	1.13*	67	0.60
Wkiv	10	0.95*	56	0.56
Wkv	132	1.28	377	0.29
Wkv	127	1.28	377	0.30
Wkv	131	1.25	368	0.28

Table 3.6(d) Dry cell mass, lycopene mass and percentage lycopene per cell dry weight values for *B. linens* mutant strains Wki, Wkii, Wkiii, Wkiv and Wkiiv in 50ml culture volumes. (*A_{472} for values with * suffix are taken from pigment dissolved in 20ml diethyl ether).

The combination of reasonable biomass and lycopene accumulation values directed further mutation using *B. linens* strain Wkiii. With a maximum cell dry mass value of 91mg and maximum percentage lycopene per cell dry mass value of 0.39%, it appeared that the antecedent strain merited further investigation by manipulations other than mutation (Table 3.6(e)) (see Chapters 4 and 5).

Mutant designation	Dry cell mass (mg)	A_{472} in 50ml diethyl ether*	Total lycopene (μg)	Percentage lycopene per cell dry mass
WkiiiM4	36	2.02*	119	0.33
WkiiiM4	33	2.20*	129	0.39
WkiiiM4	36	0.60	88	0.25
WkiiiM5	87	1.21	178	0.20
WkiiiM5	84	2.45*	144	0.17
WkiiiM5	91	1.32	194	0.21
WkiiiM2	9	0.74†	22	0.24
WkiiiM2	14	0.56*	33	0.24
WkiiiM2	16	1.17†	34	0.22

Table 3.6(e) Dry cell mass, lycopene mass and percentage lycopene per cell dry mass values for *B. linens* mutant strains WkiiiM4, WkiiiM5, and WkiiiM2 in 50ml culture volumes. (*A_{472} for values with * suffix are taken from pigment dissolved in 20ml diethyl ether; values with the suffix † are taken from pigment dissolved in 10ml diethyl ether).

A striking exception to the values obtained for lycopene accumulation in Wkiii strains M2, M4 and M5, are the values obtained for mutant WkiiiM1 (Table 3.6(f)). Whilst mean dry cell mass only reaches 29mg under standard growth conditions, percentage lycopene per cell dry mass values reach a maximum of 0.84%, which represents a yield increase of 265% compared with strain 'MR'.

Mutant designation	Dry cell mass (mg)	A_{472} in 50ml diethyl ether	Total lycopene (μg)	Percentage lycopene per cell dry mass
WkiiiM1	29	1.66	244	0.84
WkiiiM1	29	1.64	241	0.83
WkiiiM1	28	1.18	174	0.62

Table 3.6(f) Dry cell mass, lycopene mass and percentage lycopene per cell dry mass values for *B. linens* mutant strain WkiiiM1 in 50ml culture volumes.

3.7. Morphological changes in the mutants

When grown for a long period after stationary phase, clustering in *B. linens* grown in liquid culture is apparent. Cells of the relatively slow growing *B. linens* NCIMB 'Pink' mutant (see Section 3.8.1) formed clusters of 2-3mm in diameter after nine days of growth. Cells of the faster growing *B. linens* mutant BL2 formed clusters over four days of growth. Associated changes which appeared to be absent in wild type strains were also observed on solid medium; plate 3.7 shows 'colony bunching' whereby small areas of solid YGA medium display confluent growth in which pigment variation exists among colonies. After mutagenic treatment, some progeny colonies also displayed distinctive sectored growth (Plate 3.7(b)).

At a microscopic level, dramatic morphological differences appeared when wild type *B. linens* from Wiesby and mutant Wkiii were compared. Plates 3.7(c) and 3.7(d) show low power transmission electron micrographs (TEMs) of sections through wild type and mutant *B. linens*. Many more ghost cells are evident amongst the mutant cell population compared with the wild type cells. Mutant cells can also be seen to be far more irregular, displaying pleomorphic characteristics. Plates 3.7(e) and 3.7(f) also show wild type cells, one of which (Plate 3.7(f)) is undergoing division. These images contrast sharply with similar high magnification images of mutant Wkiii (Plates 3.7(g), 3.7(h) and 3.7(i)). In all cases, cell division appears to be impaired and often abortive as seen in Plate 3.7(h) where a single cell of *B. linens* strain Wkiii appears to have divided unsuccessfully at least five times. This gives

Plate 3.7 *B. linens* mutant BL2, derived from the wild-type strain from Christian Hansen showing colony morphology. Cells were grown on YGA medium.

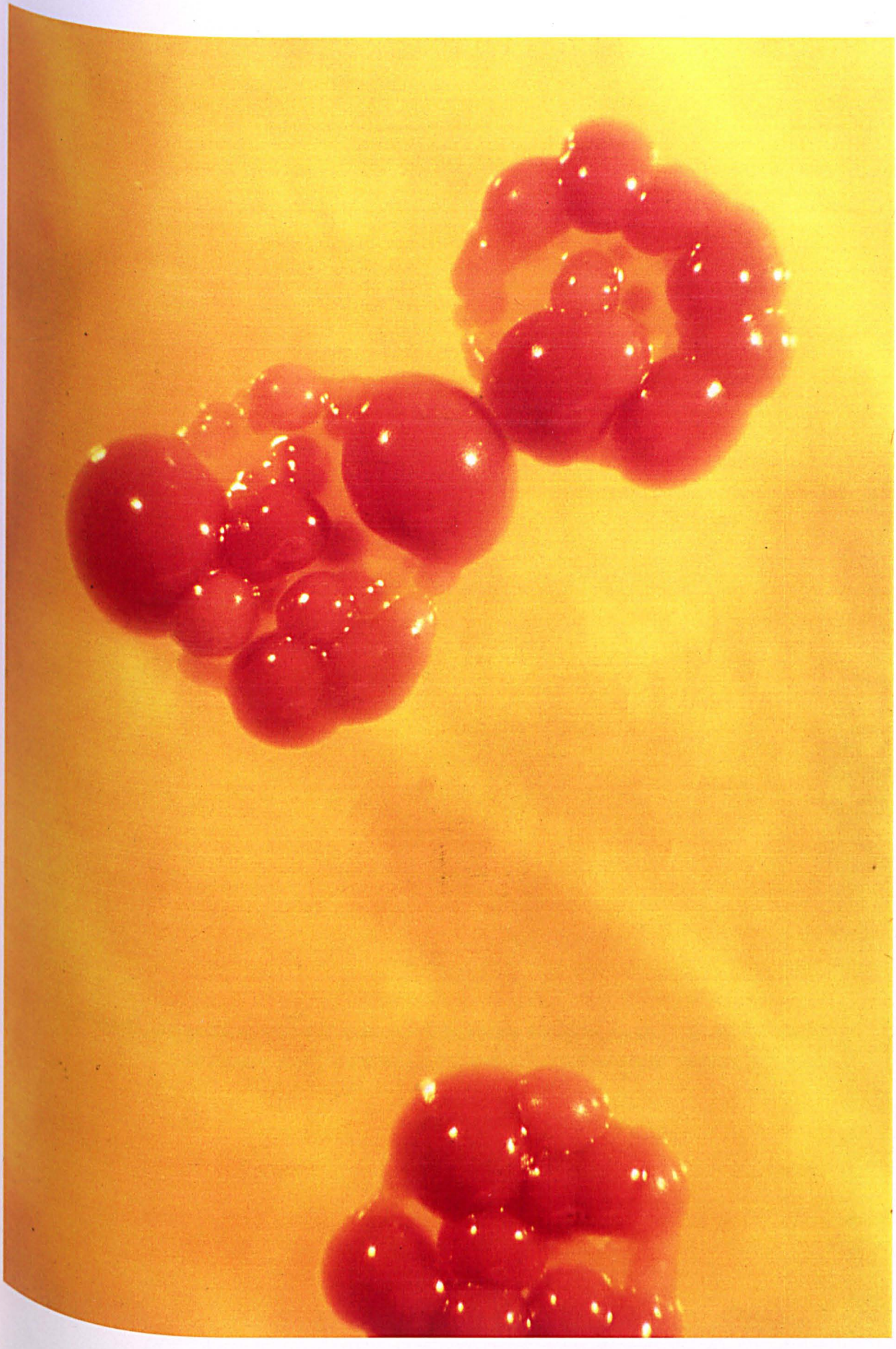


Plate 3.7(b) ***B. linens*** **mutant derived from the Wiesby wild-type, showing the sectored colony morphology. Cells were grown on YGA medium**

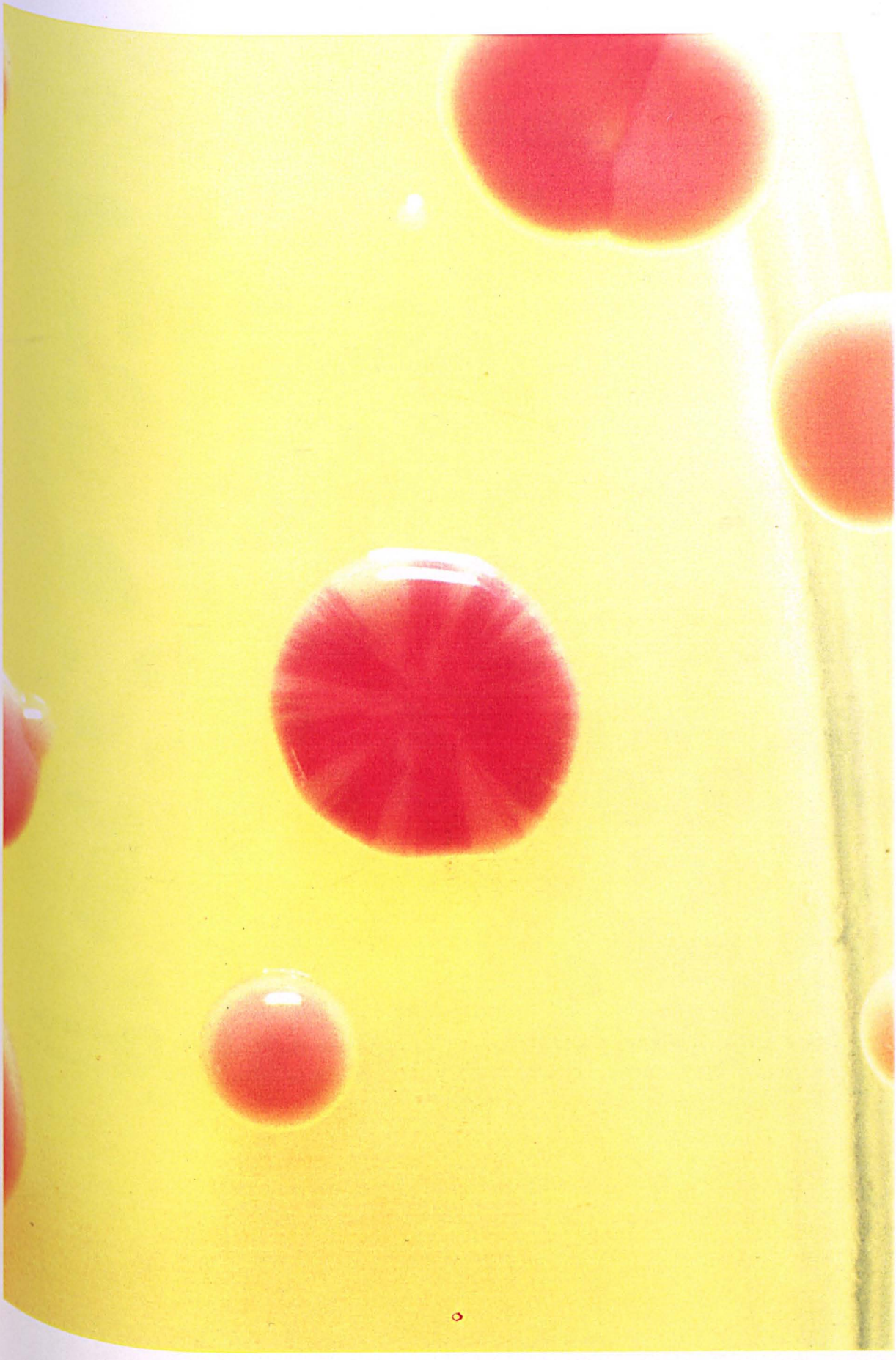


Plate 3.7(c) Transmission electron micrograph of the *B. linens* mutant Wkiii grown on YGA medium. Cells show considerable pleomorphism and abortive cell division (Bar = 1μm).

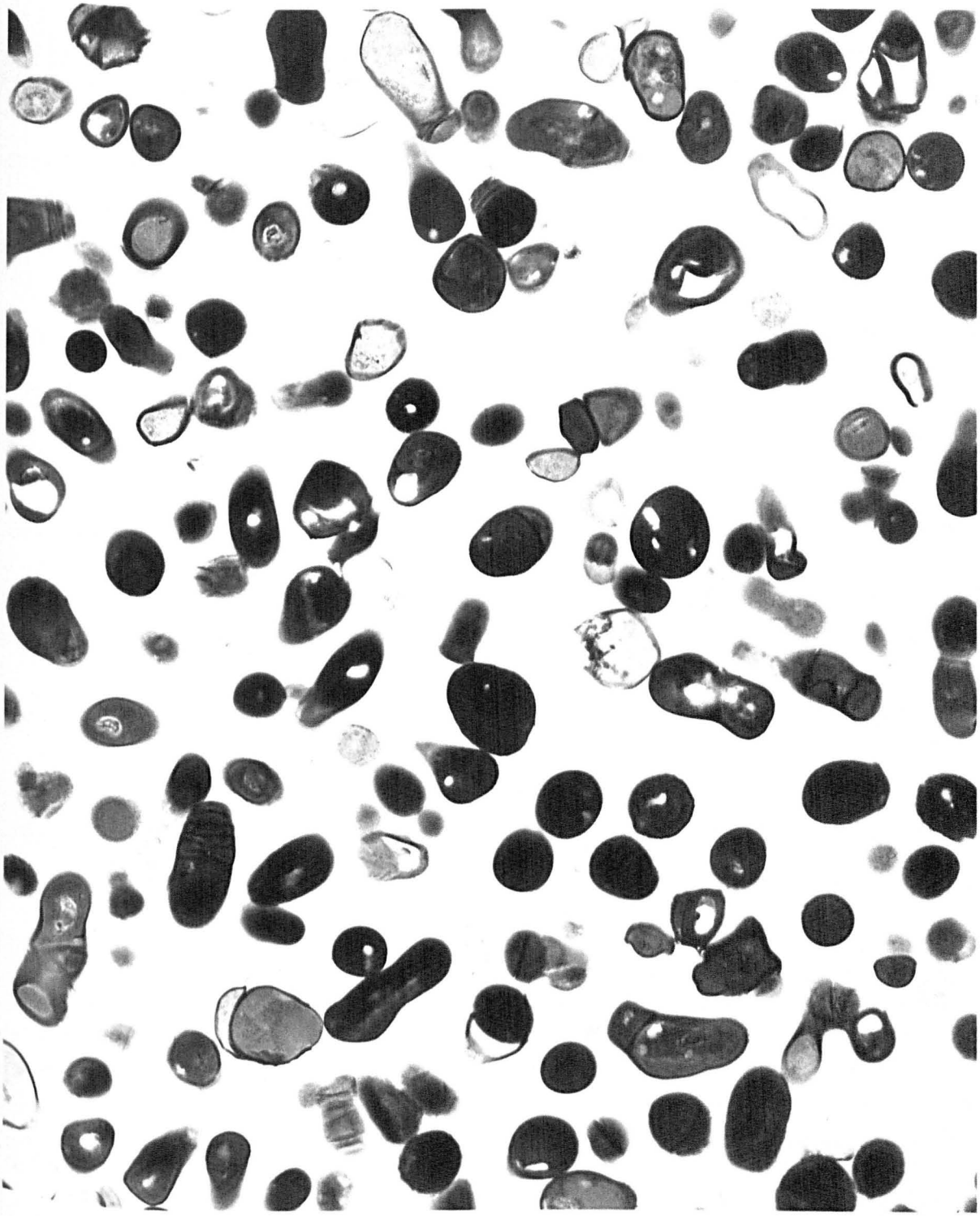


Plate 3.7(d) Transmission electron micrograph of the *B. linens* wild-type from Wiesby grown on YGA medium. Cells show considerably less pleomorphism than in mutant Wkiii (Bar = 1μm).

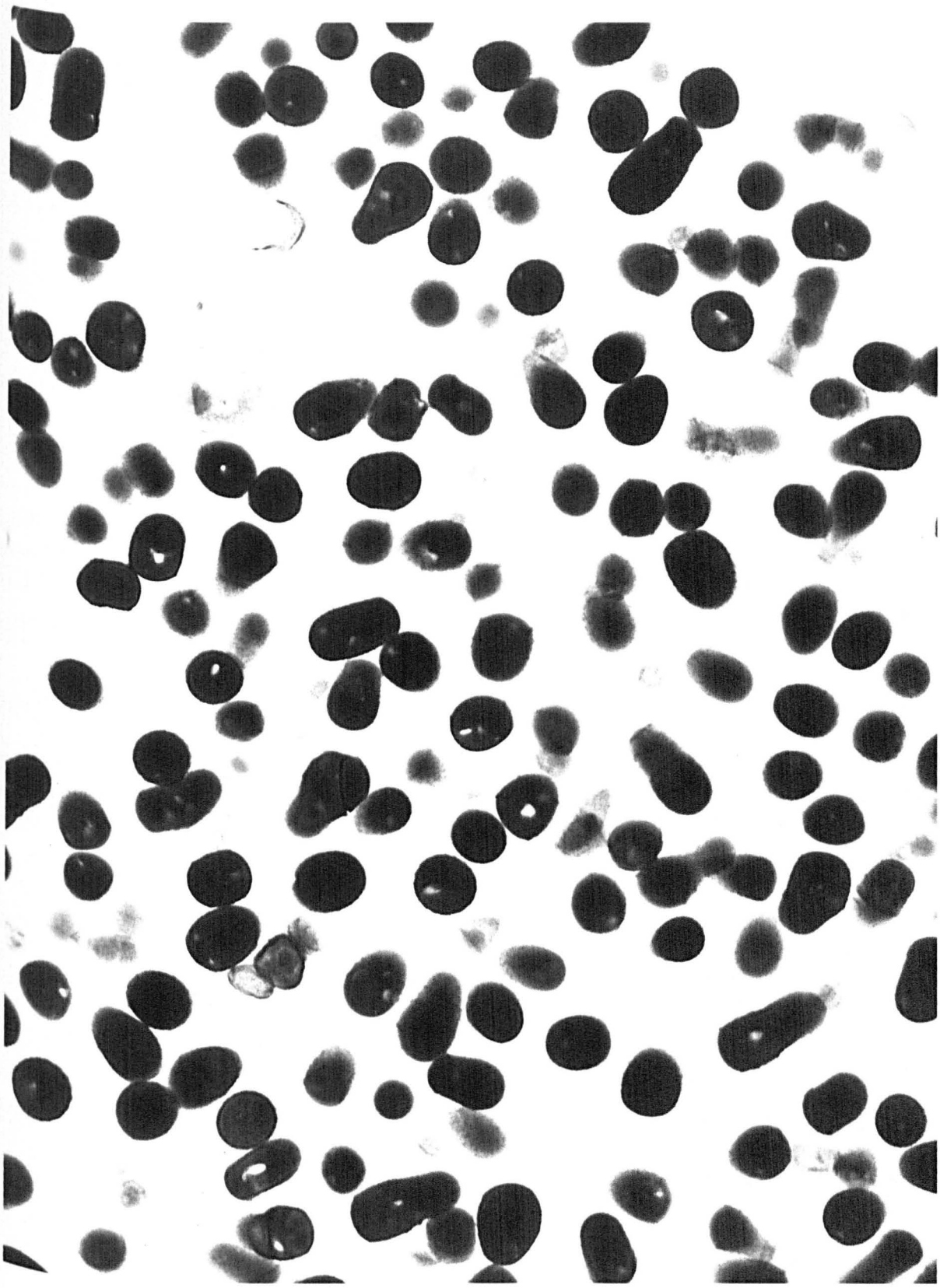


Plate 3.7(e) Transmission electron micrograph of *B. linens* wild-type (Wiesby) undergoing cell division. Cells were grown on YGA medium. (Bar = 0.1μm)

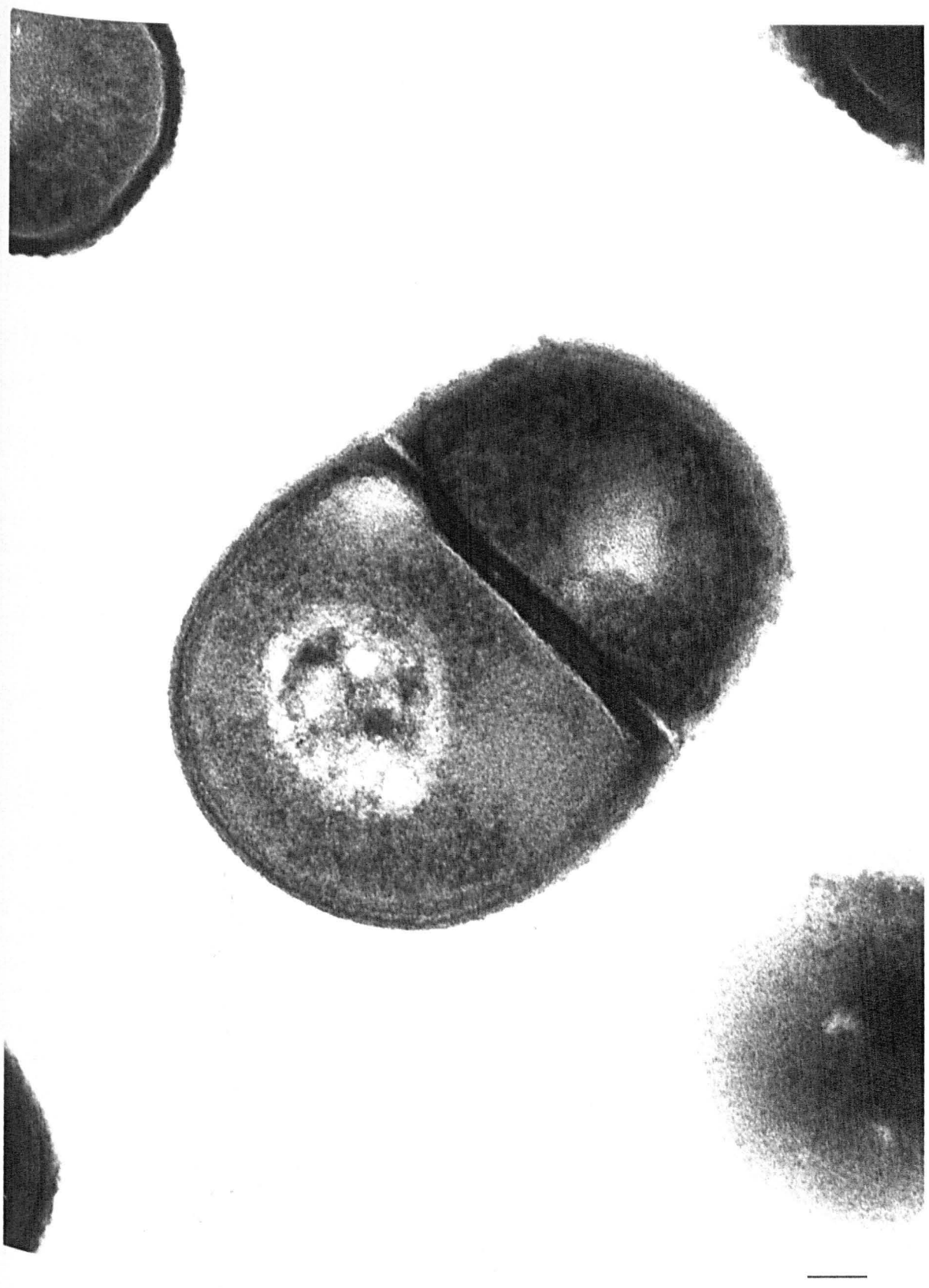


Plate 3.7(f) Transmission electron micrograph of *B. linens* wild-type (Wiesby) grown in YGA medium. The small projections on the edges of cells may be wall bands, the sites of new wall synthesis (Bar = 0.1μm)

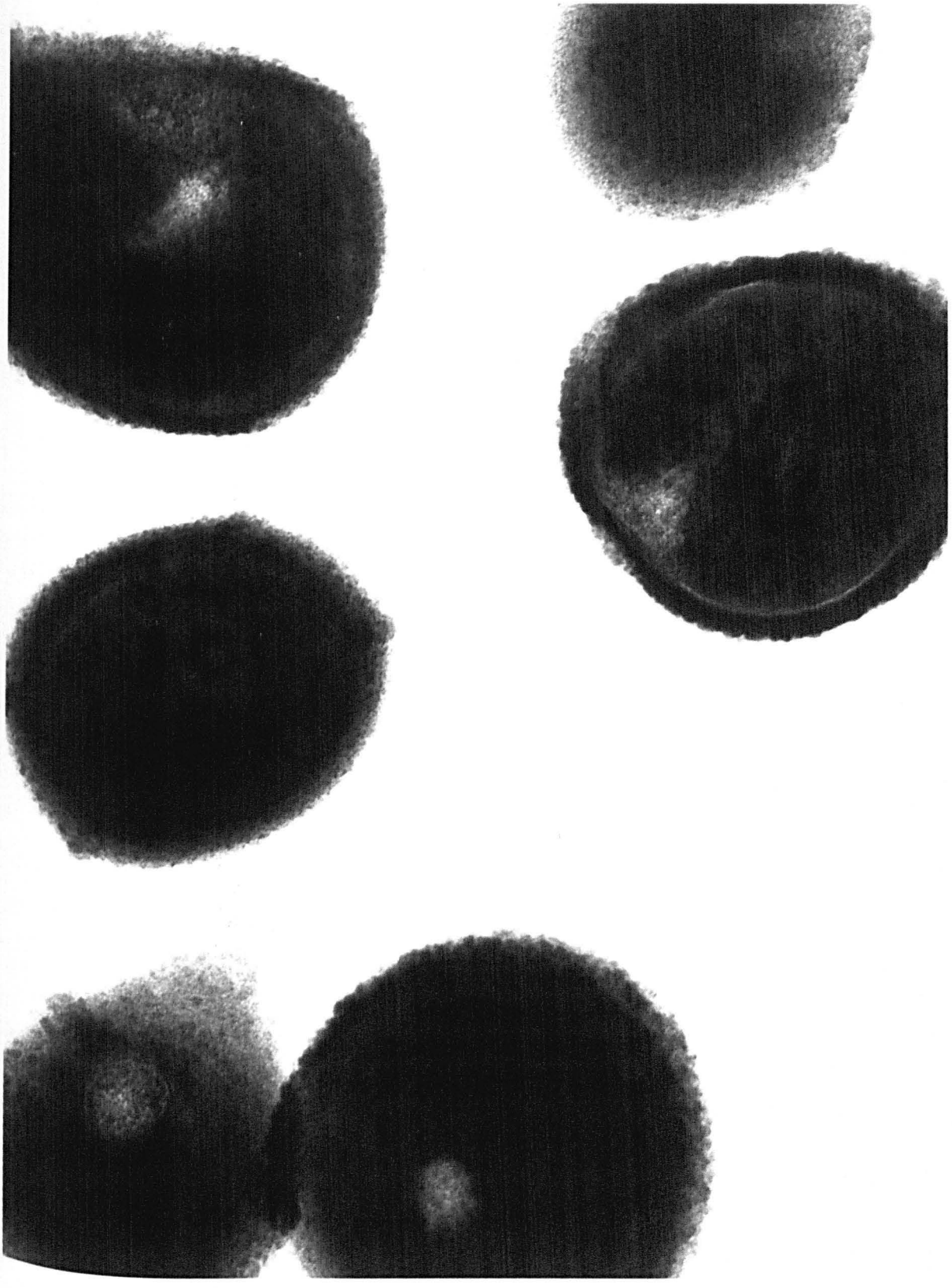


Plate 3.7(g) Transmission electron micrograph of *B. linens* wild-type (Wiesby) grown on YGA medium. The structure connecting the two cells may be a pilus or an artefact. (Bar = 0.1μm)

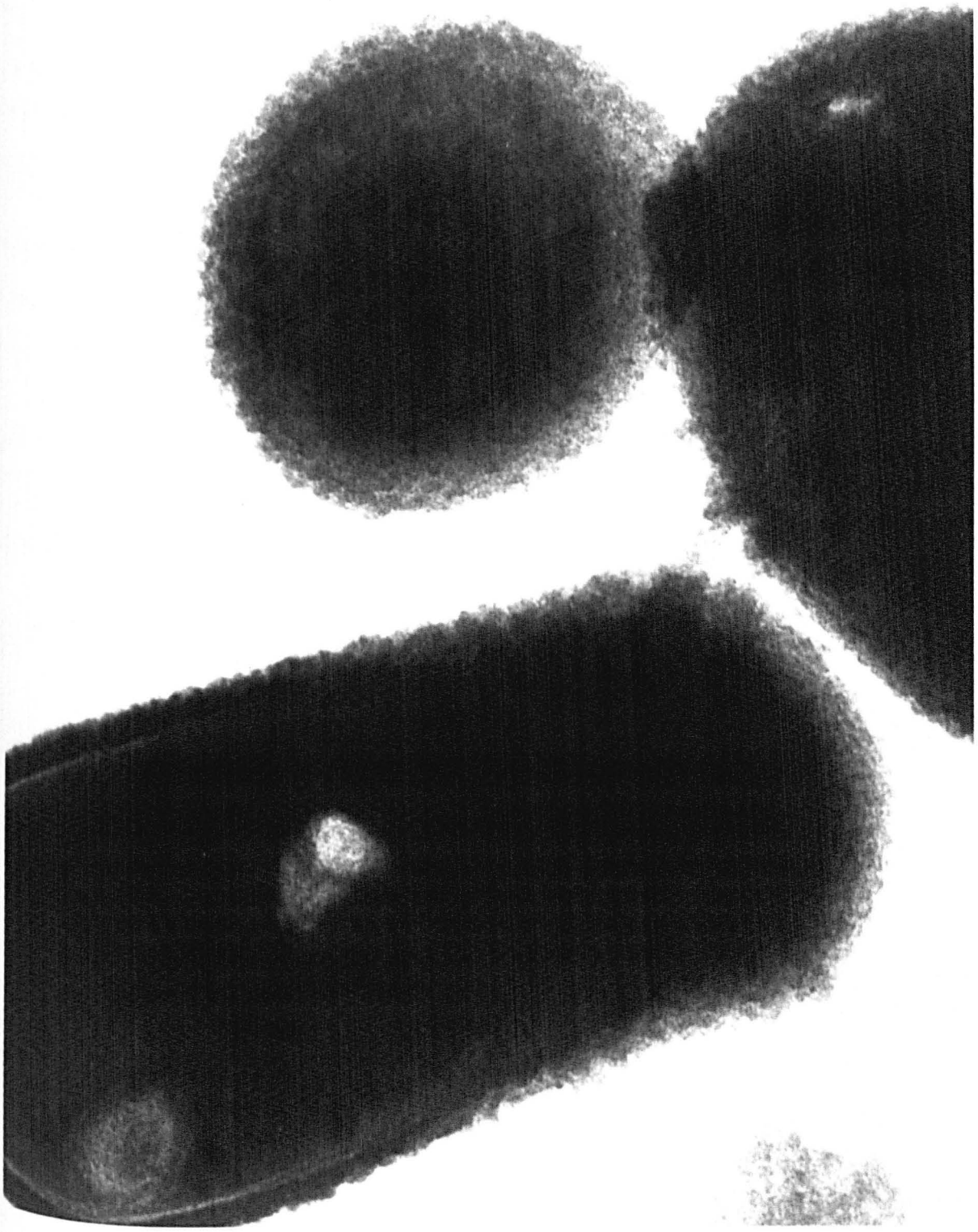


Plate 3.7(h) Transmission electron micrograph of *B. linens* mutant Wkiii showing abortive cell division. Cells were grown on YGA medium. (Bar = 0.1μm)

Plate 3.79(i) *B. linens* mutant Wkiii (Bar = 0.1µm)

Plate 3.7(j) Transmission electron micrograph of *B. linens* mutant Wkiii showing abortive cell division and pleomorphic changes. Cells were grown on YGA medium. (Bar = 0.1μm)

increased membrane proliferation at the expense of cell volume (and therefore biomass) and may account for the high levels of lycopene accumulation observed in mutant Wkiii. Pleomorphism is known to occur on nutritionally poor media along with multicellularity (Römer and Herbert, (1982)) suggesting that mutations in strain Wkiii have affected its ability to synthesise or take up sufficient quantities of growth substrates or that non-essential substrates cannot be assimilated because of the extent of the mutations carried by this strain. Multicellularity is also concordant with the purple membrane proliferation seen in *Halobacterium* during halted aeration (Stanier *et al.*, 1987).

3.8.1 Comparison of growth of mutant and wild type strains of *B. linens*

Amongst the strains of *B. linens* examined in this work, growth rates displayed variation such that some wild type strains reached stationary phase before others. The same was observed in mutant strains where lycopene-accumulating mutants took a longer time to reach stationary phase compared with the wild-type strain. The effect of the mutations in strain Wkiii which affect growth can be seen clearly in comparison with the Wiesby wild-type growth curve (Figure 3.8); strain Wkiii takes at least twice as long to reach stationary phase as does the wild type. The TEM images discussed in Section 3.7 would appear to support this finding, since they show a reduced capacity for complete cell division amongst strain Wkiii cells compared with wild type Wiesby *B. linens* cells.

Brevibacterium linens strain 'Pink' is the slowest growing of all the mutants tested here, reaching stationary phase after approximately 55 hours of incubation (Figure 3.8(b)), contrasting with the Wiesby wild type which reaches the same growth phase after approximately 40 hours of incubation and wild type strain CECT75 which grows at a similar rate (Figure 3.8).

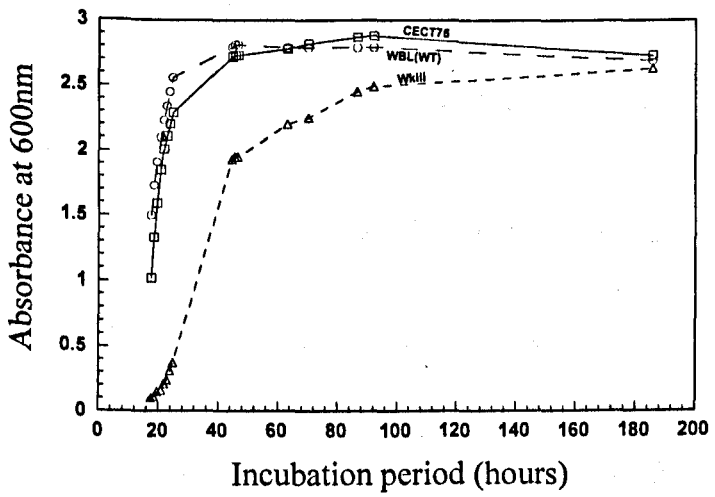


Figure 3.8 Growth curves for wild type *B. linens* CECT75 and from Wiesby. The growth curve for mutant Wkiii is also shown. (Cultures were incubated in standard conditions, see Materials and Methods Section 2.9.1).

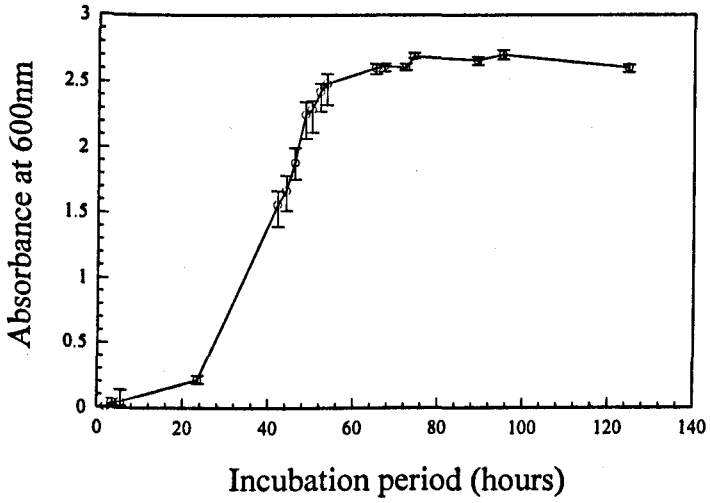


Figure 3.8(b) Growth curve for *B. linens* NCIMB 8546 strain 'Pink' grown under standard conditions

3.8.2 Accumulation of lycopene during growth of mutant strains

Observation of lycopene accumulating strains when grown on solid YGA medium suggested that the most significant level of pigmentation occurs during the stationary phase. Quantitative analysis of lycopene during the growth of strain Wkiii in liquid culture confirms this (Figure 3.8(c)), in agreement with the findings of Tanaka, Kato and Fukui (1971), who tested *Brevibacterium* KY4313 on a hydrocarbon medium.

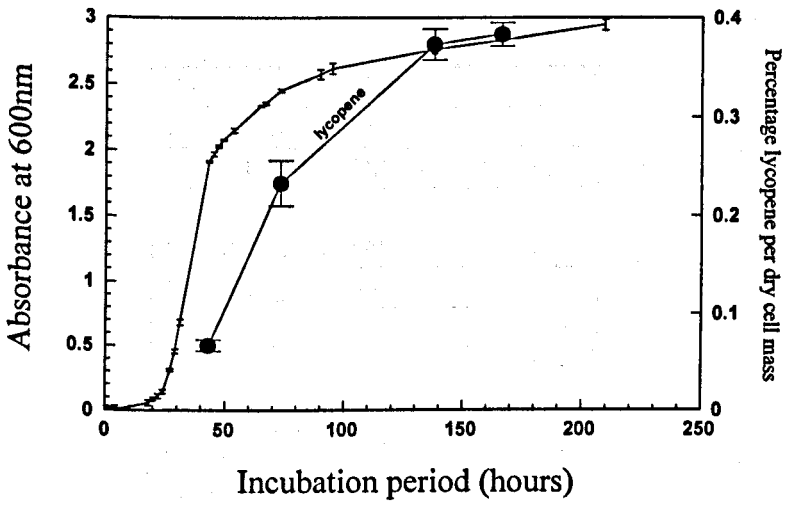


Figure 3.8(c) The accumulation of lycopene during growth of *B. linens* strain Wkiii.

3.8.3 Confirmation of identities of mutant strains

All mutants isolated were found to be of the genus *Brevibacterium* based upon Gram stained morphological studies and biochemical (API Coryne) analyses. (See Materials and Methods Section 2.12). By revealing enzymic activities or the fermentation of sugars through the presence of detectable metabolic end products, together with the application of Gram staining and examination of microscopic morphology, the genus or species was confirmed. The reactions undertaken in the API test strip are given in Table 3.8, together with the results obtained for the wild-type and mutant strains of *Brevibacterium linens*.

Nitrate Reduction	-
Pyrazinamidase	+
Pyrrolidinoyl Arylaminadase	-
Alkaline Phosphate	-
beta Glucuronidase	-
alpha Glucosidase	-
N-acetyl-β-glucosaminadase	-
Esculin (β-Glucosidase)	-
Urease	-
Gelatine hydrolysis	-
Fermentations:	
Glucose	-
Ribose	-
Xylose	-
Mannitol	-
Lactose	-
Sucrose	-
Glycogen	-
Catalase	+

Table 3.8 The results of the test strip API Coryne using *B. linens* and its lycopene accumulating mutants

3.9 Conclusions

The development and selection of strains that accumulate lycopene are shown graphically in Figures 3.9(a) (percentage lycopene per dry cell mass) and (b) (total culture lycopene) whilst the corresponding biomass values are given in Figure 3.9(c). A summary of strain derivations, showing the origin of the mutants examined above, is given in Table 3.9.

A scatter plot, which shows the linear regression between dry cell mass and lycopene concentrations for the above mutants is given in Figure 3.9(d), Figure 3.9(e) shows total culture lycopene against dry cell mass. Clearly, there is an overall negative correlation between dry cell mass and lycopene concentration (value -0.33 (to 2 d.p.)), though this is not entirely consistent with the analysis shown in Figure 3.9(e), where a stronger, positive correlation (value 0.59 (to 2 d.p.)) is evident when total culture lycopene is compared with dry cell mass values. This suggests that the improvement in lycopene concentration is a less important factor, in terms of commercial considerations, than the improvement in cell mass yield provided strains make a sufficient concentration of lycopene per cell.

When attempting to assign the likely causes of these patterns, several factors must be considered. First, the mutagen used, MNNG, is effectively random, so there is a strong probability that the lycopene-accumulating mutants examined here may have mutations elsewhere in their genomes, which might affect cellular metabolism. Secondly, although the alkylation caused by activated MNNG favours certain regions

of the DNA molecule (see Section 3.4), the possibility exists that it will not have affected all mutants in the same manner (contributing to differences in genotype-environmental interactions). Thirdly, there is a likelihood, supported by the data obtained from these mutants, that either the direction of cellular resources away from growth towards lycopene production and/or the membrane proliferation seen in mutant Wkiii (Plate 3.7(h)), or both, may contribute to the negative correlation between pigment concentration and cell mass.

In terms of the efficient conversion of medium solids, total culture lycopene values tend to be highest when cell dry mass values are highest (Figure 3.9(b)), so that in commercial terms, conditions that produce the most highly pigmented cells may not be the most economically viable. At the same time, low lycopene accumulators do not sufficiently compensate by increased cell mass. The compromise which combines both reasonably high dry cell mass and percentage lycopene per dry cell mass values is best seen in strain Wkiii.

The evidence for membrane proliferation is important because it suggests an environment in which the abnormally high levels of lycopene could accumulate. It is impossible to ascertain from these data whether the increased amount of lycopene produced is as a result of increased membrane per cell, or increased lycopene per unit membrane mass. Centrifugation experiments would help in assigning the presence of lycopene to membrane or other cytosolic fractions (see Jones, Watkins and Erickson (1973), mentioned above).

Commercially, data which prove that *B. linens* mutants can produce and accommodate large amounts of lycopene are important. Figure 3.9(a) shows the notable increases in lycopene accumulation as strains have been selected for colour intensity. Mutant WkiiiM1 is particularly highly pigmented, though, as shown in Figure 3.9(c), there is a concomitant decrease in cell dry mass values as lycopene concentrations increase. When taking into account the concentrations of 3,3'-dihydroxyisorenieratene in the wild-type, lycopene concentration values in the mutant strains show that cells are not restricted to producing quantities of pigment which do not exceed those of the wild-type (Section 3.2). The results obtained here also seek to prove that visual selection of mutants with increased pigmentation concentrations is a valid method.

As mentioned above, total culture cell yields are seen to fall as lycopene concentrations increase. These findings are also matched by the change in growth rate of the mutant Wkiii compared with the wild-type (Section 3.8.1). TEM images of Wkiii show that cell division appears to be abortive in some cases (Plate 3.7(h)), which would inevitably reduce culture growth rate. Whether or not the increased production of lycopene or the effects of mutations affecting the cellular metabolism elsewhere is responsible for this, these data do not make clear. As discussed in Section 3.8.2, lycopene accumulates at its greatest concentrations as cultures reach stationary phase, in the manner of a secondary metabolite. It might be argued that the reduced growth rate seen in mutant culture Wkiii is representative of individual cells which actually reach levels of impaired growth consistent with stationary or senescent cultures, more rapidly than the wild-type. In this way cells may be producing large

amounts of lycopene at an earlier stage of the growth of the culture and continue to produce it as culture growth proceeds. The reduced growth rate of individual cells is consistent with the finding that the growth curve for mutant Wkiii does not reach a period of senescence in the time period in which growth curves for the wild-type do.

In terms of the anabolic pathway which leads to the production of the cyclic carotenoids present in wild-type *B. linens*, there is evidence for a series of reactions in which individual ring formation is followed by sequential additions of functional groups (see Section 3.3 for example). The characterisation of the pigments in mutants tested here also lends support to the notion that a lycopene cyclase gene exists in *B. linens* along with a phytoene desaturase gene (Chapter 5 discusses an attempt at characterisation of the mutation in the lycopene cyclase gene).

Many of the mutagens tested here did not yield altered pigment phenotypes, which is at first surprising. However, it must be borne in mind that *B. linens* is a relatively slow growing organism compared with *Eschericia coli* for example, so there will be a smaller number of cells in any culture which are at a point in their division in which they are susceptible. Furthermore, in the case of UV mutagenesis, it is highly probable that 3,3'-dihydroxyisorenieratene affords protection both in light absorption and in the quenching of free radical species. It might also be possible that the mechanism of DNA repair in *B. linens* is superior to that in some other organisms.

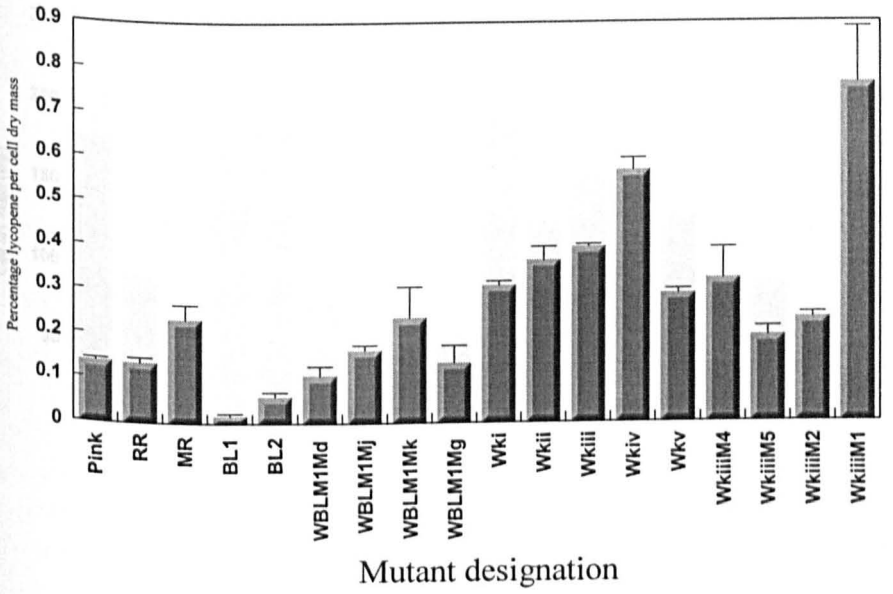


Figure 3.9(a) Percentage lycopene per dry cell mass values in a series of mutants

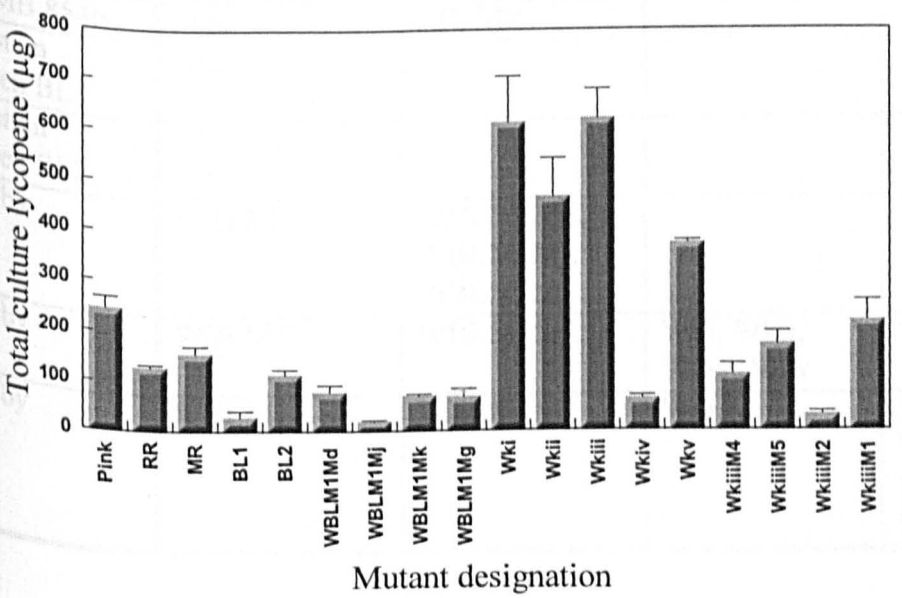


Figure 3.9(b) Total culture lycopene in the mutants

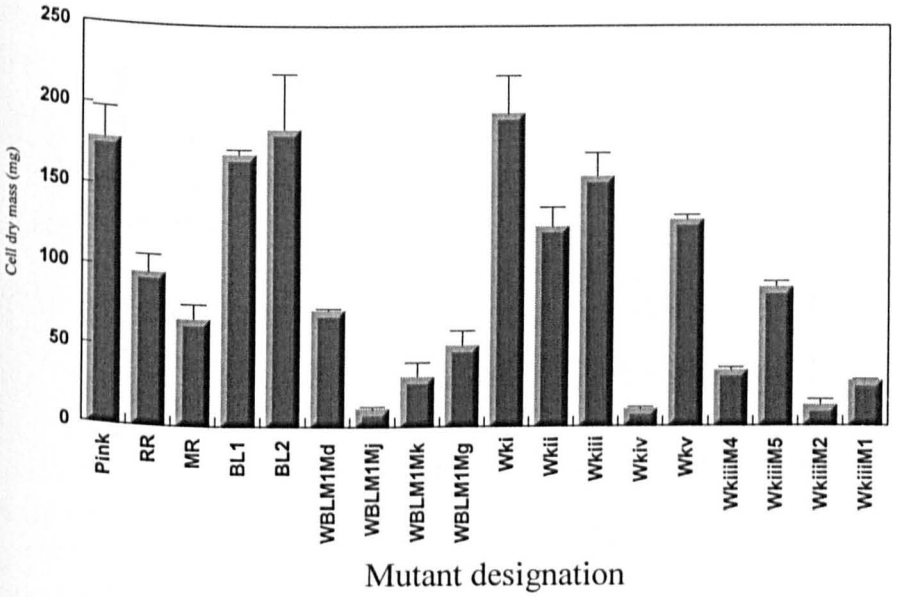


Figure 3.9(c) Dry cell masses of mutants

Parent Strain	Generations mutated to arrive at strain			
	1	2	3	4
NCIMB 8546	Pink	RR, MR		
Christian Hansen BL1	BL1			
Christian Hansen BL2	BL2			
Wiesby	WBLM1	WBLM1Md, WBLM1Mj, WBLM1Mj		
Wiesby	WBLM1	WBLM1Mk	Wki, Wkii, Wkiv, Wkv	
Wiesby	WBLM1	WBLM1Mk	Wkiii	WkiiiM1, WkiiiM2, WkiiiM4, WkiiiM5

Table 3.9 The derivation of strains of mutant *B. linens* examined in Chapter 3.

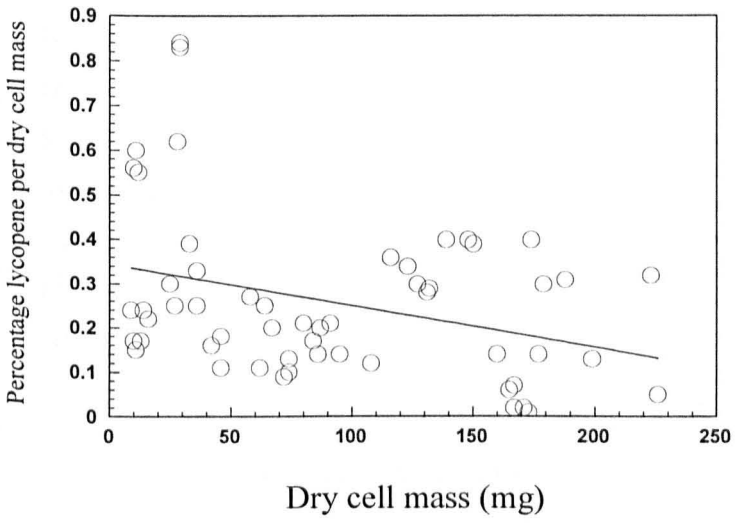


Figure 3.9(d) Scatter plot showing the linear regression between dry cell mass and percentage lycopene per dry cell mass values for mutants accumulating lycopene derived from wild-type strains of *B. linens*.

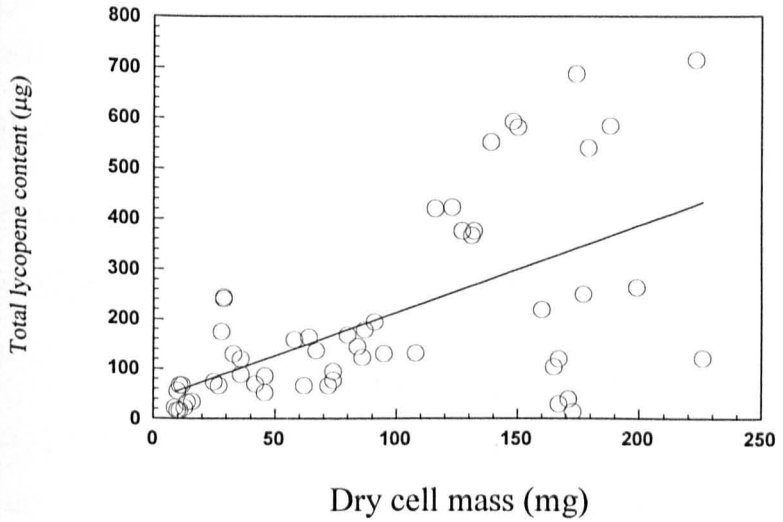


Figure 3.9(e) Scatter plot showing the linear regression between dry cell mass and total lycopene content values for lycopene accumulating mutants derived from wild-type strains of *B. linens*.

CHAPTER 4

AN EMPIRICAL APPROACH TO OBTAIN OPTIMAL LYCOPENE AND CELL MASS YIELDS THROUGH THE MANIPULATION OF GROWTH MEDIUM COMPOSITION AND GROWTH CONDITIONS

4.1 Introduction

An examination of the effects of medium composition and growth conditions on the accumulation of lycopene and on biomass yield for some of the mutants of *B. linens* allows the investigation of genomic-environmental interactions. Thus, for example, if there is a reduction in lycopene yield in high cell yield medium, this suggests that the available medium resources favour the path of cell growth over the path of lycopene production. If the production of lycopene poses a selective disadvantage then, over a number of generations, low lycopene accumulating progeny will predominate. Such behaviour is already implied by the values obtained for dry cell mass when mutants 'Pink' and 'MR' are compared, for example (see Section 3.9). Depending upon the number of generations produced in any closed fermentation system, the application of continuously selective conditions, for example the use of UV light to provide a selective advantage to more highly pigmented cells, may be merited.

Production levels of natural carotenoids are affected by a number of factors including carbon and nitrogen ratio and source, minerals, vitamins, aeration, pH and temperature. Although mutation is probably the primary method for arriving at higher yielding producers, quite significant increases in yield can be achieved by media optimisation. The carotenoid content of another *Brevibacterium* sp., for example, can be doubled or trebled by substituting fumaric acid molasses medium in place of a brain heart infusion or a hydrocarbon medium (Nelis and DeLeenheer, 1989).

All strains of *B. linens* tested in this work were seen to grow well in YGB medium. Commercially however, this medium is too expensive. By examining the capability of a mutant to utilise a medium and then the effects of this medium upon pigmentation levels and biomass yield, this work has been directed towards the identification of a cheap, cost effective source of carbon, nitrogen and essential growth factors. By means of an empirical approach, factors such as these were tested for their effects on lycopene accumulation and cell mass yield with the aim of achieving the best compromise between cost, pigmentation and growth.

4.2 The effects of variation in the carbohydrate composition of growth media

Since cost savings were a major consideration behind the development of a growth medium for *B. linens*, the effects of predominantly carbohydrate medium compositions were first investigated because carbohydrates are generally less expensive than sources of proteins, peptides and amino acids. Ideally, mutant strains of *B. linens* would be capable of obtaining their carbon requirements from these sources. Even if the strains tested here were found to grow poorly upon predominantly carbohydrate media, this finding would at least suggest the possibility of enhancing the carbohydrate utilising capabilities of the strains in future.

4.2.1 Carbohydrate-rich by-products and their effects upon cell mass and lycopene accumulation in *B. linens* mutant strain 'Pink'

Cheap sources of carbohydrate include refinery by-products and products used by the brewing industry. Bundaberg direct consumption raw sugar (BRS) (Bundaberg Sugar, Brisbane, Australia) is an inexpensive unrefined cane sugar composing 98.5-99.7% (w/w) sucrose. As an unrefined product it contains small amounts of ash and is likely to contain nitrogenous compounds and other growth factors. Bundaberg raw sugar molasses and black strap molasses are both viscous liquids containing mainly sucrose, dextrose and laevulose. Here, non-carbohydrate material such as ashes (12% (w/w)), nitrogenous compounds (4% (w/w)) and waxes (4% (w/w)) accounts for a greater part of the dry mass than in BRS.

Brevibacterium linens mutant 'Pink' was used to inoculate sterile liquid cultures containing 3%(w/v) carbohydrate source with 0.1%(w/v) yeast extract (the latter was added since it comprises a broad mixture of minerals, amino acids and growth factors (see Appendix)). Results are shown in Figure 4.2.

No growth of any strains tested here was observed in Black strap molasses plus 0.1%(w/v) yeast extract, even when in a semi-defined medium also containing fumaric acid, ammonium dihydrogen phosphate, malt extract, vitamin B12, $NaHPO_4.12H_2O$, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $FeSO_4.7H_2O$ and $MnSO_{4-n}H_2O$ (proportions are given in Materials and Methods Section 2.8.2) (Tanaka et al., 1971), which is known to allow growth of a strain of *Brevibacterium*, KY 4313. When either

1%(w/v) urea plus 0.1%(w/v) yeast extract or 1%(w/v) yeast extract were added to the semi-defined medium along with 3%(w/v) black strap molasses in the absence of fumaric acid some growth was observed, though unquantifiable because of medium solids. (See Section 4.2.4 for conclusions)

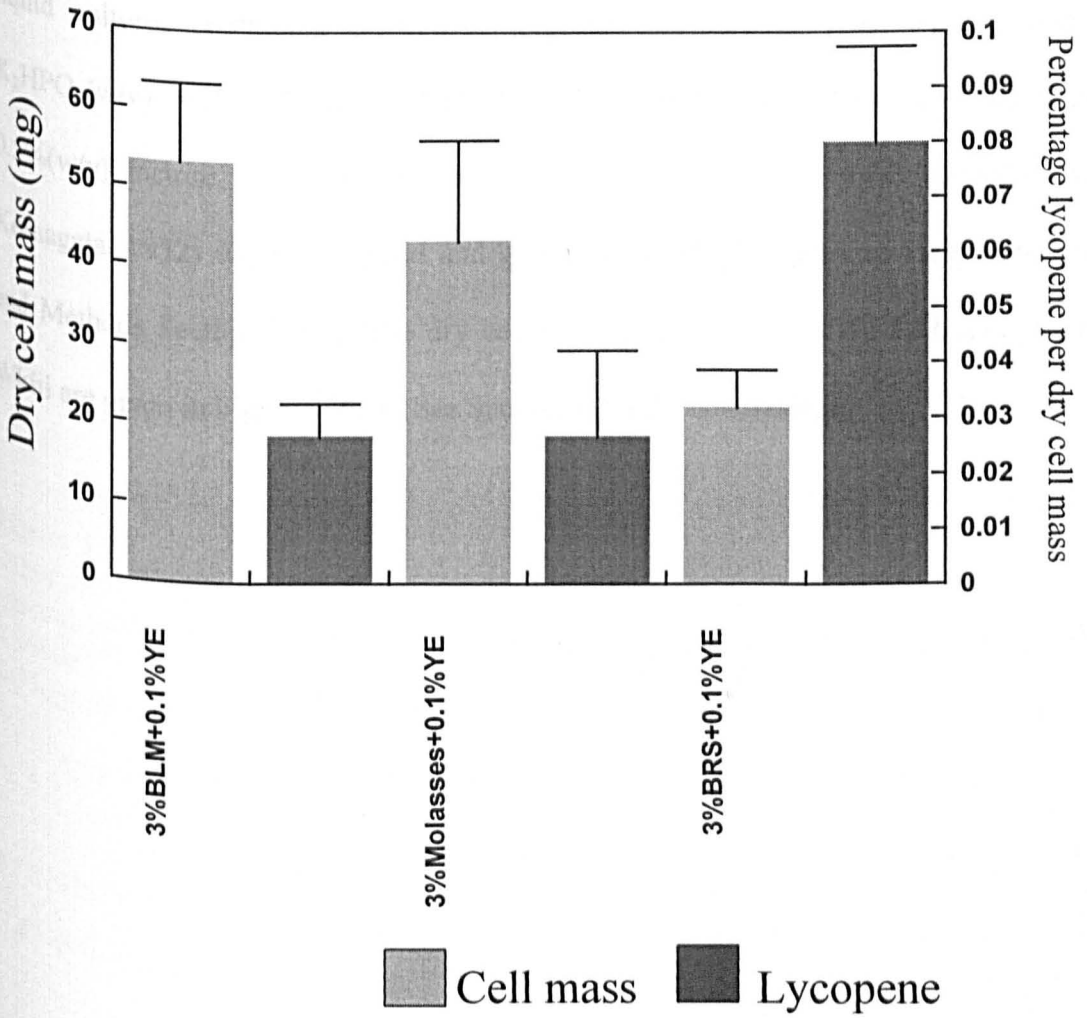


Figure 4.2 Dry cell mass and percentage lycopene per cell dry mass values for *B. linens* mutant strain 'Pink' grown in either 3%(w/v) Bundaberg raw sugar + 0.1%(w/v) yeast extract, 3%(w/v) brewing liquid maltose + 0.1%(w/v) yeast extract or 3%(w/v) Bundaberg raw sugar molasses + 0.1%(w/v) yeast extract in 50ml culture volumes. No growth was observed in 3%(w/v) black strap molasses + 0.1%(w/v) yeast extract.

4.2.2 Individual carbohydrates and their effects upon cell mass and lycopene accumulation in *B. linens* mutant strain 'Wkiii'

Since this experiment was conducted when the high yielding strain Wkiii became available, this was used as the inoculum in place of strain 'Pink'. Sterile liquid cultures containing 0.5%(w/v) tryptone, 0.01%(w/v) yeast extract, 0.1% K_2HPO_4(w/v) and 0.5%(w/v) NaCl (added to enable detectable growth) plus 0.5%(w/v) lactose, glycerol, sucrose, maltose, glucose or fructose (Yamada and Komagata, 1972) were inoculated and grown in standard conditions (see Materials and Methods Section 2.9.1). The dry cell mass values obtained for *B. linens* strain Wkiii are given in Figure 4.2(b). (See Section 4.2.4 for conclusions)

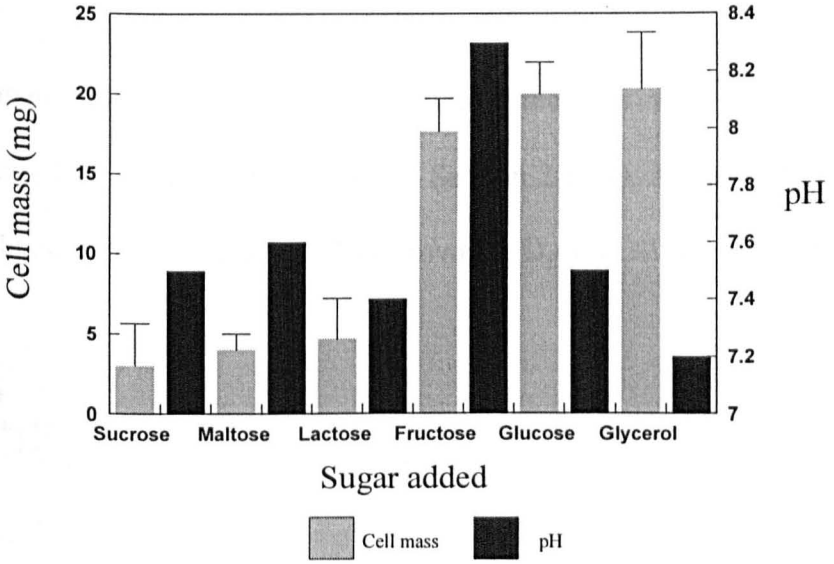


Figure 4.2(b) Dry cell mass values for *B. linens* mutant strain Wkiii grown in either 0.5%(w/v) lactose, 0.5%(w/v) glycerol, 0.5%(w/v) sucrose, 0.5%(w/v) glucose, 0.5%(w/v) maltose or 0.5%(w/v) fructose in a medium containing 0.5%(w/v) Tryptone, 0.01%(w/v) yeast extract, 0.1%(w/v) K_2HPO_4 and 0.5%(w/v) NaCl in 50ml culture volumes (Yamada and Komagata, 1972).

4.2.3 The extent of deuteration of lycopene extracted from *B. linens* grown in a D_2O containing medium

Depending on how oxidative the catabolic pathway in *B. linens* is, substrate hydrogen is subject to total depletion. In an attempt to learn more of the conversion of medium substrates into pigment, *B. linens* strain Wkiii was grown in standard YGB medium, made up in deuterium oxide rather than water, so that carbon source derived hydrogen and medium derived hydrogen were distinguishable.

Cultures took approximately two to three times longer to grow than they would have done if grown in YGB made in water (cells were otherwise grown in standard conditions).

Pigment was extracted using qualitative methods with mass spectrometry grade solvents (see Materials and Methods Section 2.17.1). A mass spectrum for the lycopene obtained from *B. linens* Wkiii grown in D_2O YGB is shown in Figure 4.2 (c,d).

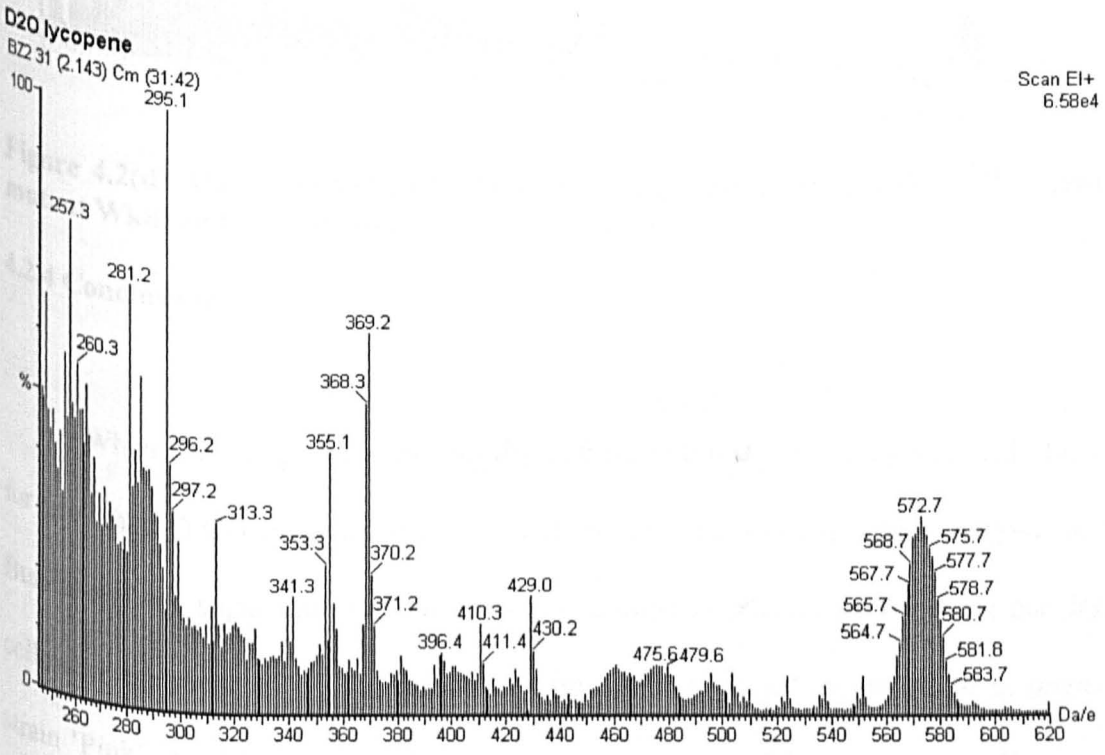


Figure 4.2(c) Mass spectrum obtained from pigment extracted from *B. linens* mutant Wkiii culture, grown in D_2O YGB medium. (The spectrum shown is an expansion of the high mass region of the spectrum given below).

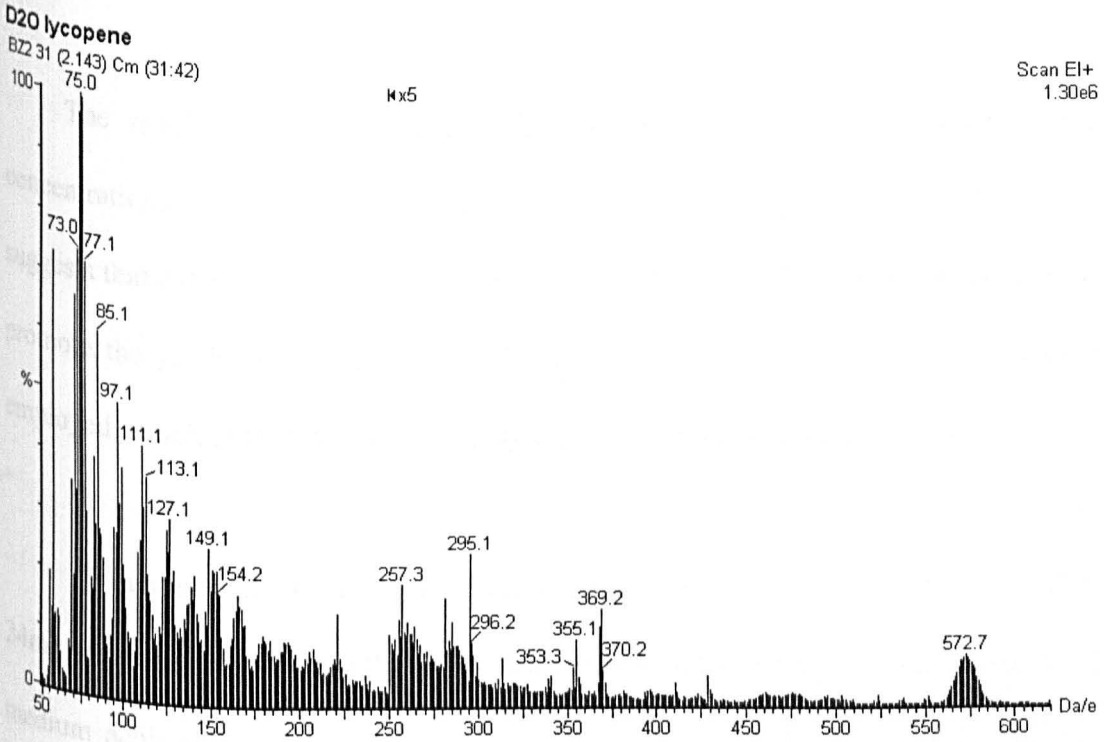


Figure 4.2(d) Mass spectrum obtained from pigment extracted from *B. linens* mutant Wkiii culture, grown in D_2O YGB medium.

4.2.4 Conclusions

When percentage lycopene per dry cell mass figures are compared with those for growth in YGB medium, the carbohydrate sources brewing liquid maltose and Bundaberg raw sugar molasses all showed a decline in percentage lycopene per dry cell mass to a mean of 0.03%, compared with 0.14% obtained by growth of *B. linens* strain 'Pink' in YGB medium. Similarly, cell mass values fell to a mean of 48mg in contrast with the mean mass in YGB medium of 179mg. 3%(w/v) BRS + 0.1%(w/v) yeast extract medium gave a mean percentage lycopene per dry cell mass value of 0.08%, however, which is rather closer to the value seen in YGB medium. Dry cell mass values followed the opposite pattern so that a mean mass of 22mg was obtained for 3%(w/v) BRS medium.

The results shown in Figure 4.2 show that the accumulation of greater concentrations of lycopene, is accompanied by a fall in dry cell mass values. This suggests that a degree of nutritional stress, which causes the decline in cell yield, may promote the production of lycopene. Though no starch utilisation test media were employed, starch is said not to be hydrolysed by *B. linens* (Crombach, 1974).

When individual carbohydrates were tested, no dry cell mass value exceeding 24mg was obtained although the same strain grown under standard conditions in YGB medium achieved a mean dry cell mass figure of 157mg. It is clear that fructose, glucose and glycerol facilitated greater cell mass accumulation than the remaining sugars lactose, maltose and sucrose (see Figure 4.2(b)). Compared with some strains of *Arthrobacter* (among which *B. linens* was originally classified), *B. linens* is noted for leaving relatively large amounts of medium glucose unchanged (Mulder, 1966). Though this pattern emerged amongst the sugars tested here, it is not entirely consistent with the values shown for final medium pH which differed significantly, e.g. pH 8.3 for fructose in contrast with the overall mean value (for the other substrates) of pH 7.4. Such a difference suggests that the presence of fructose may be encouraging greater metabolic conversion of nitrogenous compounds, yielding ammonia.

Though in Section 4.2.4 no values were obtainable for lycopene content, it is likely that lycopene yields would be low in at least the glucose medium tested here, since both in *E. herbicola* and carotenoid expressing, transformed *E. coli*, the presence

of glucose has led to repressed carotenoid pigmentation, probably through the action of cyclic AMP, which has been implicated in the regulation of carotenoid gene expression (Perry *et al.*, 1986).

Media containing only 3%(w/v) glucose + 0.1%(w/v) yeast extract or 3%(w/v) glucose + 0.05%(w/v) yeast extract in the absence of tryptone, K_2HPO_4 and added NaCl showed no signs of cell growth. In spite of this and in agreement with the results of the previous experiment, Mulder (1966) found that in *B. linens* (strain unspecified) glucose and glycerol served as good carbon sources. Sucrose and lactose served as moderate or unutilised sources depending upon the strain tested. These data suggest the importance of sufficient quantities of yeast extract being present in the medium or show that substrates present in the carbohydrate utilisation assessment medium are essential.

In a medium designed to test for the cleavage of carbohydrates containing 3g(w/v) peptone; 2.5g(w/v) NaCl and 5g(w/v) carbohydrate (per dm^3) at pH7.2, 28 carbohydrates and the total of eight strains of *B. linens*, formed no observable acid though a considerable number of strains caused an increase in pH in the absence of citrate (Yamada and Komagata, 1972).

The mass spectrum of lycopene obtained from *B. linens* grown in D_2O YGB medium (Figure 4.2(c)) suggests that the metabolic processes leading to the formation of lycopene are highly oxidative, with an average level of deuteration of approximatley 36 out of a possible maximum of 56 (see the distribution of relative

mass values around the molecular ion *m/z* 572). Fragments of the lycopene molecule are also more difficult to define, this is shown, for instance, by the cluster of fragments surrounding *m/z* 75, which contrasts with the corresponding undeuterated fragment at *m/z* 69 (see Figure 3.1).

4.3 The effects of individual organic and amino acids upon cell mass of *B. linens* mutant strain 'Wkiii'

After studying the sugar utilisation of *B. linens* mutant Wkiii, the next factor to be investigated was the consumption of individual amino acids and organic acids.

Many bacteria require a variety of amino acids for their growth and metabolism and these amino acids should normally be present in the growth medium as small peptides (Ziska, 1968, Kihara and Snell, 1960, Payne and Gilvarg, 1968, Payne, 1971). It is known that there is variation amongst *B. linens* strains in their nutritional requirements, where one or more amino acids are often specifically required (Mulder, 1966). Depending upon the cost of the 'best' amino or organic acid, a possibility exists to develop a defined medium. Cultures of *B. linens* strain Wkiii were used to inoculate sterile media (Yamada and Komagata, 1972) containing approximately 0.03M organic acid or amino acid, glucose 0.02%(w/v), yeast extract 0.01%(w/v), tryptone 0.01%(w/v), K_2HPO_4 0.1%(w/v) and NaCl 0.5%(w/v) (see Materials and Methods Section 2.8.4). Liquid cultures were grown in otherwise standard conditions (see Materials and Methods Section 2.9.1). Dry cell mass values obtained are given in Table 4.3.

Added organic/amino acid	Final pH	Dry cell mass (mg)
Valine	7.7	3
Valine	7.8	4
Valine	7.7	1
Serine	7.6	9
Serine	7.6	5
Serine	7.6	7
Citric acid	7.7	3
Citric acid	7.7	2
Citric acid	7.7	2
Fumaric acid	7.1	6
Fumaric acid	7.0	6
Fumaric acid	7.0	8
α-Ketoglutarate	8.4	10
α-Ketoglutarate	8.4	8
α-Ketoglutarate	8.4	4
Pyruvic acid	8.2	5
Pyruvic acid	8.2	5
Pyruvic acid	8.2	6
Glutamic acid	7.9	6
Glutamic acid	7.8	5
Glutamic acid	7.8	4
Leucine	6.7	2
Leucine	6.8	3
Leucine	6.8	5
Methionine	7.1	1
Methionine	7.2	<1
Methionine	7.2	<1
Glutamine	7.0	4
Glutamine	7.0	2
Glutamine	7.0	3
Proline	6.7	1
Proline	6.8	9
Proline	6.8	5
Histidine	7.0	1
Histidine	7.1	1
Histidine	7.0	4
Lysine	6.9	3
Lysine	6.9	1
Lysine	6.9	1
Phenylalanine	7.0	2
Phenylalanine	6.9	4
Phenylalanine	7.1	2

Table 4.3 (Previous page) Dry cell mass values for *B. linens* mutant strain Wkiii grown in media containing added organic acids or amino acids (see text). (Percentage lycopene per dry cell mass values were unquantifiable). Culture volumes were 50ml.

4.3.1 Conclusions

With a dry cell mass maximum of 10mg when strain Wkiii is grown in a medium containing α-ketoglutarate, values compare very poorly with those obtained in YGB medium (174mg maximum dry cell mass). Since both the sugar utilisation test medium and medium YGB contain 0.5%(w/v) glucose compared with 0.02%(w/v) in the organic acid assimilation test medium, then the poor cell growth seen may be attributable to a shortage of carbohydrate. YGB medium also contains a much higher proportion of peptide and protein sources at 2.5%(w/v), together with yeast extract at 0.3%(w/v) compared with the organic acid assimilation test medium which contains approximately only 0.5%(w/v) organic or amino acid plus 0.01%(w/v) tryptone. Cell mass values resulting from this experiment were too low to justify any conclusions as to the utilisation of any individual amino acids or organic acids.

In view of the possibility that specific routes through the cytoplasmic membrane are available for single amino acids and peptides (Ziska, 1968, Kihara and Snell, 1960, Payne and Gilvarg, 1968, Payne, 1971), the generally auxotrophic phenotype of the mutants examined here may partly or wholly be the effect of the gross morphological changes (including changes to the membrane) present in cells of strain Wkiii (see Section 3.7).

Certain unpigmented cheese *Arthrobacters* required 3%(w/v) L-methionine but not the addition of extra vitamins in a medium containing 0.5%(w/v) glucose, 0.5%(w/v) $CaCO_3$ and 0.025%(w/v) $(NH_4)_2SO_4$ (Mulder, 1966) though there is no reason to suggest that the strains of *B. linens* tested here would respond in the same way. In the case of Brevibacterium KY4313, added individual amino acids exhibited no positive effects upon carotenoid concentration or cell yield, when present at concentrations between 1-2mM, apart from L-histidine which promoted carotenoid concentration slightly, though not the dry cell mass yield (Tanaka, Kato and Fukui, 1971).

4.4 The effects of amino acids mixtures in the presence or absence of glucose upon growth of *B. linens* strain Wkiii

Since amino acids, organic acids and carbohydrates have been investigated, to some degree, individually, the effects of mixtures of amino acids and peptides, such as are found in digests such as tryptone and casamino acids were examined in the presence and absence of glucose.

Casamino acids (Difco Labs. Limited, Surrey, England) or tryptone at a concentration of 0.5%(w/v) were inoculated with *B. linens* strain Wkiii in liquid cultures in the presence or absence of 0.5%(w/v) glucose (yeast extract was absent, in order to assess the capability of the digests (casamino acids and tryptone) to supply essential growth factors). Dry cell mass values are given in Figure 4.4. Cultures were

grown under otherwise standard conditions (see Materials and Methods Section 2.9.1).

As in the previous experiment, dry cell mass values were too low and the level of dry cell mass value variation too high to draw any justifiable conclusions.

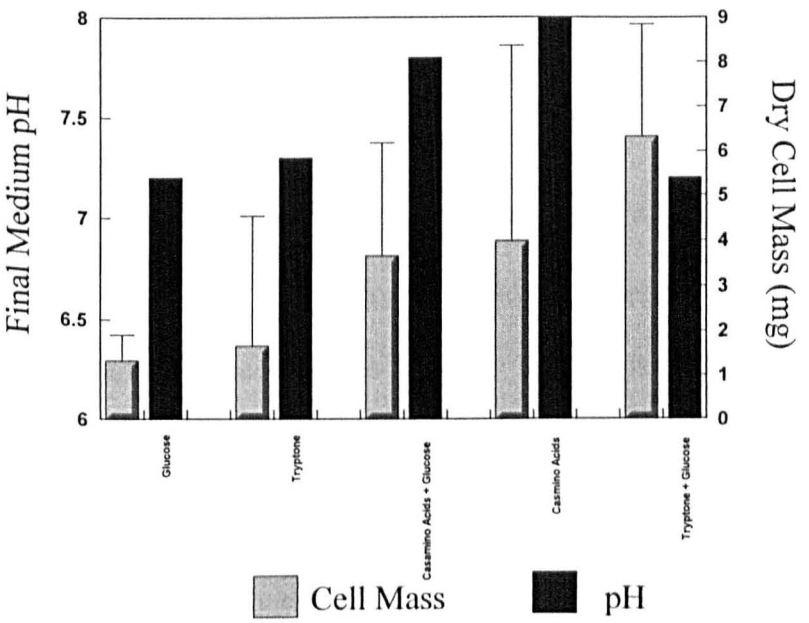


Figure 4.4 Dry cell mass, and final medium pH values for *B. linens* mutant strain Wkiii grown in 0.5%(w/v) glucose, 0.5%(w/v) casamino acids, 0.5%(w/v) tryptone or in combinations of 0.5%(w/v) casamino acids + 0.5%(w/v) glucose or 0.5%(w/v) tryptone+0.5%(w/v)glucose. Culture volumes were 50ml.

4.5.1 The effects of addition of the vitamins thiamine and B_{12} upon cell mass and lycopene accumulation in *B. linens* strain Wkiii

In cell free extracts of *Flavobacterium* R1560, phytoene formation is enhanced by the presence of cofactors (Brown *et al.,* 1975). In growth experiments using cheese coryneforms, Mulder (1966) found that more than half of the organisms tested had no requirement for added vitamins, 7% needed only biotin and the remainder required a mixture of vitamins. Thiamine with or without added biotin was often an essential growth factor, though nicotinic acid and vitamin B_{12} were sometimes required.

Here, a vitamin utilisation test medium consisting of 3% (w/v) neutralised soya peptone (Oxoid, Unipath, Hampshire, England) (a vegetable protein digest (see Section 4.8)) plus various concentrations of either vitamin B_1 (thiamine) or vitamin B_{12} was inoculated with *B. linens* strain Wkiii and incubated under standard conditions (see Materials and Methods Section 2.9.1). Neutralised soya peptone supplemented with 0.1%(w/v) yeast extract served as a comparative growth medium, in which yeast extract was present to ensure adequate cell growth.

Cultures of *B. linens* strain Wkiii grown in 3%(w/v) neutralised soya peptone plus either vitamin B_1 or vitamin B_{12} were assayed for dry cell mass and lycopene accumulation. Results are shown in Figure 4.5.

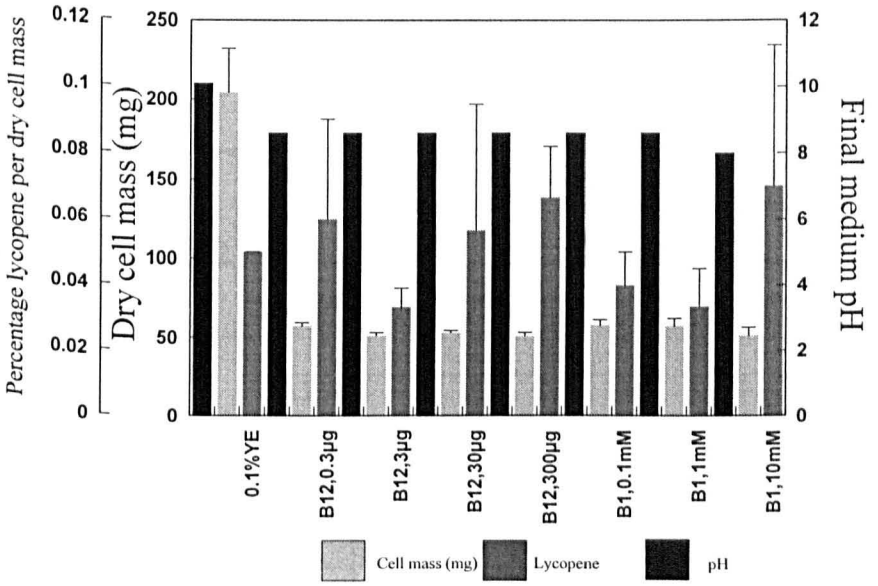


Figure 4.5 Dry cell mass, final medium pH and percentage lycopene per dry cell mass values for *B. linens* strain Wkiii grown in 3%(w/v) neutralised soya peptone plus concentrations of vitamin B_1 or B_{12} (or yeast extract) shown. (*A_{472} for values with values with the suffix † are taken from pigment dissolved in 20ml diethyl ether). Legend labels refer to the concentration of vitamins B_1 or B_{12} in 150ml culture volumes.

4.5.2 The effects of low concentrations of thiamine in combination with carbohydrates and a protein digest, tryptone, upon lycopene accumulation and dry cell mass yield of *B. linens* strain 'Pink'

The previous experiment (Results and Discussion Section 4.5.1) concerned levels of vitamin B_1 in excess of those recommended. In this experiment an attempt was made to evaluate the effects of the presence of recommended thiamine concentrations (see below) upon the utilisation of carbohydrate (Bundaberg raw sugar) (see Results and Discussion Section 4.2.1) alone and carbohydrate plus

tryptone. Strain 'Pink' was used as the inoculum because of its availability at the time of the experiment. The results are given in Table 4.5.

Medium	Final pH	Dry cell mass (mg)	A_{472}* in 10ml diethyl ether	Total lycopene (µg)	Percentage lycopene per cell dry mass
6%(w/v)Bundaberg Raw Sugar + 0.1µgml-1 thiamine	8.1	3	-	-	SQ
	8.1	10	-	-	SQ
	8.1	3	-	-	SQ
6%(w/v)Bundaberg Raw Sugar	8.1	6	-	-	SQ
	8.1	SQ	-	-	SQ
	8.1	7	0.08	2.3	0.03
6%(w/v)BRS + 3%(w/v)tryptone + 0.1µgml-1 thiamine	9.6	196	2.15†	158.1	0.08
	9.6	176	2.13†	156.6	0.09
	9.6	92	-	-	SQ
6%(w/v)Bundaberg Raw Sugar + 3%(w/v)tryptone	8.6	134	1.47†	108.09	0.08
	8.6	157	1.53†	112.5	0.07
	8.6	184	1.92†	141.18	0.08

Table 4.5 Dry cell mass, lycopene mass and percentage lycopene per cell dry mass values for *B. linens* mutant strain 'Pink' grown in the media shown. (*A_{472} for values with the suffix † are taken from pigment dissolved in 25ml diethyl ether). SQ denotes less than quantifiable.

4.5.3 Conclusions

Figure 4.5 shows little contrast between the dry cell mass values obtained across all the vitamin concentrations employed whether vitamin B_1 or B_{12} were added, though they clearly pose a disadvantage when compared with the medium containing neutralised soya peptone with yeast extract. Pigmentation values display more variation however, such that the highest levels of lycopene coincide with the highest vitamin concentrations, whether B_1 or B_{12}, peaking at 0.08 and 0.1 % lycopene per dry cell mass for 300µg of vitamin B_{12} in 150ml culture volume and 10mM vitamin

B_1 respectively. However, the peak value for lycopene per dry cell mass in the medium containing 0.3μg vitamin B_{12} in 150ml culture volume reached 0.09% compared with 0.1% in the 300μg containing medium. The lowest values for lycopene accumulation in vitamin B_1 containing media corresponded with 1mM vitamin B_1 with a mean percentage lycopene per dry cell mass value of 0.03.

The single most important factor affecting the results obtained here is likely to be the concentrations of vitamins used, which in some cases may have been in excess of the amounts required as proposed by Mulder (1966), who suggested 2μgdm^{-3} vitamin B_{12} (equivalent to 0.3μg in 150ml culture volume and 1nM) and particularly in the case of vitamin B_1, where 100μgdm^{-3} (equivalent to 15μg in 150ml culture volume (equivalent to 0.3μM)) was suggested. In light of the excesses used, a one hundred times excess of vitamin B_{12} and a three hundred to 30,000 times excess of vitamin B_1 appear to have a significant detrimental effect upon the cell masses achieved when compared with the neutralised soya peptone and yeast extract medium. This is consistent with the potentially harmful effects in mammalian systems when the thiamin:energy ratio of foods is highly unbalanced, but in the case of this strain of *B. linens*, an excess of either vitamin appears to be unacceptable. In the case of *Brevibacterium* KY4313, thiamine concentrations of up to 200μgdm^{-3} were not inhibitory to cell growth, though carotenoid yield fell on the addition of vitamin B_{12} (Tanaka, Kato and Fukui 1971). The failure of additions of cobalt or methionine to reproduce these effects suggests that *Brevibacterium* KY4313, at least, is not readily able to synthesise vitamin B_{12}. No other water-soluble vitamins exhibited any growth- or pigmentation-enhancing effects.

The addition of thiamine at a concentration of 0.1μgml^{-1} appears not to significantly improve either biomass yield or percentage lycopene per dry cell mass when both carbohydrate and protein digest are present (see Table 4.5). In Section 4.4.2, when the utilisation of individual sugars was examined, fructose was notable in that the final pH of the medium was noticeably greater than that seen in the glucose and glycerol media, despite there being only small differences in culture dry cell mass yields. An interesting, though not perfect, parallel is seen when comparing 6%(w/v) Bundaberg raw sugar + 3%(w/v) tryptone with the same medium plus thiamine; here the final medium pH is a full one pH unit higher in the thiamine-containing medium. It is perhaps the case that the presence of thiamine promotes a particular form of metabolism which encourages the formation of ammonia over and above the levels consistent with the amount of growth promotion achieved. Cell growth in Bundaberg raw sugar medium in the presence or absence of thiamine was minimal.

In animals, thiamine serves as a coenzyme in its active form of thiamine diphosphate (TDP), which functions as a decarboxylase enzyme cofactor. Because C-2 in the TDP thiazole ring is highly acidic, it ionises and is thus able to combine with keto acids such as pyruvic acid *via* a carbonyl group. Through the action of pyruvate decarboxylase, acetyl CoA is finally formed which yields energy in the TCA cycle. The conversion of α-ketoglutarate into succinyl CoA is also TDP-dependent as is the conversion of branched chain amino acids for fatty acid synthesis. TDP also activates the transketolase enzyme of the hexose monophosphate shunt required in the synthesis of nucleic acids (Basu and Dickerson, 1996). The most important source of vitamin

B_{12} (cobalamin) in mammals is bacterial. In the mammalian metabolism, methionine generation, succinyl CoA synthesis and the conversion of leucine are the most significant reactions in which B_{12} has a role as coenzyme.

4.6 The effect of sodium chloride concentration upon cell mass and lycopene accumulation in *B. linens* strain 'Pink'

Brevibacterium linens is considered to be a halotolerant organism, surviving up to 3M sodium chloride (Bernard *et al*, 1993). Whilst salt concentrations high enough to alter cell growth may physically affect the cell through water and nutrient uptake mechanisms, it appears that enzyme activity rather than permeability is affected by sodium chloride. Work by Mulder (1966) showed that sodium chloride may protect the glutamate uptake mechanism of *B. linens* at low pH, even when permeability barriers were disrupted by freeze-thaw cycles. At concentrations greater than 1M sodium chloride, growth of *B. linens* slows but can be restored by the addition of an osmoprotectant such as glycine betaine or its precursor choline at 1mM concentration. Under osmotic stress, ectoine accumulates in cells of *B. linens* together with potassium ions (Bernard *et al*, 1993). An ability to grow in acid pH has also been noted in the presence of high sodium chloride concentrations where normal *B. linens* growth took place at pH6 (Mulder, 1966).

Cells of *B. linens* strain 'Pink' were inoculated into modified sterile YGB medium (which already contains sodium chloride at a concentration of 0.09M) containing added sodium chloride at the concentrations 0.0, 0.2, 0.5 and 1.0M. Cells

were grown under otherwise standard conditions (see Materials and Methods Section 2.9.1). Data are displayed in Figure 4.6.

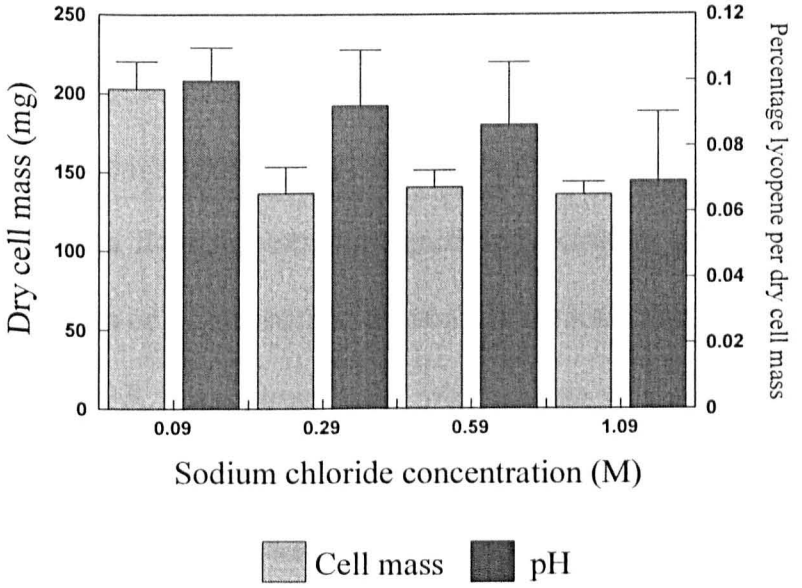


Figure 4.6 Dry cell mass, and percentage lycopene per dry cell mass values for *B. linens* mutant strain 'Pink' grown in modified YGB medium containing sodium chloride to 0.09, 0.29, 0.59 and 1.09M

4.6.1 Conclusions

Clearly optimum growth appears to be achieved at 0.09M sodium chloride which is in general agreement with the work of Bernard (1993) discussed above. At 0.29, 0.59 and 1.09M sodium chloride, there appears to be no significant difference between the mean dry cell mass values obtained, with an overall mean value of 137mg at 0.08 percent lycopene per dry cell mass (mean), compared with the mean

value of 203mg at 0.10 percent lycopene per dry cell mass (mean) obtained from cells grown in YGB medium containing no added salt (0.09M sodium chloride present).

4.7 Examination of proteolysis in mutants and wild-type strains of *B. linens*: Attempts to grow *B. linens* strain 'Pink' in medium containing defatted soya flour

Defatted soya flour represents a significantly cheaper growth substrate than meat or milk proteins or digests such as tryptone. A medium was formulated in which to test the growth of *B. linens* strain 'Pink' which, along with strain Wkiii, has been shown to benefit from the presence of peptide or amino acid rich media rather than predominantly carbohydrate substrates (see Section 4.5.2 for example).

3%(w/v) defatted soya flour, 0.3%(w/v) yeast extract plus 1%(w/v) urea was used as a medium to test for growth of *B. linens* strain 'Pink' as well as lycopene accumulation. Urea was added in order to ensure that a non-protein nitrogen source was available. Cultures were grown under standard conditions.

4.7.1 Conclusions

The defatted soya flour medium was seen to have very poor characteristics at 3%(w/v) in terms of pigment yield, which came to a mean value of 23μg (S.D.= ± 3.6) in 50ml total culture volume. This was in spite of supplementation with 1% (w/v) urea and 0.3%(w/v) yeast extract. Biomass was observed but not measured because of

the presence of medium solids, suggesting the failure of this mutant to perform proteolysis. Though all Brevibacteria are considered proteolytic (gelatin, milk and casein are hydrolysed by most strains (Bousfield, 1972), it would appear that this ability is not present in the strain tested here. Further experiments in which all mutant and wild-type strains used in this work were incubated in a medium containing 3%(w/v) sodium caseinate with or without the addition of 0.1%(w/v) yeast extract or 3%(w/v) sodium caseinate plus semi-defined medium (see Materials and Methods Section 2.8.2) all failed to show growth (results not shown). This indicates that the lack of proteolysis is not unique to the mutant strains tested.

Lycopene accumulation values for the 3%(w/v) soya flour plus 0.3%(w/v) yeast extract and 1%(w/v) urea medium compare poorly with those obtained for the strain 'Pink' grown in YGB medium, which gave a mean total lycopene mass value of 244µg. The failure of urea to promote growth is consistent with the findings of Yamada and Komagata, (1972) who suggested that ureases are absent from *B. linens*. This is further supported by growth experiments in which *B. linens* strains 'Pink' and Wkiii demonstrated no detectable growth in media consisting of 3%(w/v) urea plus 0.1%(w/v) yeast extract and 3%(w/v) urea dissolved in tap water (for the purpose of providing minerals).

4.8 The utilisation of neutralised soya peptone by *B. linens* strains 'Pink' and Wkiii

The above experiment demonstrated the lack of proteolysis in the strains of *B. linens* tested here. Extracellular proteinases are rare in cultured bacteria (Bridson (1994)) and since the proteolytic digestion of defatted soya flour is commercially viable (together with the added benefit that vegetable protein extracts tend to contain high levels of carbohydrates, which have been seen to benefit culture yields (see Section 4.4)), pre-digested 3% (w/v) Oxoid neutralised soya peptone (Unipath, Hampshire, England) (plus 0.1% (w/v) yeast extract), was tested for its effects on biomass and lycopene accumulation in *B. linens* strains 'Pink' and Wkiii. Cultures were grown under otherwise standard conditions. Results are shown in Table 4.8.

Mutant designation	Dry cell mass (mg)	A_{472} in 100ml diethyl ether*	Total lycopene (μg)	Percentage lycopene per cell dry mass
'Pink'	232	1.88*	111	0.05
'Pink'	177	1.30†	96	0.05
Wkiii	53	0.94	277	0.52
Wkiii	52	0.93	274	0.53
Wkiii	52	0.81	238	0.46

Table 4.8 Dry cell mass, lycopene mass and percentage lycopene per cell dry mass values for *B. linens* mutant strains 'Pink' and Wkiii grown in 3% Oxoid neutralised soya peptone (Unipath, Hampshire, England) plus 0.1% Yeast extract under standard growth conditions. Mean final pH values were 10.1 for 'Pink' and 8.7 for Wkiii. (*A_{472} for values with * suffix are taken from pigment dissolved in 20ml diethyl ether; values with the suffix † are taken from pigment dissolved in 25ml diethyl ether).

4.8.1 Conclusions

Values for percentage lycopene per dry cell mass for strain 'Pink' fell by approximately 50% compared with values for this strain grown in YGB medium (Table 3.4(b)), though when peak cell mass values are compared, a rise of 17% is noticeable (Table 4.8).

Strain Wkiii attained a mean percentage lycopene per dry cell mass value of 0.50% in contrast with the mean value obtained in YGB medium of 0.40% (see 3.6(d)). Values for cell mass yield follow an opposing pattern such that the strain Wkiii grown in YGB medium produces over 300% more biomass when compared with growth in the Oxoid neutralised soya peptone medium.

In spite of the differences in growth of each strain when compared with their growth in YGB medium, both are clearly able to utilize the digested soya flour. Strain Wkiii is less able to utilise the digest than strain 'Pink', though with the advantage that lycopene concentration is seen to increase. These data suggest, in light of the commercial viability of this medium, that the use of digested soya flour might be merited if its utilisation by strain Wkiii can be improved.

4.9.1 The effects of medium glutamate upon dry cell mass and lycopene accumulation in *B. linens* strain Wkiii

Wiley (1962) and Stokes (1963) found, after keeping grey-white cheese *Arthrobacter* strains (originally thought to be related to *Brevibacterium*) for a number of years at room temperature on yeast extract agar slopes, that several strains lost the ability to utilize ammonium nitrate and had to be supplied with a mixture of an ammonium salt and glutamic acid. With glutamic acid as the sole nitrogen source growth was poor though growth became proportional to the amount of glutamic acid added when the medium was supplemented with ammonium salts.

In the following experiment, monosodium glutamate, a very common and relatively inexpensive food ingredient, was combined with neutralised soya peptone and yeast extract. The rationale for the inclusion of neutralised soya peptone along with yeast extract, was that the yeast extract would provide the source of growth factors otherwise likely to be absent, but that because of the expense of yeast extract, neutralised soya peptone was added as a cheap source of amino nitrogen likely to be present in insufficient quantity in the amount of yeast extract used.

Cell mass and pigmentation values for *B. linens* strain Wkiii grown in differing proportions of monosodium glutamate, neutralised soya peptone and yeast extract are shown in Figure 4.9. Note that 13.5% and 14.7% of the weight of yeast extract and neutralised soya peptone, respectively, consists of glutamic acid (see

Appendix). Cultures were grown under otherwise standard conditions. Conclusions are given in Section 4.9.3.

4.9.2 The effects of medium glutamate upon dry cell mass and lycopene accumulation in *B. linens* strain Wkiii when additional carbohydrates are present

Because of the comparatively good cell mass and pigmentation values seen in the above experiment, the carbohydrates Bundaberg raw sugar and glucose were combined with neutralised soya peptone and monosodium glutamate in the absence or presence of yeast extract, in case their presence may enhance cell mass and pigment yields. Lycopene accumulation and cell mass values are shown in Figure 4.9.

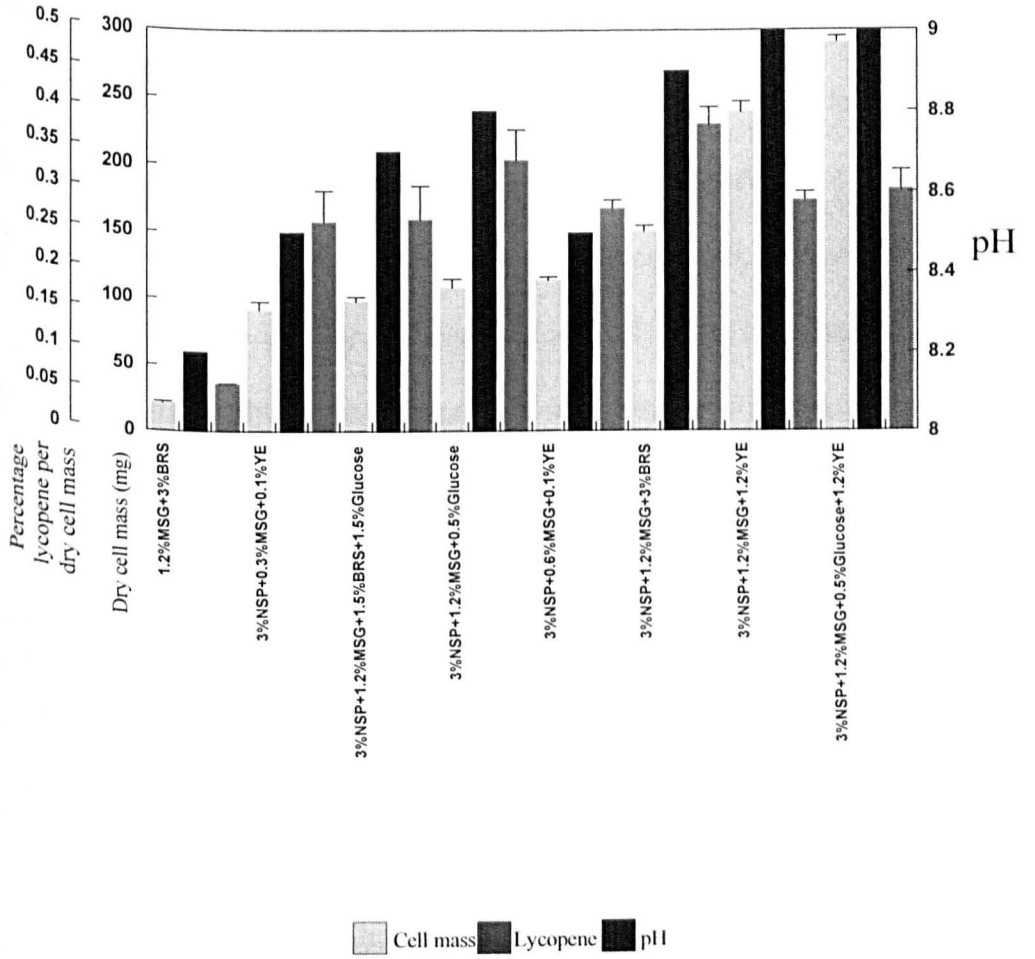


Figure 4.9 shows final medium pH, dry cell mass and percentage lycopene per dry cell mass values obtained from the media shown inoculated with *B. linens* strain Wkiii. All media values shown are %(w/v). Abbreviations refer to the following substrates: NSP Neutralised soya peptone, MSG monosodium glutamate, YE yeast extract, BRS Bundaberg Direct Consumption Raw Sugar

4.9.3 Conclusions

Figure 4.9 shows the results of experiments conducted using media containing monosodium glutamate in combination with a number of other substrates. When media containing 3%(w/v) neutralised soya peptone + 0.1%(w/v) yeast extract +

0.3%(w/v) or 0.6%(w/v) MSG are compared (see Figure 4.9), there is no significant increase in cell mass and pigmentation, despite a doubling in the concentration of MSG. When 1.2%(w/v) monosodium glutamate was combined with 3%(w/v) Bundaberg Direct Consumption Raw Sugar (BRS) only, the lowest dry cell mass and pigmentation values of the whole experiment were obtained, with means of 21mg and 0.06 percent lycopene per dry cell mass respectively. Cell mass and lycopene yields improved dramatically when neutralised soya peptone (NSP) at 3%(w/v) was combined with the same concentrations of BRS and monosodium glutamate as in the previous medium, with mean values of 151mg and 0.38 percent lycopene per dry cell mass. The addition of 3%(w/v) NSP at the sugar concentration of 3%(w/v) promoted an increase in mean dry cell mass of 719% and in percentage lycopene per dry cell mass of 633% when compared with the 1.2%(w/v) MSG plus 3%(w/v) BRS medium.

Where the 3%(w/v) sugar content was provided by a mixture of BRS and glucose at 1.5%(w/v) each, whilst NSP and MSG remained at 3%(w/v) and 1.2%(w/v) respectively, mean cell mass and percent lycopene per dry cell mass values both fell (from 151 to 98mg and 0.38 to 0.27 percent lycopene per dry cell mass) when compared with the medium in which BRS acted as the sole carbohydrate source. When BRS was absent in the 3%(w/v) NSP + 1.2%(w/v) MSG medium and glucose concentration reduced to 0.5%(w/v), mean dry cell mass was 109mg, whilst mean percentage lycopene per dry cell mass was 0.34. When the same medium was altered to contain 3%(w/v) BRS in place of 0.5% glucose, mean dry cell mass and percentage lycopene per dry cell mass values were 151mg and 0.38% respectively. When the 3%(w/v) NSP + 1.2%(w/v) MSG + 0.5%(w/v) glucose medium is compared with the

1.5%(w/v) glucose + 1.5%(w/v) BRS containing medium, no significant difference is evident in either percentage lycopene per dry cell mass or dry cell mass values.

Substitution of the glucose in the 3%(w/v) neutralised soya peptone + 1.2%(w/v) monosodium glutamate + 0.5%(w/v) glucose medium with 1.2%(w/v) yeast extract promoted a dramatic increase in cell dry mass yield from a mean of 109mg to a mean value of 239mg, representing a yield increase of 219%. As noted in previous experiments, the improvement in cell mass yield is often accompanied by a drop in the degree of pigmentation, thus the mean value of 0.34 percentage lycopene per dry cell mass drops to a mean of 0.29, a fall of 15%. The addition of yeast extract is examined more closely in the next section.

The addition of 0.5%(w/v) glucose to the 3%(w/v) neutralised soya peptone + 1.2%(w/v) monosodium glutamate + 1.2%(w/v) yeast extract medium increases mean dry cell mass to 291mg with a mean percentage lycopene per dry cell mass value of 0.30. Thus mean dry cell mass increases by 22% with the addition of medium glucose at 0.5%(w/v). Mean percentage lycopene per dry cell mass does not increase significantly, though it fails to reach the value of 0.34 which is achieved in the 3%(w/v) neutralised soya peptone + 1.2%(w/v) monosodium glutamate + 0.5%(w/v) glucose medium.

Comparing the values obtained here with those obtained for *B. linens* strain Wkiii grown in the standard YGB medium of 157mg mean dry cell mass and mean percentage lycopene per dry cell mass of 0.4, the highest dry cell mass values with a

mean of 291mg from the 0.5%(w/v) glucose + 3%(w/v) neutralised soya peptone + 1.2%(w/v) monosodium glutamate + 1.2%(w/v) yeast extract medium are some 185% greater based upon the mean. The peak pigmentation values obtained occur with the 3%(w/v) neutralised soya peptone + 1.2%(w/v) monosodium glutamate + 3%(w/v) BRS and 3%(w/v) neutralised soya peptone + 1.2%(w/v) monosodium glutamate + 0.5%(w/v) glucose media with values of 0.40 and 0.38 percent lycopene per dry cell mass respectively. The peak pigmentation value for the 0.5%(w/v) glucose + 3%(w/v) neutralised soya peptone + 1.2%(w/v) monosodium glutamate + 1.2%(w/v) yeast extract medium of 0.33 percent lycopene per dry cell mass is lower than the peak value obtained with YGB by a factor of 1.2, though in terms of total lycopene yield from 50ml culture volumes, mean total lycopene for the 0.5%(w/v) glucose + 3%(w/v) neutralised soya peptone + 1.2%(w/v) monosodium glutamate + 1.2%(w/v) yeast extract medium was 885µg compared with 621µg for the YGB medium, an increase of 43%.

In this experiment final medium pH values are generally correlated with the cell mass values obtained, rising when cell mass values rise (see Figure 4.9). It is interesting to note however that, until yeast extract is added at a concentration of 1.2%(w/v), the presence of carbohydrate appears to promote an increase in pH when dry cell masses achieved are otherwise very similar. When the dry cell mass and pH values for 3%(w/v) neutralised soya peptone + 1.2%(w/v) monosodium glutamate + 0.5%(w/v) glucose and 3%(w/v) neutralised soya peptone + 0.6%(w/v) monosodium glutamate + 0.1%(w/v) yeast extract for example, are compared, these give mean dry cell masses of 109 and 114mg respectively but final medium pH values of 8.5 and 8.8

respectively. The increase in pH does not accompany any significant increase in mean dry cell mass.

4.10 The effect of varying concentrations of yeast extract upon dry cell mass and lycopene accumulation in *B. linens* strain Wkiii

Yeast extract is added frequently throughout this work because it is likely to supply amino acids, and especially minerals (see Appendix) and vitamins which may be otherwise absent or present in insufficient quantities in the other growth substrates used. The previous experiment shows that, at the appropriate concentration, yeast extract is an important medium component. Whilst yeast extract is rather expensive and thus less desirable as a commercial substrate, it was important to establish its impact upon cell growth and pigment accumulation.

Neutralised soya peptone was kept constant at 3%(w/v) concentration throughout, whilst yeast extract levels were changed. The effect of these changes upon cell mass, lycopene accumulation and final medium pH are shown in Figure 4.10.

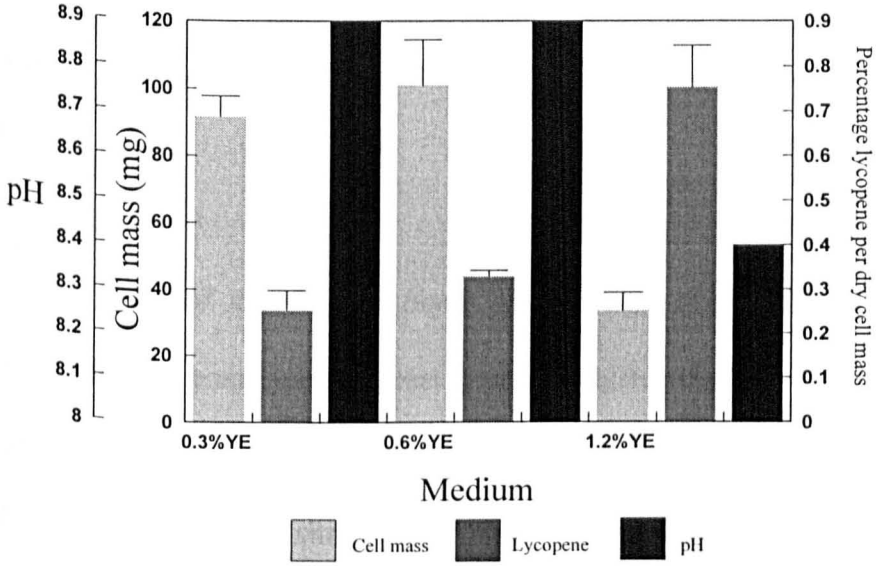


Figure 4.10 Dry cell mass, lycopene mass, final medium pH and percentage lycopene per cell dry mass values for *B. linens* mutant strain Wkiii grown in 3%(w/v) Neutralised soya peptone plus either 0.3%(w/v), 0.6%(w/v) or 1.2%(w/v) yeast extract.

4.10.1 Conclusions

The combination of 3%(w/v) neutralised soya peptone plus 0.3%(w/v) yeast extract yielded a mean dry cell mass value for mutant Wkiii of 92mg with a corresponding mean percentage lycopene per dry cell mass value of 0.25 (see Figure 4.10). This contrasts with the respective values 157mg and 0.4%, obtained from the same strain grown in YGB medium. By doubling the concentration of yeast extract, mean dry cell mass and mean lycopene yield did not increase significantly. At 1.2%(w/v) yeast extract, mean dry cell mass fell to 34mg, but lycopene accumulation was enhanced very significantly, up to a maximum of 0.83% and a mean value of

0.75%. When comparing mean percentage lycopene per dry cell mass values for strain Wkiii grown in YGB medium and in 3%(w/v) neutralised soya peptone plus 0.3%(w/v) yeast extract medium, values are seen to rise by 88%. The final medium pH value for the 1.2%(w/v) yeast extract medium is consistent with its low cell mass yield.

The *Table of Analysis for Some of the Proprietary Media Employed* (see Appendix) shows the analysis for yeast extract alongside that for tryptone, neutralised soya peptone, 'Lab Lemco' powder and bacteriological peptone. In most cases values of components fall within a similar range, with the exceptions of tin and serine, where the values in yeast extract are notably higher and proline, where the yeast extract value is much less. It is difficult to suggest an explanation for the very high pigmentation values achieved with 1.2%(w/v) yeast extract, based on the presence of these constituents, though the presence of yeast extract vitamins may be of relevance. The results shown in Section 4.5, however, suggest that an excess of either vitamin B_1 or vitamin B_{12} is unlikely to be responsible, although the possible effects of the different test medium employed must be borne in mind.

4.11.1 The effects of altered medium carbon/nitrogen ratios upon lycopene accumulation and cell mass in lycopene accumulating mutants of *B. linens*: Strain 'Pink' grown in tryptone and Bundaberg Raw Sugar

As mentioned in Section 4.2, above, the ratio of carbon to nitrogen in a growth medium is known to affect carotenoid production. *B. linens* strain 'Pink' was used as the test inoculum in a number of growth media containing differing mass ratios of tryptone and Bundaberg raw sugar. Results are shown in Figure 4.11(a). Conclusions are given in Section 4.11.3.

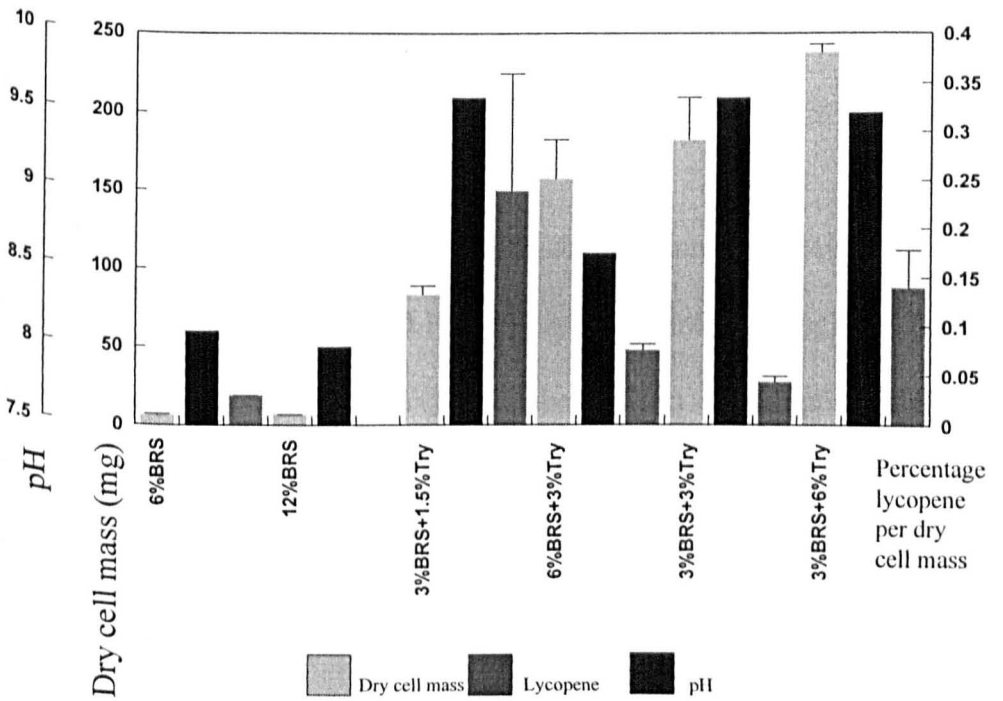


Figure 4.11(a) Dry cell mass, lycopene mass and percentage lycopene per cell dry mass values for *B. linens* mutant strain 'Pink' grown in the media shown.

4.11.2 The effects of altered medium carbon/nitrogen ratios upon lycopene accumulation and cell mass in lycopene accumulating mutants of *B. linens*: Strain Wkiii grown in neutralised soya peptone, yeast extract and Bundaberg raw sugar

A similar study to that shown above was conducted using differing ratios of neutralised soya peptone (Oxoid) and, as before, Bundarberg direct consumption raw sugar in the presence of a low concentration of yeast extract. The strain of *B. linens* used in this case was Wkiii because of its availability and superior pigmentation values. neutralised soya peptone was used also because its ability to allow growth had been proven, although it was supplemented with yeast extract to provide extra or otherwise absent growth substrates and growth factors.

Combinations of 2.5%(w/v) neutralised soya peptone + 0.5%(w/v) Bundaberg direct consumption raw sugar + 0.1%(w/v) yeast extract through to 0.5%(w/v) neutralised soya peptone + 2.5%(w/v) Bundaberg direct consumption raw sugar + 0.1%(w/v) yeast extract were made up as sterile liquid media and inoculated with *B. linens* strain Wkiii. Cultures were incubated under standard conditions. Values obtained for lycopene accumulation, dry cell mass and final medium pH are shown in Figure 4.11(b).

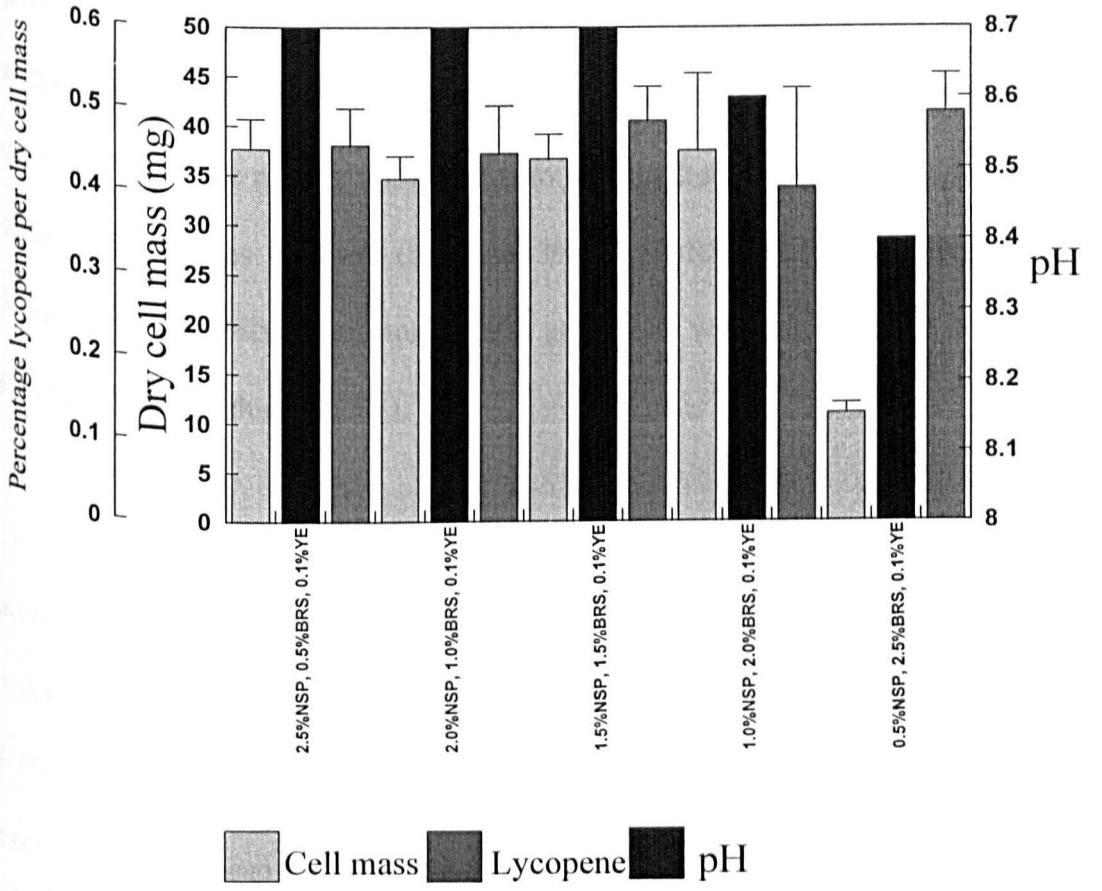


Figure 4.11(b) Dry cell mass, final medium pH and percentage lycopene per cell dry mass values for *B. linens* mutant strain Wkiii grown in concentrations of neutralised soya peptone (NSP) from 2.5 to 0.5%(w/v) plus opposing concentrations of Bundaberg direct consumption raw sugar (BRS) ranging from 0.5 to 2.5%(w/v) in 0.1%(w/v) yeast extract (represented here, by '0.1%').

4.11.3 Conclusions

The results of experiment 4.11.1, shown in Figure 4.11(a), show that *B. linens* strain 'Pink' is essentially unable to utilise Bundaberg raw sugar solution when present at 6 or 12%(w/v) sugar. When 3%(w/v) BRS is supplemented with 1.5%(w/v) tryptone, growth improves, with a mean value of 83mg in a 50ml culture volume. When both of the above substrate concentrations are doubled, mean dry cell mass also

increases to 158mg though the lowest lycopene accumulation value is the same as that seen in the 3%(w/v) BRS +1.5%(w/v) medium. Overall, a slight increase is accrued in dry cell mass as the ratio of amino acids to carbohydrate increases, though lycopene accumulation values fluctuate (both the 3%(w/v) BRS + 1.5%(w/v) tryptone and 3%(w/v) BRS + 6%(w/v) tryptone media grew cells with base percentage lycopene per dry cell mass values of 0.07).

In experiment 4.11.2 (see Figure 4.11(b)), values for cell mass and lycopene accumulation do not alter significantly over the media concentrations 2.5%(w/v) NSP + 0.5%(w/v) BRS + 0.1%(w/v) yeast extract to 1.0%(w/v) NSP + 2.0%(w/v) BRS + 0.1%(w/v) yeast extract. In the 0.5%(w/v) NSP + 2.5%(w/v) BRS + 0.1%(w/v) yeast extract medium, mean dry cell mass falls dramatically from 38mg in the 1.0%(w/v) NSP + 2.0%(w/v) BRS + 0.1%(w/v) yeast extract medium to 11mg, although there is no significant difference in lycopene accumulation.

4.12 Attempts to adapt and select cells of Wkiii for efficient utilisation of high carbohydrate, low protein media

Bacteria are known for their ability to adapt over a number of generations to environments which are barely able to sustain their growth. In some cases, it is possible to 'wean' bacteria from a rich medium to one which is minimal or near minimal and yet maintain good cell yields or cell product yields. By selecting for colonies which grow more rapidly than others and subculturing from these onto a poorer medium, then repeating the process of selection and subculturing, the

possibility exists of obtaining generations which may thrive on a medium which is lower in protein, peptides or amino acids and for the purposes of commercial culture, less expensive.

A single colony of *B. linens* mutant Wkiii was diluted in 250ml YGB medium. Aliquots were spread onto solid medium containing 2.5%(w/v) neutralised soya peptone, 0.5%(w/v) Bundaberg raw sugar plus 0.1%(w/v) yeast extract in 1.5%(w/v) agar. After 3.5 days of dark incubation at 30°C, the largest colonies were selected.

Passage of selected large cultures through progressively carbohydrate-rich and amino acid-poorer media, through increments of 0.5%(w/v) substrate (culminating in the poorest medium containing 0.5%(w/v) neutralised soya peptone plus 2.5%(w/v) BRS plus 0.1%(w/v) yeast extract), failed to increase lycopene content and may have favoured selection of low lycopene yielding strains more able to cope with the increasingly stringent growth conditions (since cell resources may be targeted away from pigment production when nutrients are scarce). Percentage lycopene values were obtained between 0.4% for the richest medium down to 0.01% for the poorest (values not shown). Dry cell mass values in 50ml cultures peaked over the range 130-149mg in the richest medium and fell to approximately 10mg in the poorest medium.

4.13 Growth on solid media

Visual evidence suggested that lycopene concentration in cells grown on a solid medium may be greater than that seen in liquid cultures incubated in the same medium, perhaps through an increased level of aeration resulting from direct contact with the atmosphere. Lycopene in stationary phase, fully coloured cultures of *B. linens* strain Wkiii was analysed quantitatively. No significant difference was found between mutant cell lycopene accumulation when grown on either solid or liquid YGB medium. This finding may be consistent with the appearance of colonies on solid medium when observed from below which appear to display greater peripheral pigmentation, thereby maintaining overall culture pigmentation at a lower concentration.

4.14.1 The effects of two different growth temperatures upon the growth rate of *B. linens* strain 'Pink'

Since large scale fermentation can generate considerable levels of heat, it is important to evaluate the effects of temperature upon the growth rate of *B. linens*. In this experiment the growth temperature normally used for the incubation of the strains tested here was compared with a lower temperature, to establish the effects upon growth rate.

A small number of measurements of growth were taken by absorbance at 600nm over a period of nearly three days (66.5 hours), using cultures of *B. linens* strain 'Pink' grown at either 30°C or 20°C in YGB medium in otherwise standard conditions. Results are shown in Figure 4.14(a).

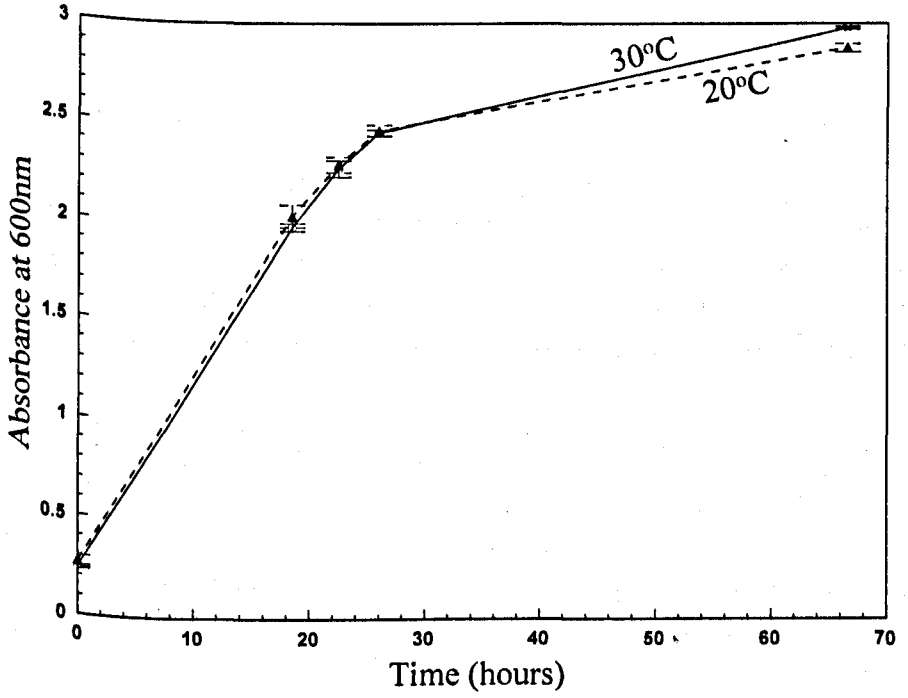


Figure 4.14(a) Growth curves for *B. linens* strain 'Pink' grown at 30°C and at 20°C

4.14.2 Comparative cell mass and lycopene yields for the *B. linens* strains 'Pink' and Wkiii grown at 33°C

In the context of its industrial application, temperatures greater than 30°C are preferred because of the cost constraints involved in cooling plant. According to work by Keddie and Jones (1981) maximum growth temperatures amongst strains of *B. linens* tend to be in the range 30-33°C. The maximum growth temperature of *B. linens* is raised when the medium contains 4%(w/v) sodium chloride. Certain strains of *B. linens* are known to grow well at 37°C; these include ATCC8377, ATCC21330. Strain ATCC21330 grows at 40°C in Oxoid Nutrient Broth medium. The following experiment examined the effects of an incubation temperature of 33°C upon cell mass yield and lycopene accumulation in the *B. linens* strains Wkiii and 'Pink'. Results are shown in Figure 4.14(b).

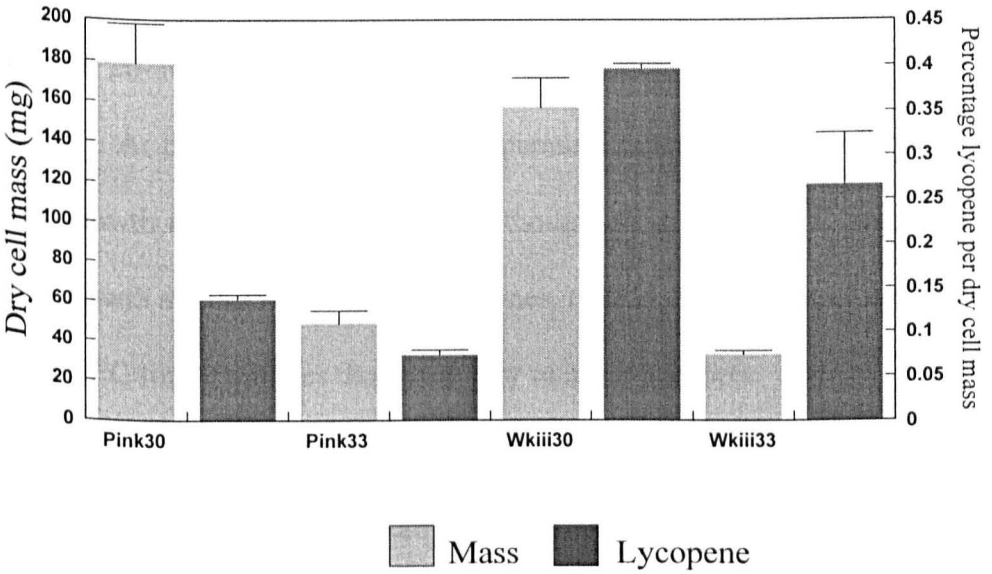


Figure 4.14(b) Dry cell mass, and percentage lycopene per dry cell mass values for *B. linens* mutant strains 'Pink' and Wkiii grown at 30 and 33°C.

4.14.3 Comparative dry cell mass yields of *B. linens* strain Wkiii grown at 27°C and at 30°C

When the growth temperature of *B. linens* strain Wkiii is reduced to 27°C from 30°C (see also Section 4.16.2), mean dry cell mass values changed dramatically, at 185mg (S.D.=13.3) and 122mg (S.D.= 5.6) respectively. The reduction in growth temperature by 3°C resulted in a 52% increase in mean dry cell mass. Cells were grown under otherwise standard conditions (see Materials and Methods Section 2.9.1).

4.14.4 Conclusions

As shown in Figure 4.14(a), the difference of 10°C between incubation temperatures 20-30°C appears to have little effect upon the growth rate of *B. linens* strain 'Pink'. At the lower end of the temperature range, some *Brevibacteria* show moderate growth after two weeks at 5°C, though all grow at 30°C and most show optimum growth at 20-25°C (Keddie and Jones, (1981). Most *B. casei* strains survive heating at 60°C for 30 minutes though at very reduced numbers.

All strains of *B. linens* tested here appeared to be unable to grow at 35 or 37°C, which is consistent with the majority of *B. linens* strains (see above). In Figure 4.14(b), the dry cell mass and lycopene accumulation values for *B. linens* strains 'Pink' and Wkiii are shown for the growth temperatures 30 and 33°C. The mean dry cell mass value for 'Pink' of 49mg compares with 179mg when grown at 30°C under standard conditions (see Section 2.9.1). In the case of strain Wkiii, the value fell from 157mg to 33mg. Thus reduction in incubation temperature caused a decline in mean dry cell mass of 73% in the case of strain 'Pink' and 79% in the case of strain Wkiii. Mean pigmentation values also fell, from 0.14% to 0.07% lycopene per dry cell mass in the case of strain 'Pink' and 0.4% to 0.3% lycopene per dry cell mass in the case of strain Wkiii, equivalent to declines of 50% in strain 'Pink' and of 25% in strain Wkiii.

It is clear, therefore, that in respect of incubation temperature, a declining cell mass and an increase in cellular stress are not coincident with increased lycopene yield, which is perhaps the opposite case to that which occurs in some medium

comparisons (see Chapter 4). Indeed, in the case of *Rhodococcus ruber* carotenogenesis was seen to be enhanced at reduced growth temperature (Takaichi and Isshidsu, 1993).

4.15 The effect of cold shock upon lycopene accumulation and dry cell mass yield in *B. linens* strain 'Pink'

Occasionally, cold shock treatments applied to carotenogenic cells may increase the intensity of pigmentation. The mechanism by which this might occur is unknown, though it may form part of a stress response.

Cultures of *B. linens* strain 'Pink' were subjected to cold shock treatment at 23.5, 53.5 and 89.0 hours post inoculation. Cells to be shocked were exposed to a 4°C incubation temperature for a period of two hours. Results are shown in Figure 4.15.

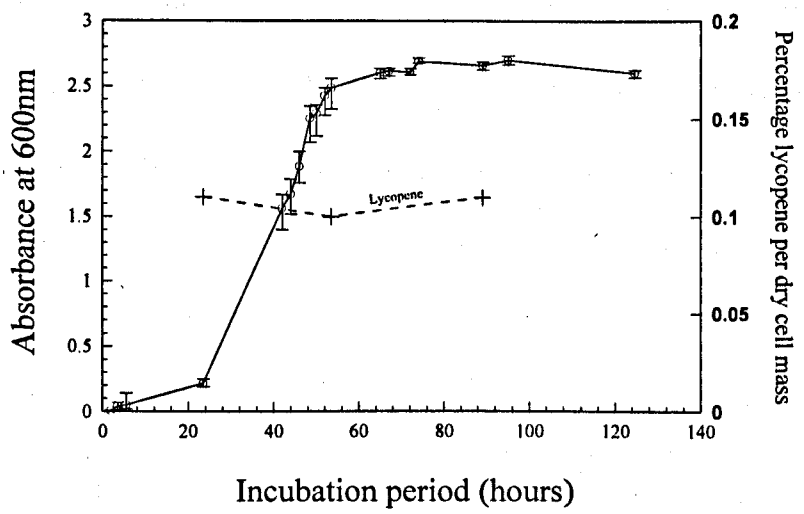


Figure 4.15 Dry cell mass, total lycopene and percentage lycopene per dry cell mass values obtained from cultures of *B. linens* strain 'Pink' cold shocked for two hours at 4°C at the times post medium inoculation shown.

4.15.1 Conclusions

The mean lycopene accumulation value of the cold-shock treated cells is 21% less than for cells that have been incubated normally. Similarly, mean dry cell mass values are seen to fall from 179mg obtained by standard growth conditions to 125mg for cold shocked cells. It would appear therefore, that cold shock treatment of *B. linens* strain 'Pink' as conducted here, serves only to slow or impair both cell growth and pigmentation.

4.16.1 The effects of illumination upon colony growth and pigmentation of Wild type and 'Pink' strains of *B. linens*

Pigmentation amongst *B. linens* strains is often induced by illumination, so that more than half of all strains tested (Mulder *et al*, 1966) produce orange pigment only when exposed to light. In the non-photosynthetic, Gram-negative bacterium *Myxococcus xanthus*, down-regulation of light induced carotenogenesis is thought to occur once carotenoids begin to accumulate (Hodgson, 1993). So in spite of their protective role, carotenoid production appears to be under the control of a feedback mechanism once the damaging effects of illumination are controlled (Hirschberg, 1998). Certain fungi also display photoregulated carotenogenesis. Thus *Neurospora crassa* undergoes light-induced altered transcription of its carotenogenesis genes during conidiation (Arpaia, Carattoli and Macino, 1995).

In order to establish initial parameters relating to the influence of illumination upon lycopene production in cells of mutant 'Pink' and wild type NCIMB8546 *B. linens*, YGA plates containing confluent growth were exposed at a distance of 20cm from a bank of fluorescent lights, with control plates covered with aluminium foil and test plates covered likewise though with windows cut in the foil to allow colony illumination.

Plates 4.16 and 4.16(b) show *B. linens* strain 'Pink' and wild type respectively after exposure to a bank of 40W fluorescent lamps for five days. Conclusions are given in Section 4.16.3.

Plate 4.16 *B. linens* mutant strain 'Pink' grown in darkness (bottom) and under illumination (top) on YGA medium

Plate 4.16(b) *B. linens* wild-type strain NCIMB8546 grown in darkness (bottom) and under illumination (top) on YGA medium

4.16.2 The effects of illumination upon the cell mass yield of *B. linens* strain Wkiii at 30°C and at 27°C

A separate experiment examined the effects of varying growth temperatures and light intensity in case cells should be subject to any synergistic effects involving both of these factors.

Cultures of *B. linens* strain Wkiii were grown in otherwise standard conditions in liquid medium and subject to illumination as in the previous experiment. Dark grown cultures were grown alongside but were shielded using aluminium foil. Results are shown in Figure 4.16.

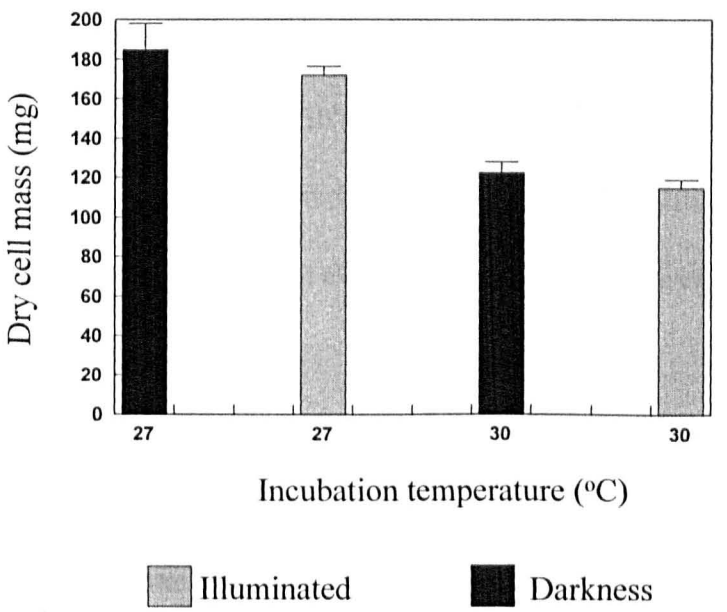


Figure 4.16 The effects of illumination and temperature upon the dry cell mass of *B. linens* strain Wkiii.

4.16.3 Conclusions

Carotenogenesis appears to have been inhibited in the illuminated cells compared with those maintained in darkness (see Plates 4.16 and 4.16(b)), possibly due to growth impairment induced by high light intensity. This is consistent with the finding that freshly inoculated YGA plates failed to flourish under the same levels of illumination and that only confluent plates were able to be used in their place (results not shown). Plates 4.16 and 4.16(b) also suggest photobleaching so that carotenoid production whilst not impaired, was followed by carotenoid destruction, producing a false estimate of the amount of pigment actually produced.

Results of the incubation of *B. linens* strain Wkiii at 27 and 30°C with or without illumination are shown in Figure 4.16. As discussed in Section 4.14.3, mutant Wkiii demonstrates greater growth at the lower temperature (27°C) compared with growth at the higher temperature of 30°C, in contrast with the growth seen in *B. linens* strain 'Pink' at 20°C when compared with growth at 30°C. Illumination does not however, appear to have a significant effect upon dry cell mass (see Figure 4.16).

4.17.1 The pH profile of a growing *B. linens* strain Wkiii culture

Hydrogen ion content is known in to be an important factor affecting both growth and carotenogenesis in bacteria. Growth between pH6.5 and pH8.5 has been reported amongst *B. linens* strains (Mulder, 1966), though this range broadens to

pH5.5 in the presence of 4%(w/v) sodium chloride. The pH profile of a liquid culture of *B. linens* Wkiii grown under standard conditions was measured.

YGB medium was inoculated with *B. linens* strain Wkiii and incubated in an aerated and agitated fermenter vessel, without pH control (see Materials and Methods Section 2.9.2). Culture pH was measured over the growth periods of the culture. pH values obtained over time are shown in Figure 4.17(a). Conclusions are discussed in Section 4.17.4.

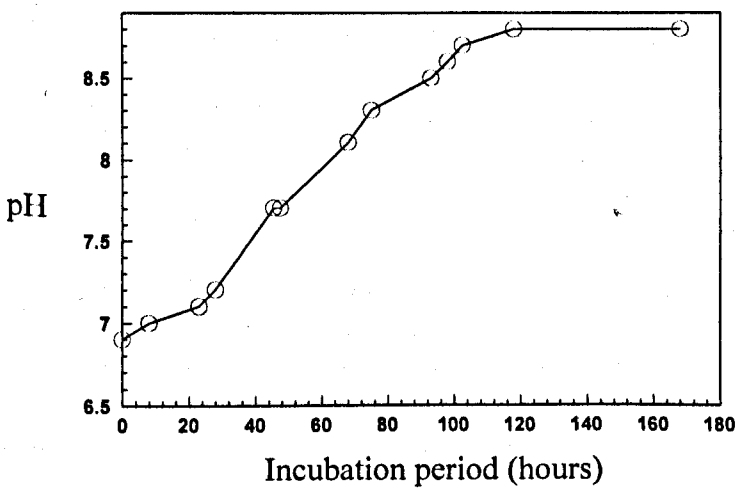


Figure 4.17(a) pH measurements taken at the times shown, during *B. linens* Wkiii fermenter growth in standard YGB medium with aeration and agitation.

4.17.2 Establishing the initial medium pH which gives optimal cell growth and pigmentation in *B. linens* strain 'Pink'

In order to establish the pH in which cell mass and pigmentation are optimal, a simple experiment which controlled only the initial medium pH at the point of inoculation was conducted.

Standard YGB medium was provided at pHs 6.4 through to 7.6 in increments of 0.2 pH units (see Materials and Methods Section 2.9.3) and inoculated with *B. linens* strain 'Pink'. Cultures were incubated in otherwise standard conditions (see Materials and Methods Section 2.9.1). After incubation, cell mass and pigmentation were assessed as shown in Figure 4.17(b). Conclusions are discussed in Section 4.17.4.

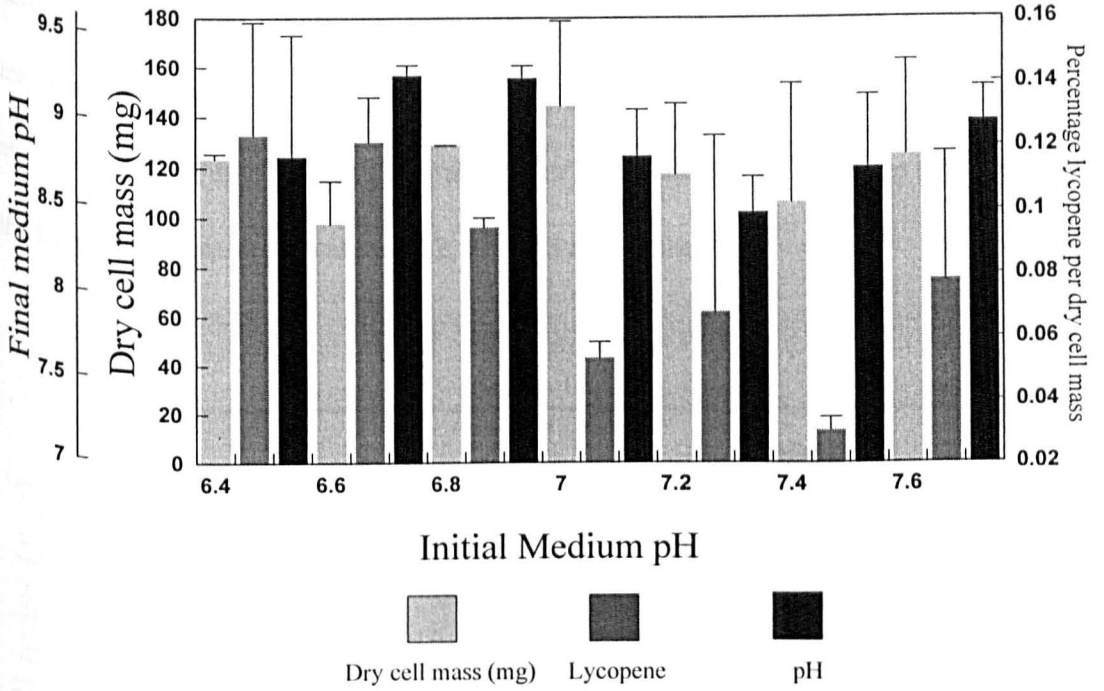


Figure 4.17(b) Initial medium pH and subsequent cell dry mass, pigmentation and final pH values for *B. linens* strain 'Pink' grown under otherwise standard conditions.

4.17.3 The effect of pH controlled fermentations upon dry cell mass yield and lycopene accumulation in *B. linens* strain Wkiii

With a fermenter vessel, it was possible to control the maximum pH of a culture of *B. linens* strain Wkiii by using dilute hydrochloric acid, the application of which was controlled by a pH controller (see Materials and Methods Section 2.9.2). In this way, it was possible to determine if growth and pigmentation were limited by pH.

YGB medium was inoculated with *B. linens* strain Wkiii and incubated under aerated and agitated conditions (see Materials and Methods Section 2.9.2) with pH maxima controlled with a pH controller with hydrochloric acid (7M). Dry cell masses and lycopene accumulation values are given in Figure 4.17(c). Values correspond to the following limited pH values: 7.2, 8.1, 8.5 and unlimited.

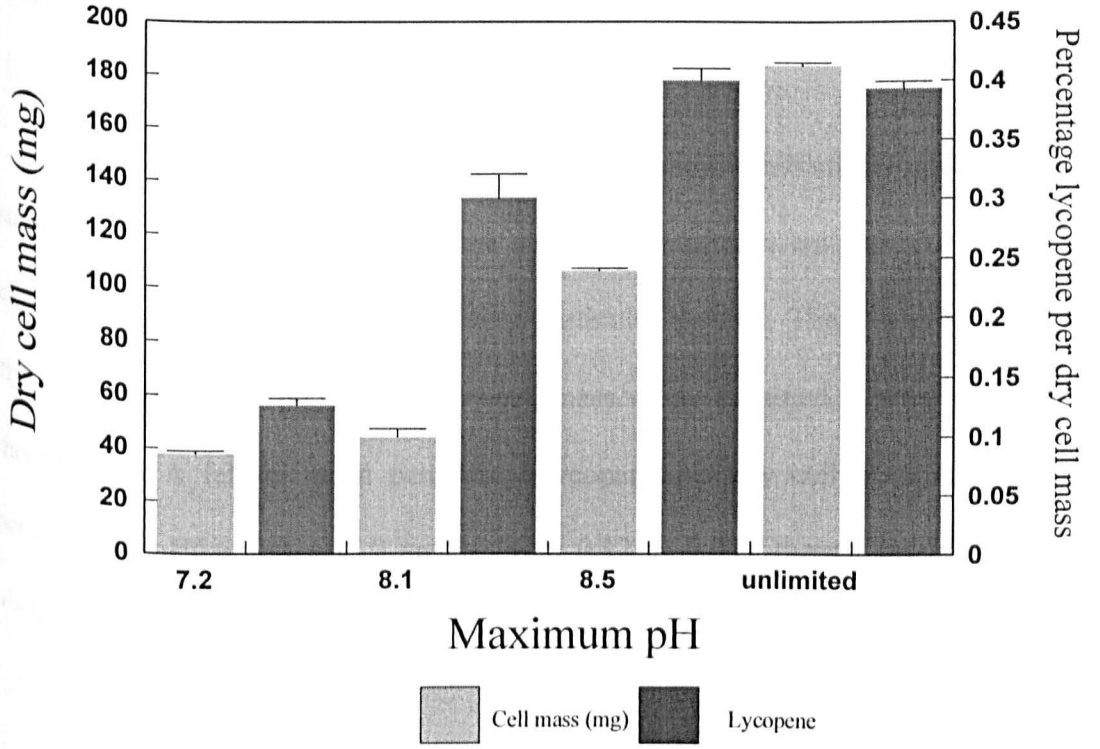


Figure 4.17(c) Dry cell mass and percentage lycopene per dry cell mass values for *B. linens* strain Wkiii grown under agitated and aerated conditions in pH controlled fermentations. pH values shown represent pH maxima employed. Cells were grown in 1dm^3 YGB medium. The dry cell mass values shown are per 50ml culture.

4.17.4 Conclusions

As Figure 4.17(a) shows, pH rises steadily over the incubation period until it reaches the maximum value corresponding to approximately 120 hours growth. The final pH values are consistent with the odour of ammonia detectable during growth in YGB medium and the apparent high amino acid requirement of the *B. linens* strains tested here.

Experiment 4.17.2 examines the effects of initial medium pH upon *B. linens* strain 'Pink'. However, because of the amount of variation amongst each triplicate experiment, it is difficult to assign any particular pattern. There appear to be no significant differences between the dry cell mass values obtained, whatever the initial medium pH. A fall in mean percentage lycopene per dry cell mass can be seen between the pH6.6 and pH7.0 media (from 0.12 to 0.06), though the lowest value obtained (0.03) occurs with the pH7.4 medium.

As Figure 4.17(c) shows, there are considerable contrasts both in terms of cell dry mass and pigmentation, when the pH maxima of fermentations are controlled. *B. linens* strain Wkiii grew to a mean dry cell mass value of 38mg when medium pH was controlled so that it did not exceed 7.2. The concentration of lycopene under these conditions was equivalent to 0.13% (mean) per dry cell mass. In contrast, when pH was allowed to reach a value of 8.5, mean cell dry mass rose to 106mg (an increase equivalent to 179%) and percentage lycopene per dry cell mass rose to a mean value of 0.40 (an increase equivalent to 208%). Ultimately, though mean percentage

lycopene per dry cell mass did not increase over the pH8.5 maximum medium value, mean dry cell mass in the medium in which pH was uncontrolled reached 184mg, compared with 106mg in the highest pH, pH controlled medium.

These results provide strong evidence that the growth of *B. linens* strain Wkiii is not limited by alkaline pHs, where these values have been achieved through the growth of the culture and not through the setting of medium pH at the time of inoculation, though this contrasts significantly with the growth characteristics of *B. linens* CNRZ 918, which is optimal when pH is maintained at neutral (Ferchichi, Hemme and Boullanne, 1986). The reduced growth and pigment production associated with the lower pH maxima suggest the possibility that a requirement of confluent, late exponential growth, is the associated increase in alkalinity and that this is not merely an unnecessary process which occurs as the result of waste ammonia. Ultimately, a biphasic growth process may have occurred in the examined culture whereby at inoculation a low pH is suggested ((see Section 4.17.2) as applied to *B. linens* strain 'Pink') which is followed later by a high pH requirement. It is interesting to note that similar attempts to control pH in a hydrocarbon medium inoculated with Brevibacterium KY4313 also led to the inhibition of carotenoid production (Tanaka, Kato and Fukui, 1971), though acidic pH values were in this case controlled by the addition of $CaCO_3$.

4.18.1 The effects of aeration on the growth and lycopene accumulation of *B. linens* strain 'Pink'

An additional factor which has been investigated is aeration. Previous work has reported good pigment production by *B. linens*, especially in an oxygen-rich atmosphere (Keddie and Jones, 1981; Albert *et al.*, 1944). It has also been noted (Keddie and Jones, 1981; Albert *et al.*, 1944) that oxygen uptake is affected by the growth medium employed, such that in the case of media containing glutamic acid as the growth substrate, or where sodium chloride is absent, or if glucose is used as the growth substrate, oxygen uptake is lowered. The level of culture aeration can be adjusted crudely by altering the volume of medium in agitated shake flasks.

Figure 4.18 indicates the effects of the different culture volumes 12.5, 25, 50 and 100ml in 250ml baffled shake flasks upon the pigment production and cell dry mass of *B. linens* strain Wkiii. Cells were grown in YGB medium at 30°C in darkness, with agitation at 100rpm. Conclusions are discussed in Section 4.18.3.

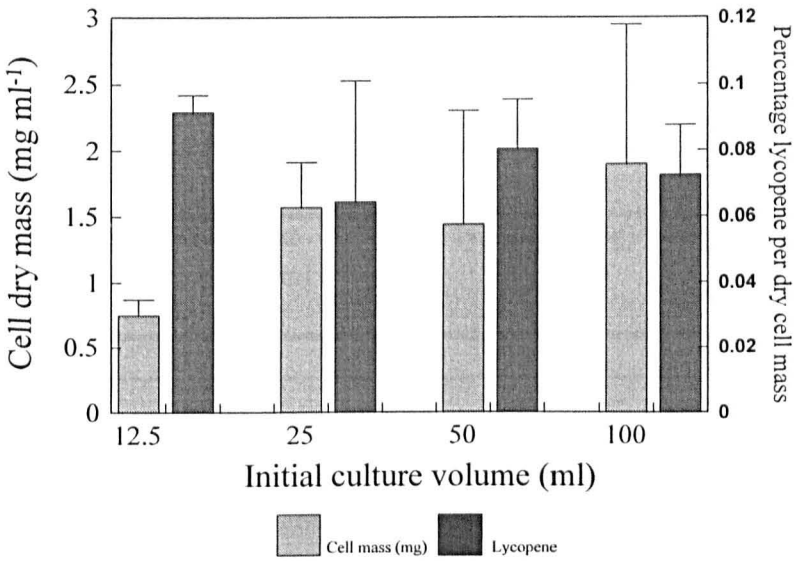


Figure 4.18 The effect of initial medium volume upon dry cell mass density and lycopene accumulation in *B. linens* strain 'Pink'.

4.18.2 The effects of direct aeration of cultures of *B. linens* strain Wkiii grown in an agitated fermenter vessel

Sterile filtered compressed air was used to aerate cultures of *B. linens* strain Wkiii grown in YGB medium (see Materials and Methods Section 2.9.2), to establish the effects of aeration supplied directly into the medium under conditions of agitation which would also encourage the disruption of air bubbles. In contrast with the previous experiment, culture volumes were maintained at constant levels. The aim of this experiment was also to establish the value of fermenter vessel conditions compared with those of the shake flask.

1 dm^3 of sterile YGB medium was inoculated with *B. linens* strain Wkiii in a $2.5dm^3$ fermenter vessel. The culture was agitated at 500 rpm during growth and aerated at a rate of one volume per minute. Culture dry mass, total lycopene and lycopene per dry cell mass values are given in Table 4.18.

Cell dry mass per 50ml culture (mg)	Absorbance at 472nm in 200ml diethyl ether	Total lycopene (μg)	Percentage lycopene per dry cell mass
184	1.25	735	0.40
182	1.21	710	0.39
184	1.22	718	0.39

Table 4.18 Dry mass and pigmentation values for *B. linens* mutant Wkiii grown in YGB medium in $1dm^3$ culture volume in a $2.5dm^3$ capacity fermenter vessel with agitation rate set at 500rpm and one volume per minute aeration rate.

4.18.3 Conclusions

The effects of culture volume upon lycopene accumulation show considerable variation, so that it is not possible to assign any pattern (see Figure 4.18). Dry cell mass values are also highly variable, though the smallest culture volume yields a dry cell density of 0.75 $mgml^{-1}$, whilst the next smallest dry cell density value is 1.24 $mgml^{-1}$, in the 25ml culture volume. Whilst the lycopene accumulation in the 12.5ml culture is not significantly different to that of the other culture volumes, it is notably high with respect to dry cell mass density. Since the 12.5ml initial culture volume yields the lowest cell density in the experiment it is perhaps the case that it will reach stationary phase most rapidly. This may lead to prolonged cell stress and relatively

high levels of pigmentation. In the case of Brevibacterium KY4313, increased aeration, achieved *via* culture volume changes was seen to have a detrimental effect both upon cell yield and pigmentation (Tanaka, Kato and Fukui, 1971).

When grown under direct aeration in a one litre culture volume, *B. linens* mutant Wkiii yielded cell mass and pigmentation values as shown in Table 4.18. Compared with the values obtained in 50ml cultures in 250ml shake flasks (see Table 3.6(d)) mean dry cell mass increases from 157mg to 183mg (per 50ml of culture), an increase equivalent to 17%. Mean percentage lycopene per dry cell mass does not alter significantly. The conditions of agitation and aeration employed (500rpm and one volumes per minute) would therefore appear to favour the more efficient consumption of the growth medium without significant pigmentation concentration losses, indeed optimal cell growth was seen in cultures of *B. linens* CNRZ 918 at 50% oxygen saturation (Ferchichi, Hemme and Boullanne, 1986).

4.19 Conclusions to Chapter 4

As was seen in Chapter 3, lycopene accumulating *B. linens* mutants are not restricted to accumulating concentrations equivalent to those of 3,3'-dihydroxyisorenieratene in the wild-type. However, in terms of the commercial production of lycopene, the total amount in the culture as well as in each cell, is important.

In some experiments (see Section 4.17.2 for example), there is evidence of an inverse relationship between cell mass and lycopene concentration, such that media which appear to do worst in terms of the promotion of growth, are often in fact, better at yielding cells which contain more lycopene. A set of scatter plots (Figures 4.19 (a) to (h)) shows the relationships between lycopene concentration and cell mass or total culture lycopene and cell mass for the data from Chapter 4.

Figure 4.19(a) compares the total lycopene values for the media in which *B. linens* strain Wkiii was grown with the corresponding cell mass values. It is clear that there is a positive linear relationship between the two, highlighted by the plot of linear regression shown and a correlation coefficient of 0.9. The data in Figure 4.19(a) show that media which improve cell mass also improve in total culture lycopene.

In Figure 4.19(b) the corresponding data for lycopene concentration and cell mass amongst the media tested are plotted, again for *B. linens* strain Wkiii. These data show that there is essentially no relationship between cell mass and lycopene

concentration (correlation coefficient 0.02 (2 d.p.)), suggesting that growth media that improve cell mass do not generally improve lycopene concentration, compared with the case seen with total culture lycopene. This is at least a better scenario in commercial terms than that noted in some experiments, where an inverse relationship between the two was observed.

When factors other than those directly attributable to the constituents of the medium are tested, there is once again a close, positive, linear relationship between total culture lycopene and cell mass when *B. linens* strain Wkiii is examined (Figure 4.19(c)). Though the number of samples is small, the correlation coefficient between the two factors is 1.0 thus factors, such as pH and temperature, which strongly affect dry cell mass, strongly affect total culture lycopene as well.

As was the case with lycopene concentration and media factors, there is once again a weaker relationship when lycopene concentration is compared with total culture lycopene, when non-media factors are examined (see Figure 4.19(d)). In this case however, there is a much stronger correlation coefficient of 0.7 (1 d.p.), though, once again, a quite broad spread of data.

Since *B. linens* strain 'Pink' yielded lower concentrations of lycopene than those seen in Wkiii, it might be expected that improvements which improve cell mass yield have less impact than is the case with Wkiii. This assumption is borne out in Figure 4.19(e), where total culture lycopene and dry cell mass values are plotted. The

correlation value of 0.8 (to 1 d.p.) is less than that seen with the equivalent values for Wkiii, though it is important to consider that the media employed were not usually the same. However, it appears that improvements in media which result in increased cell mass do increase total culture lycopene, but to a lesser degree than seen with Wkiii.

Figure 4.19(f), as opposed to the situation seen with Wkiii, shows a positive relationship between percentage lycopene per dry cell mass and cell dry mass for *B. linens* 'Pink'. This suggests that improvements in media which result in increased cell proliferation, also improve the concentration of lycopene in cells. This appears to differ from *B. linens* Wkiii. The correlation shown in Figure 4.19(f), is however smaller than that seen when total culture lycopene and cry cell mass values are compared (0.5 to 1 d.p.) and again, it is important to remember that comparisons between Wkiii and 'Pink' are not usually based upon the same medium.

When the effect of non-media factors are considered in *B. linens* 'Pink', a positive relationship is again seen between culture lycopene and dry cell masses (see Figure 4.19(g)), though, compared with Wkiii, the correlation coefficient is less (0.4 to 1 d.p.) and the distribution of data broader. Once again, this suggests that improvements in cell yield in *B. linens* 'Pink' have less of an impact upon total lycopene than is the case with Wkiii.

Figure 4.19(h), which shows the relationship between dry cell mass and percentage lycopene per dry cell mass for *B. linens* 'Pink' subject to non-media factors, confirms many casual observations made when cell mass values are seen to improve, *i.e.* there is a negative relationship between cell mass and lycopene

concentration. This shows that factors which are aimed at improving total culture lycopene tend to have the opposite effect upon percentage lycopene per dry cell mass values. These data provide evidence which suggests that the degree of stress to which cells are subjected has an important effect upon their requirement to accumulate lycopene, but at the same time, in terms of maximising the total yield of lycopene from a culture, the employment of factors which are not stressful and which lead to an improvement in cell yield, is of more importance.

Though these data do not provide explanations for the relationships between total lycopene, percentage lycopene per dry cell mass and dry cell mass, there are a number of possibilities which might help to explain them. Cell masses which are low under certain media or other conditions, for instance, may promote a reduction in the metabolic activity of cells. Though not necessarily reflected in total culture growth by the presence of stationary or senescent phases, this reduction in the growth rate of individual cells may promote the accumulation of lycopene (as discussed in Section 3.9). Referring to the TEM images of mutant cells (Plates 3.7(c), 3.7(f), 3.7(h) and 3.7(i)), it is perhaps the case that mutants which endure conditions which are stressful are less able to divide properly. This lack of division may promote an increase in membranation per cell, consistent with slowed growth and increased lycopene per cell.

In summary of the findings especially concerning mutants Wkiii and 'Pink', it is clear that mutant Wkiii grows more slowly than its wild-type ancestor (Section 3.8.1) and this appears to be the casual observation with all other mutants examined.

Furthermore, as the growth of a culture slows, lycopene accumulation increases (Section 3.8.2) (this is seen not only in Wkiii, but also as a casual observation in colonies grown on solid medium).

Concerning some of the findings when carbohydrate substrates are employed, it is interesting to note that in the case of strain 'Pink', the ratio of cell mass to percentage lycopene per dry cell mass is reversed in the presence of Bundaberg Direct Consumption Raw Sugar, compared with the other by-products tested (Section 4.2.1). In contrast, peak total lycopene per culture values remain similar (at 17,21 and 18μg), reflecting the compensation effect of high lycopene concentration per cell despite the lack of cell growth. Such findings suggest that the goal of increased lycopene production per cell may be a misleading one once the level of lycopene per cell reaches a certain threshold because it will tend to result in a diminished cell mass and thus little improved or reduced total culture lycopene. The poor cell growth seen with Bundaberg Direct Consumption Raw Sugar is borne out by the findings concerning the growth of strain Wkiii in a variety of carbohydrate test media, found to grow most poorly in the presence of sucrose (of which Bundaberg Direct Consumption Raw Sugar is largely composed), though best with (Section 4.2.2) fructose, glucose and glycerol (though there is an interesting anomaly in the final medium pH seen with the fructose medium). Though the lycopene accumulation values are not available, the scatter plot shown in Figure 4.19(a) strongly suggests that the best utilised of these sugars would promote the greatest culture lycopene values.

In the case of protein digests containing amino acids and peptides, it is interesting to note that Wkiii tends to produce more growth when tryptone is combined with glucose than when tryptone is alone (Section 4.4). This is echoed in section 4.9.2, where 3%(w/v) neutralised soya peptone + 1.2%(w/v) monosodium glutamate + 1.2%(w/v) yeast extract medium performs better, in terms of cell mass, when glucose is present at 0.5%(w/v). The mass spectrum obtained from lycopene from *B. linens* grown in D_2O also shows the metabolic pathway in *B. linens* that leads to lycopene to be highly oxidative (Section 4.2.3).

The concentrations of thiamine and vitamin B_{12} added to the test medium in Section 4.5.1 appear to be detrimental in terms of cell mass and therefore total lycopene, but have no significant effect upon lycopene concentration per dry cell mass. When thiamine is added, but not in such excess, in the presence of Bundaberg direct consumption raw sugar and tryptone (Section 4.5.2), it does not significantly improve either lycopene accumulation per cell or culture lycopene mass for strain 'Pink' when compared with the same medium in which it is absent.

When neutralised soya peptone is present in a medium it appears to be well utilised (Section 4.8) although peak culture lycopene is still less than that seen in YGB medium for strain Wkiii (277 and 688 μg respectively). The addition of large amounts of monosodium glutamate and yeast extract to a neutralised soya peptone containing medium (Section 4.9.1) produces a Wkiii culture with a peak total lycopene mass of 956μg though the conversion of medium solids to lycopene at 0.03% is less than that seen in the YGB medium of 0.04%, although the difference in

medium solids to cell mass conversion is not significant. Of all media tested, the greatest percentage lycopene per dry cell mass value is seen with 3%(w/v) neutralised soya peptone plus 1.2%(w/v) yeast extract medium where strain Wkiii yields 0.83 % lycopene per dry cell mass although the conversion of medium solids to lycopene only reaches 0.01% and the conversion of medium solids to cell mass yields 1.5%. This shows that strain Wkiii has considerable capacity for lycopene accumulation.

In an experiment in which media containing neutralised soya peptone contain varying concentrations of yeast extract (Section 4.10), percentage lycopene per dry cell mass values are greatest when cell mass values are lowest. At a lower concentration of yeast extract (0.6%(w/v) compared with 1.2%(w/v)), peak total culture lycopene and dry cell mass values are greatest suggesting that yeast extract may contain a substrate or growth factor which when present in sufficient quantities may promote the production of lycopene at the expense of cell mass, perhaps through the induction of enzyme systems involved in the process.

When carbon and nitrogen ratios are altered, as is the case in media containing differing Bundaberg direct consumption raw sugar and tryptone concentrations (Section 4.11.1), the peak concentration of lycopene per cell in *B. linens* 'Pink' is seen when Bundaberg direct consumption raw sugar is present in large quantities relative to tryptone, but peak culture lycopene and cell masses are seen when the opposite is the case in the medium (181µg total lycopene for the 3%(w/v) BRS + 1.5%(w/v) tryptone medium compared with 351µg for the 3%(w/v) BRS + 6%(w/v) tryptone medium). When neutralised soya peptone and Bundaberg direct consumption raw sugar are

combined in differing ratios, percentage lycopene per dry cell mass and dry cell mass values for *B. linens* Wkiii (Section 4.11.2) remain similar until the concentration of neutralised soya peptone drops from 1%(w/v) to 0.5%(w/v) in the presence of 2.0%(w/v) and 2.5%(w/v) Bundaberg direct consumption raw sugar respectively, at which point cell dry mass falls dramatically although peak percentage lycopene per dry cell mass does not alter significantly. Peak total culture lycopene is greatest (at 179µg) when Bundaberg direct consumption raw sugar and neutralised soya peptone are present in equal concentrations, perhaps suggesting that neutralised soya peptone may be encouraging growth whilst Bundaberg direct consumption raw sugar encourages lycopene production per cell and that the two need not be antagonistic.

A 10°C drop in growth temperature appeared to have little effect upon the growth rate of *B. linens* 'Pink' (Section 4.14.1), but an increase to 33°C caused a fall in percentage lycopene per dry cell mass in both 'Pink' and Wkiii when compared with growth at 30°C (Section 4.14.2). Growth of Wkiii at 27°C increased cell dry mass and thus is likely to improve total culture lycopene values as well (Section 4.14.3). It appears, at least in the case of *B. linens* 'Pink', that cold shock treatment had no effect upon lycopene accumulation per cell. Both at 30°C and at 27°C, illumination was seen to have a slightly negative effect upon Wkiii cell mass, agreeing with the effects of illumination seen in *B. linens* strains grown on solid medium (Section 4.16.1).

As expected, the pH profile for Wkiii steadily rises as the culture grows (Section 4.17.1), reaching a high value consistent with the presence of ammonia

which is detectable by odour in all *B. linens* cultures. Ammonia is likely to be toxic to cells of *B. linens*, but attempts to control the pH of the medium had a detrimental effect upon cell mass values.

When strain Wkiii is grown in fermenter conditions with aeration and agitation (Section 4.18.2), peak total lycopene increases from 688µg for cells grown in shake flasks to 735µg per 50ml culture, suggesting that aeration favours cell proliferation and total lycopene accumulation. Peak lycopene concentrations per cell remain the same, which shows that lycopene concentration will not always diminish in conditions which favour cell growth.

Based upon generalised assumptions a medium optimised for total culture lycopene may contain fructose, glucose or glycerol as carbohydrates, though Bundaberg direct consumption raw sugar may be beneficial depending upon its interactions with the growth substrates present (in some cases it may need to be present in equal concentration). The presence of α-ketogluterate may also be beneficial but a mixture of amino acids and perhaps peptides would also be required. Depending upon the digest present the addition of a carbohydrate such as glucose may be merited. Sodium chloride concentration should be kept low. It appears that components such as monosodium glutamate and neutralised soya peptone are beneficial in large quantities but that an excess of yeast extract may diminish lycopene yield. Growth temperature should be maintained at 30°C or less and cultures grown in darkness without pH limitation. (Excessive agitation should be avoided as this

appeared to prevent growth of strain Wkiii when greater than 500rpm in a fermenter vessel, probably due to the damage caused to cells by shear forces generated).

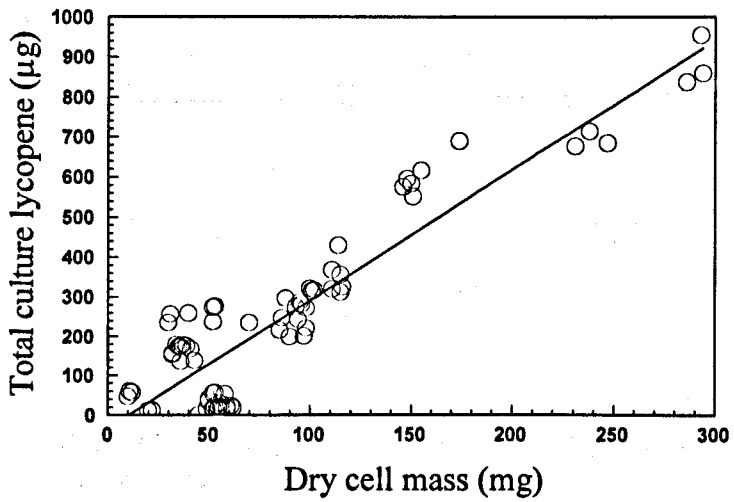


Figure 4.19(a) Total culture lycopene plotted against dry cell mass for *B. linens* mutant Wkiii grown in a variety of media

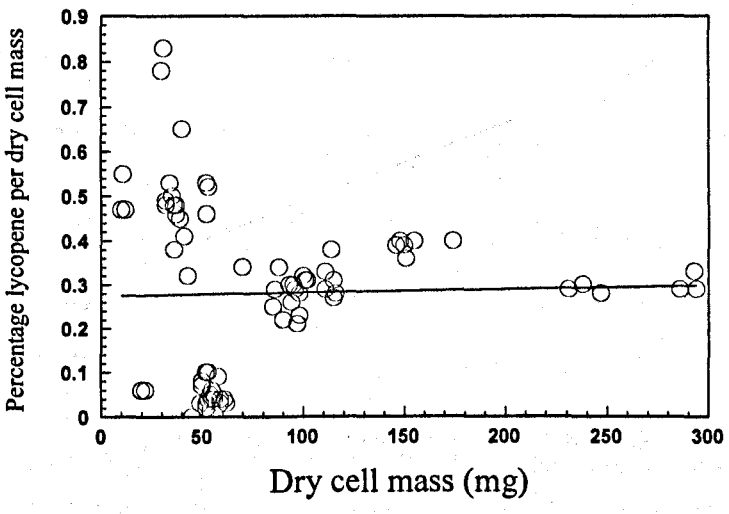


Figure 4.19(b) Percentage lycopene per dry cell mass plotted against dry cell mass for *B. linens* mutant Wkiii grown in a variety of media

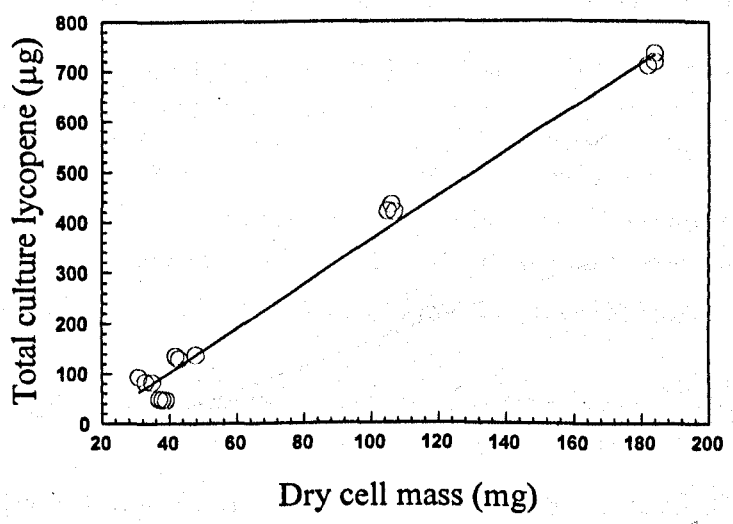


Figure 4.19(c) Total culture lycopene plotted against dry cell mass for *B. linens* mutant Wkiii grown in a variety of non-media conditions

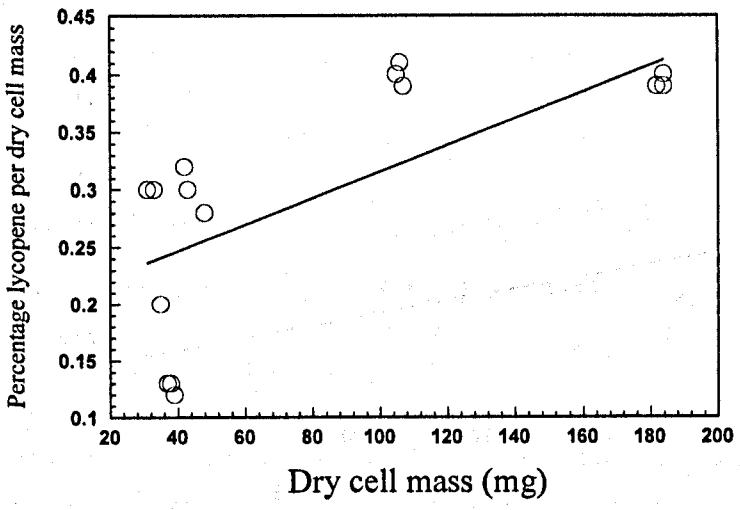


Figure 4.19(d) Percentage lycopene per dry cell mass plotted against dry cell mass for *B. linens* mutant Wkiii grown in a variety of non-media conditions

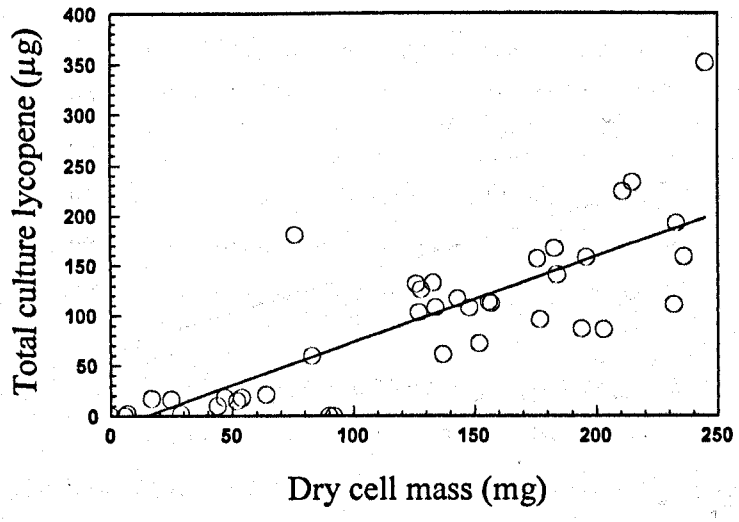


Figure 4.19(e) Total culture lycopene plotted against dry cell mass for *B. linens* mutant 'Pink' grown in a variety of media

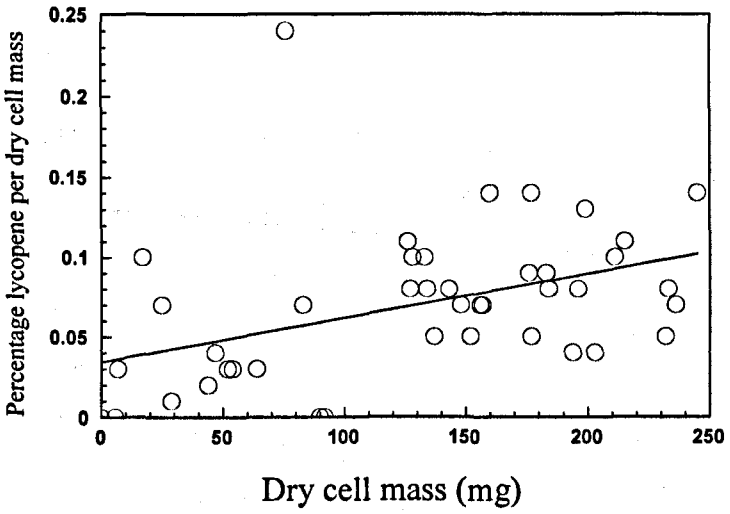


Figure 4.19(f) Percentage lycopene per dry cell mass plotted against dry cell mass for *B. linens* mutant 'Pink' grown in a variety of media

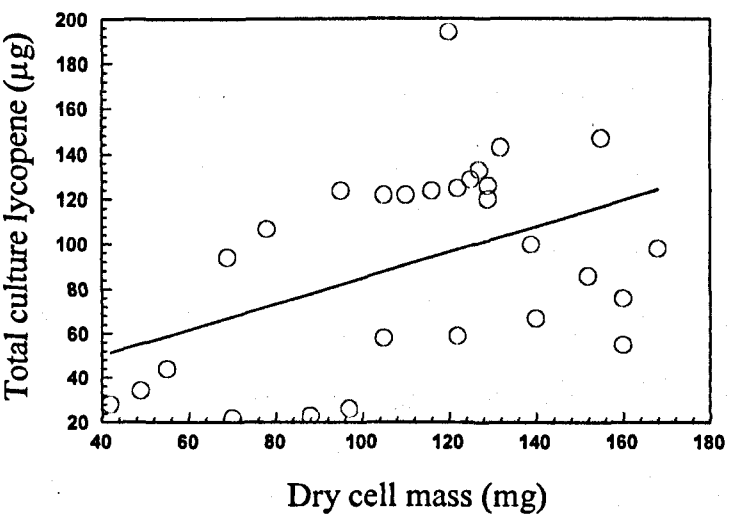


Figure 4.19(g) Total culture lycopene plotted against dry cell mass for *B. linens* mutant 'Pink' grown in a variety of non-media conditions

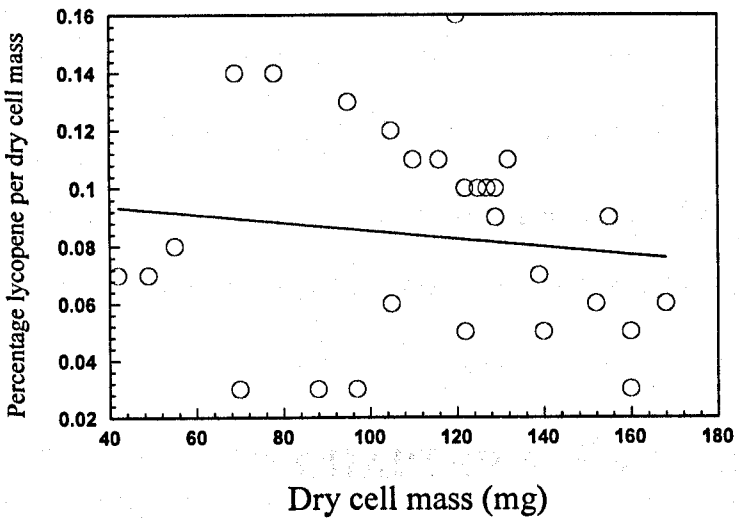


Figure 4.19(h) Percentage lycopene per dry cell mass plotted against dry cell mass for *B. linens* mutant 'Pink' grown in a variety of non-media conditions

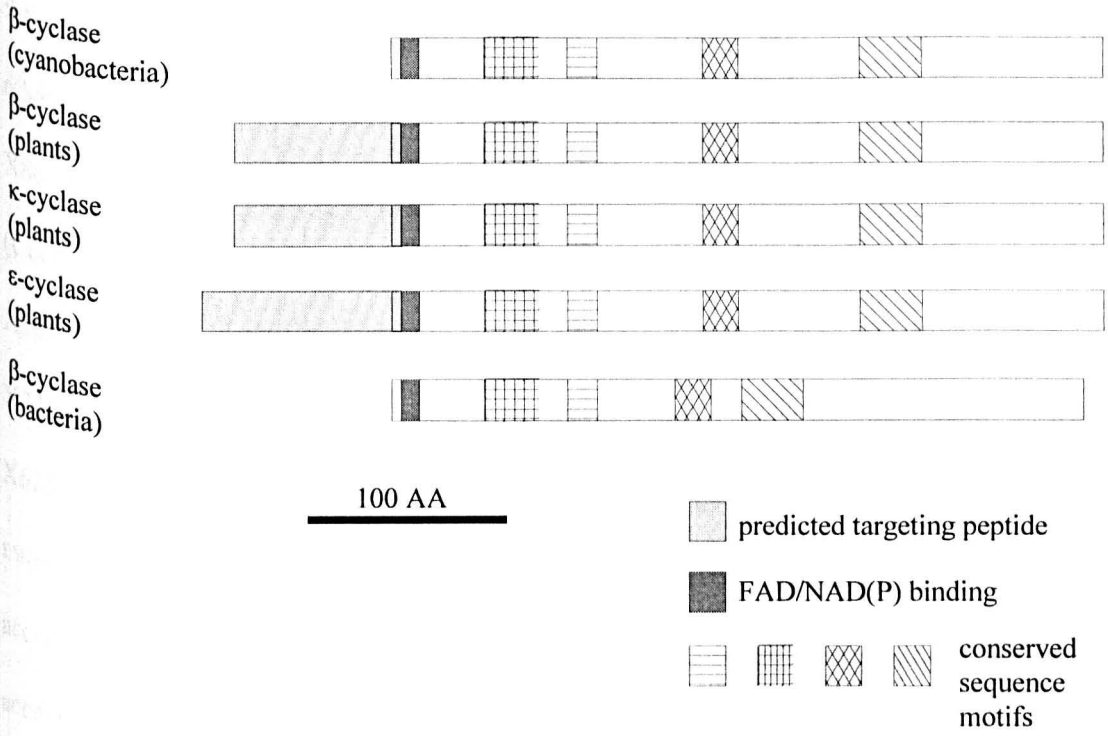
CHAPTER 5

AN ATTEMPT TO SEQUENCE AND COMPARE THE WILD-TYPE AND MUTANT LYCOPENE CYCLASE GENES OF *B. LINENS* NCIMB 8546 AND TO SEQUENCE THE *B. LINENS* CECT75 PLASMID PBL100

5.1 Introduction

In light of the aims of this work, the investigation of the biosynthesis of carotenoids at the nucleotide sequence level was merited. A study to compare the wild type and mutated lycopene cyclase genes was undertaken both in an attempt to characterise the nature of the mutation or mutations and obtain unique sequence information relating specifically to the carotenoid biosynthesis genes of *B. linens*. Consideration must, however, be given to the caveat that the lycopene cyclase gene may in fact not carry any mutation, as is the case in some phytoene accumulating mutant plants which lack mutations in their phytoene desaturase genes (Hirschberg, 1998). Since the process of mutagenesis with MNNG is random, a number of genes may have been affected, these may include a regulatory gene for example, mutated so that its product does not permit the normal action of the lycopene cyclase enzyme.

Certain regions of the lycopene cyclase genes of plants, cyanobacteria and bacteria are conserved, these include a dinucleotide binding motif and other regions of unknown function (see below). When the putative sequence of the lycopene cyclase gene of *B. linens* is compared with these sequences, it is therefore reasonable to assume that it is likely to contain similarities in some of these regions.



The conservation of amino acid regions in the carotene cyclases in plants, cyanobacteria and bacteria (After Hirschberg, 1998)

5.2 The deduced amino acid sequence of the sequenced DNA from wild-type *B. linens* : comparison with existing lycopene cyclase amino acid sequences

Genomic DNAs from *B. linens* strain 'Pink' and its wild type ancestor were extracted (see Materials and Methods Section 2.20.1). Genomic DNA was used as template material in the polymerase chain reaction (see Materials and Methods Section 2.20.4) using the primer sequences CRTYLH (ATGATGTGATGCTGGTG GGCGCT) (5') and CRTIRH (AAGCCTGCACCAATTACAATGGTT) (3') the sequences of which were based upon the 5' region of eleven aligned lycopene cyclase (*crt*Y) gene nucleotide sequences and nine nucleotide sequences of the downstream flanking phytoene desaturase gene, *crt*I respectively (a nested 5' primer was chosen due to the poor quality of potential flanking primers). The nucleotide sequences were taken from the phytoene desaturase or lycopene cyclase genes from some or all of the

following organisms: *Arabidopsis thaliana* (GenBank accession number 40176 (Cunningham *et al.*, 1996)), *Capsicum annuum* (GenBank accession number X86221(Hugueney *et al.*, 1995)), *Nicotiana tabacum* (GenBank accession number (Cunningham *et al.*, 1996)), *Lycopersicon esculentum* (GenBank accession number X86452 (Cunningham *et al.*, 1996)), *Synechococcus* sp. (GenBank accession number X74599 (Cunningham *et al.*, 1994)), *Synechocystis* (GenBank accession number X62574 (Martinez-Ferez *et al.*, 1992)), *Erwinia herbicola* (GenBank accession numbers M87280 M99707 (Armstrong *et al.*, 1993)), *E. uredovora* (GenBank accession number D90087 (Misawa 1990)), *Agrobacterium aurantiacum* (GenBank accession number D58420 (Misawa *et al.*, 1995)), *Flavobacterium* ATCC21588 (GenBank accession number U62808 (Pasamontes *et al.*, 1997)), *Neurospora crassa* (GenBank accession number M57465 and M33867 s (Schmidhauser *et al.*, 1990)), *Rhodobacter capsulatus* (GenBank accession number X52291 (Armstrong *et al.*, 1989)) and *Streptomyces griseus* (GenBank accession number X95596 (Schumann *et al.*, 1996)). Aligned sequences (using the PILEUP algorithm of the Wisconsin Genetic Computer Group's Sequence Analysis Software Package (1994)) are given in the Appendix. In both cases, primer design was biased to favour nucleotide sequences originating from prokaryotic genes. Based upon the sequence interval between the two primers in the case of *Erwinia herbicola*, the expected size of the product would be approximately 1200bp (see Appendix).

In order to facilitate easy sequencing of the PCR product, amplified DNA was ligated into a suitable cloning vector. PCR products were separated by agarose gel electrophoresis (Plate 5.1) (see Materials and Methods Section 2.20.6). The portion of

gel containing the band of the expected molecular weight was excised and the DNA purified (see Materials and Methods Section 2.20.7). The excised and purified DNA, was ligated (see Materials and Methods Section 2.20.12) into a sequencing vector, pGEM®-T Easy (Promega, UK) (see below) designed to incorporate PCR products specifically, by virtue of the presence of overhanging thymine residues in the multiple cloning site, which are compatible with the overhanging adenine residues generated by PCR. Vectors containing insert DNAs were transformed into *E. coli* XL1 Blue cells and screened in blue/white assays (see Materials and Methods Section 2.20.10 and 2.10.11). Plasmids were purified from cultures of transformed clones (see Materials and Methods Section 2.20.2) and sequenced (see Materials and Methods Section 2.20.13).

Although the primers CRTYLH and CRTIRH were designed to amplify the lycopene cyclase gene specifically (and therefore produce a product of only one size), Plate 5.1 displays agarose gels in which a minimum of three product sizes are visible in both wild-type and mutant *B. linens* PCRs. In Southern blot experiments, Haycock (1996) found multiple hybridisations when probing *Erwinia herbicola* DNA using a probe based upon the phytoene desaturase gene of the same organism, generated by PCR. The multiple banding was attributed to unspecified regions of homology within the *E. herbicola* carotenoid gene cluster. Perhaps this phenomenon is also the source of the multiple products shown in Plate 5.1.

Plate 5.1(b) shows a further agarose gel in which the transformed and cloned vector was run having at first been restriction digested (see Materials and Methods Section 2.20.3) to release the insert sequence.

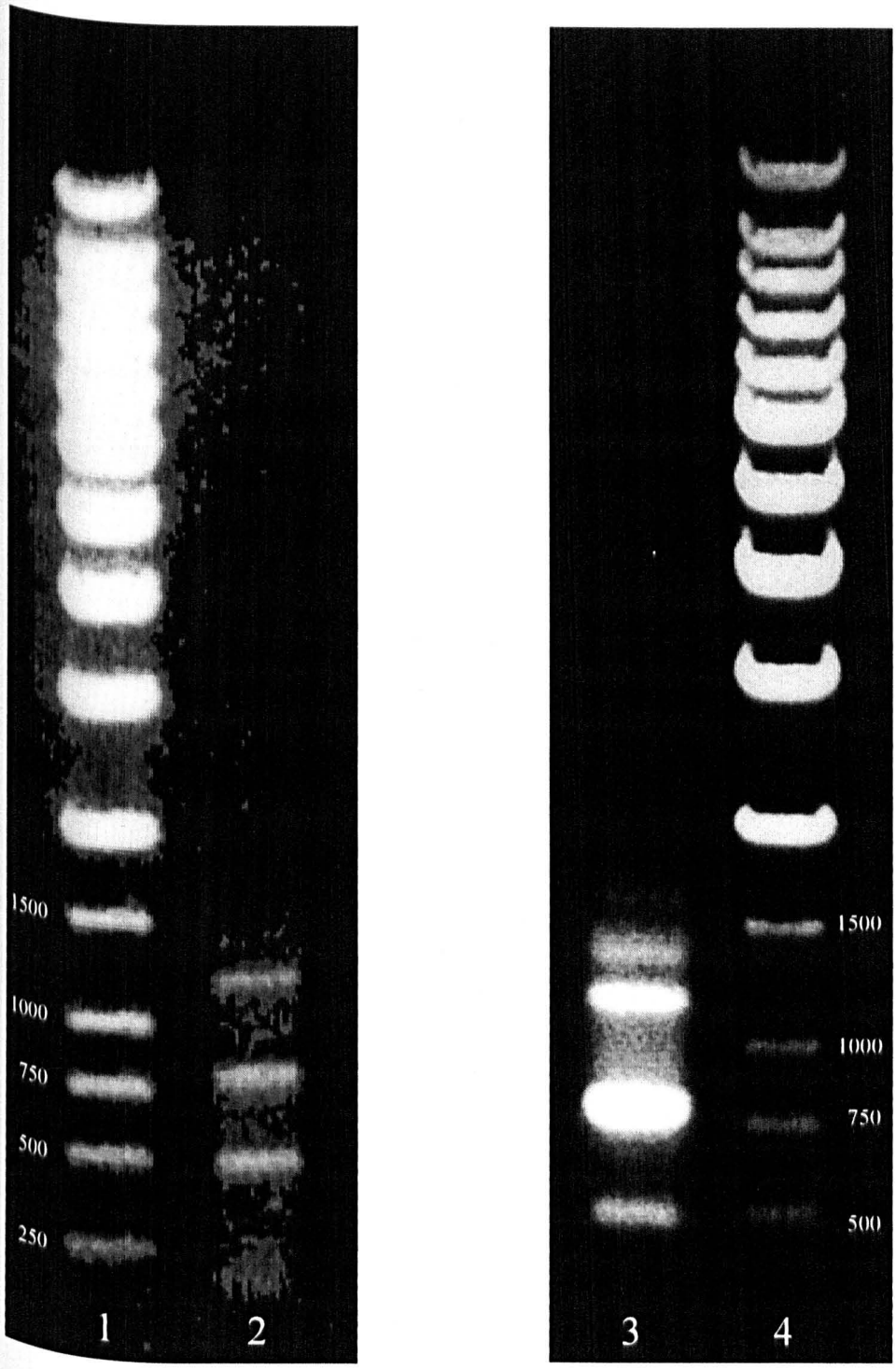


Plate 5.1 0.5%(w/v) agarose gel electrophoresis of the PCR amplified putative *B. linens* lycopene cyclase gene, from *B. linens* 'Pink' (lane 2) and *B. linens* wild-type (lane 3). Values show length of marker bands (bp) (KB Marker Ladder, Stratagene, UK) (lanes 1 and 4). In all cases, PCR negative controls (lacking template material) failed to show any amplification

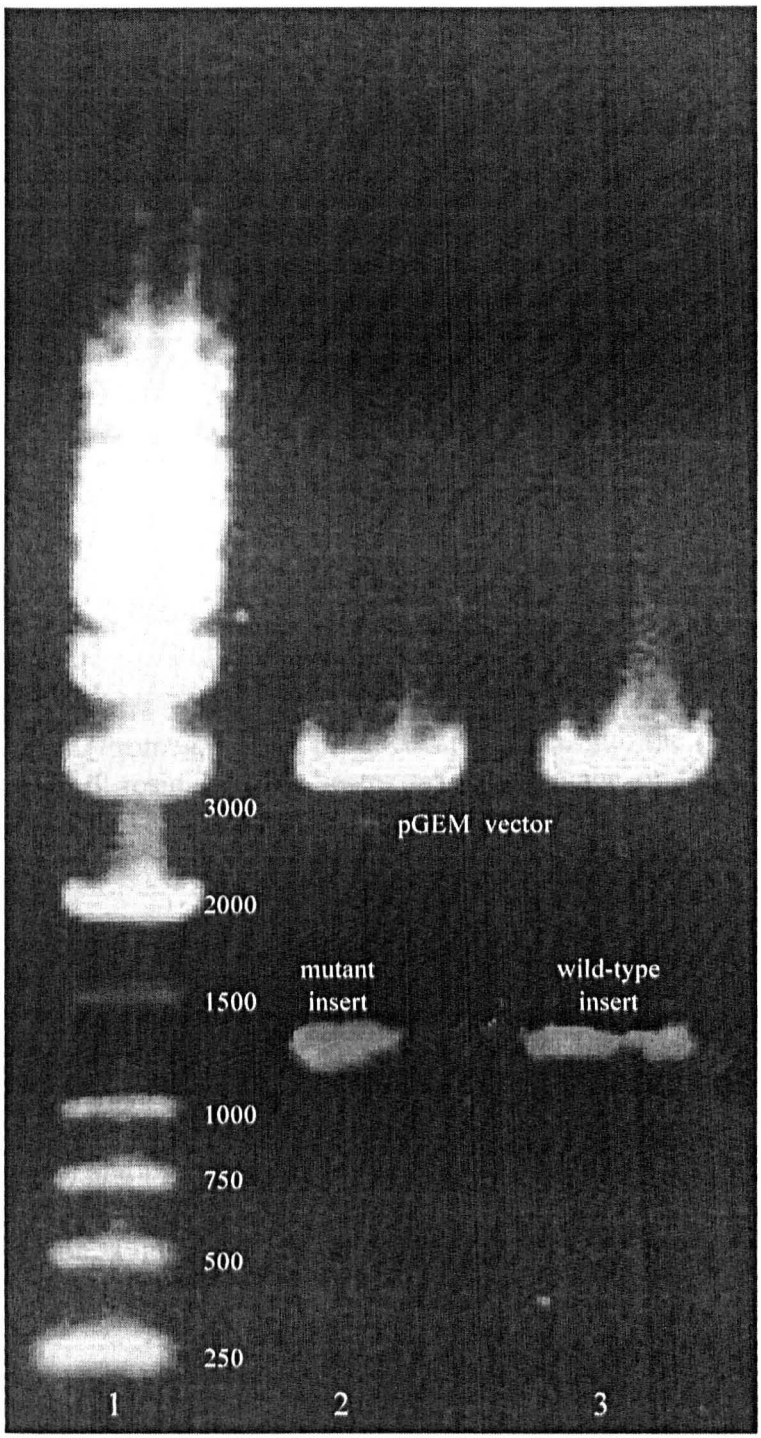
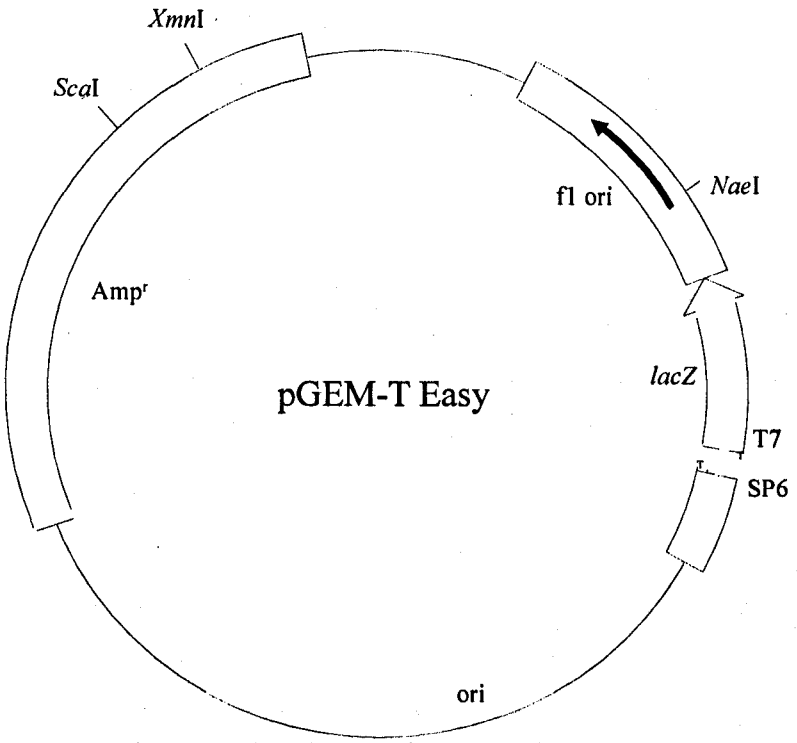


Plate 5.1(b) 0.5%(w/v) agarose gel electrophoresis of *Eco*RI digested recombinant pGEM-T Easy vector which contained the putative PCR amplified *B. linens* lycopene cyclase gene insert (mutant lane 2 and wild-type lane 3). Values show length of marker bands (bp) (KB Marker Ladder, Stratagene, UK) (lane 1) (insert band intensities have been enhanced)



pGEM-T Easy vector (Promega). PCR products are ligated into the position between the two overhanging T residues (this vector takes advantage of the fact that PCR generates products with overhanging A residues).

Having obtained sequence information using standard pGEM sequencing primers SP6 and T7 (see Appendix), further sequence data was elicited using primers based upon the obtained sequences. Primers CRTYLH2 and CRTYRH2 were designed (see below) and used to prime cycle sequencing reactions to obtain the final sequence information. Figure 5.1 shows the assembled sequences of mutated and wild-type genes of *B. linens* mutant 'Pink' (`pink.dc`) and NCIMB 8546 (`WILD.ed`) respectively. Sequences were aligned against one another using the GAP algorithm from the Wisconsin Genetic Computer Group's Sequence Analysis Software Package (1994).

```
  1  AAGCTAtGCA TCCAACGCGT TGGGAGCTCT CCCATATGGT CGACCTGCAG
 51  GCGGCCGCGA ATTCACTAGT GATTATGATG TGATGCTGGT GGGCGCTGAT
101  GAGGACCATG CCGTAGATCG TCGGGGCACC ACACGTGACA TAAACAAGCA
151  GGACAATGCC GGTGAACCAG CTGACGGTGC GTTTCCGTGG CCATTTGTCT
201  CCAGTCATGC GAGCCTTGCG CACTCCGAGG AGATAGACGG CGGCAGCAGT
251  GAGGATGAAC GCTAACCAGA GCCAGTCCCA CCGCCATTCG CTGAGCCACC
301  GGTACCAGGT CAGGGCCGGT GGCATGGGAT AGTCGGTGAG GACTTCGGCG
351  GGGGTGATCG CTGGCTCGAG TTCAAGCGGG ACAGGAGGGG CTGTTCGTCC
401  CAgTGCCGCG GTGACgCCGA TGACTGCGCC CATGATAANG ACTTCGGCAA
451  TTACCAATCG CCAAtCAAgg TAGTGTGCCG GATCTGCTTC GGACTTGGGT
501  CATTGGCCCA TTGGCGATGG GCGAATCCCG ATTGGCTCCT AGCAACNAGG
551  GTTgCCGANA ACTTGATGAA GATTAACCTG ANCGTaACCG GNTCANCAAA
601  CCCGTCCCAT TCNCCCAAGG CGGAGGGCAN CCTTNGATTA CANCCGGGNN
651  CTAAGGACCA ANGAAANACC CACGCCCGGC AATTCANAAN ACCNGGCCAA
701  TTACCGATTT TNNTNACCAA NCCGTNGAAC CCTTNTGNCA NNACTGGGAN
751  TGGTCANCGG GAAANTNCTN NAAACCCCAC CANT
```

The emboldened sequence is sequencing primer CRTYLH2, taken from *B. linens* sequence obtained using PCR and sequenced as an insert using standard primers SP6 and T7 (see above).

```
  1  TTGAGTcGNa tGCTCCGGCC GccNTGGCGG CCGCGGGAAT TCGATTATGA
 51  tgTGATGCTG GTGGGCGCTA CCGATCGCCT TGACCGTGCA CCATCTGGTG
101  TTGGGGGTGG TCGTTGGCTG CCTGATCTTC GCGGCAACAC TCGTTCCGTC
151  CAAAGACACT GACACGGCAG AAGAGGATTC GGCTGCCCAT CCGGCATTCA
201  CTCGCGTGCG GACCGTTGCC GTCGCCGCAT CGTTCGTGTG GCTGGGTTCA
251  GTGGTGGTGG TGACGGTGCT GACCTATGCC AATCTCGTGG GCCAGCCAGT
301  GTCGGGTAGT GCGGCCTTCT TAAGTCAGCT GACCTACTTC CTCACTGACC
351  TCATCGTCGG TCAAGCCTGG GGTGCAATCA CCGTCATTGC CTTCCTCGTT
401  TGCAACTTAG CCTTCTTCTT TCGTTCCACC ACCGGCCTGG CATGCACAGC
451  CCTGCTCGCA CTGACTGCCA TCGTCCCCAC TTCACTCATC GGTCATGCTG
501  CAGGCAGCGA CGACCATTAC GCCGGTGTCG GTGCGCTGGC TGTGCACTGG
551  CTCCGGAGTC CTTGTCTGGG TTGGTGGCGT TGCAGCACTT GCCGTGACCA
601  TCCCCGTGCT GGCCTCAAAC GAGTACCACG CTCGTGACAA AtTCGGTGAT
651  CGCCCGCTTC TCTGGCACTt GGcCGGCGTG GGTTCTCCTC GTCCTTAACT
701  CCgGTGTGAT CAACGCTGCN CTCNGCCTCN GTCAAANGGA CGGGCTGNTG
```

The emboldened and underlined sequence is sequencing primer CRTYRH2, taken from *B. linens* sequence obtained using PCR and sequenced as an insert using standard primers SP6 and T7.

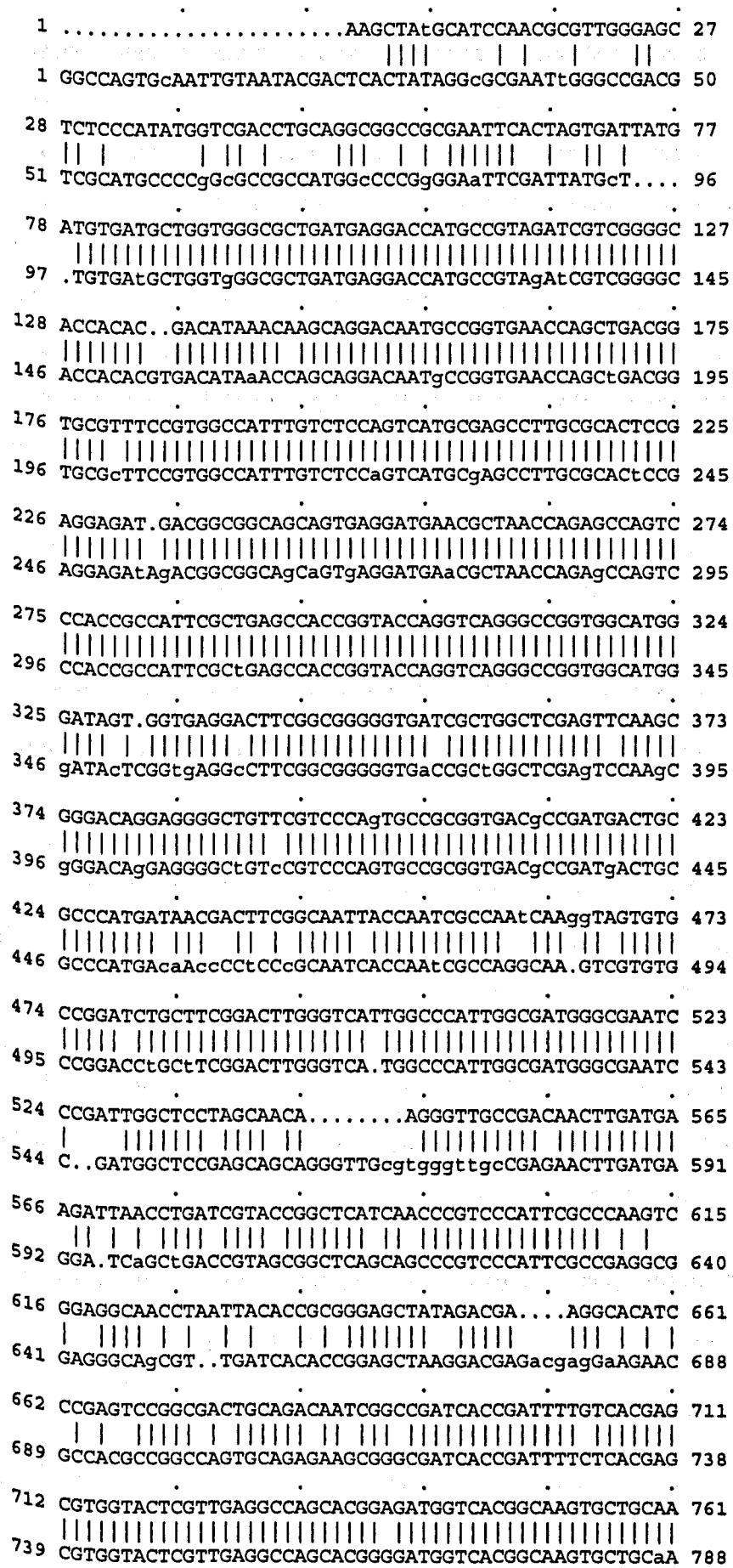

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Figure 5.1 Alignment between DNA sequences obtained from *B. linens* strains 'Pink' and wild-type NCIMB 8546 (lower sequence) using the GAP algorithm of the Wisconsin Genetic Computer Group's Sequence Analysis Software Package (1994).

The degree of similarity (88%) between the two sequences suggests that they are both from the same region of the *B. linens* genome. Whilst only one strand was sequenced in both strains, these data suggest a reasonable level of reproducibility. Although it is known that the *Taq* polymerase enzyme used in these PCR reactions has an error rate of approximately 1/10,000 bases, it is not possible to assign differences between the two sequences to either PCR errors or mutations with certainty. It is interesting to note, however, the predominance of putative deletions in the 'Pink' sequence, ranging from two of a single base in length (positions 233 and 331), through to one each of 2 (position 135), 3 (position 830), 4 (position 644) and 8 (position 544) bases. In contrast, the wild type sequence, when aligned, displays a slightly higher frequency of small gaps (3 of 1 base (positions 486, 521 and 595) and 2 of 2 bases (positions 545 and 653)) but only 1 of 5 bases (position 96). Since one of the putative deletions in the 'Pink' sequence (bases 828-831) occurs in a region where there is otherwise a very high level of homology between the two sequences, the

probability that this region may have occurred as the result of a genuine mutation rather than a sequencing or PCR error, is high.

MNNG is known as a mutagen that promotes transversions rather than deletions, and proceeding from the 5' end of the nucleotide sequence, possible transitions outnumber transversions such that 6 out of 18 possible mutations are transversions from 'Pink' sequence bases 145 to 527. The ratio gradually alters in favour of transversions between bases 528 to 704 so that 28 out of 42 possible mutations are transversions. Between bases 656-704, all 13 possible mutations are transversions. Only one more possible mutation follows, which is a transition. Possible mutations involving more than one base are noticeably rarer than those involving point mutations, indeed, only 17 out 61 possibly mutated bases are joined by another base or bases from 'Pink' sequence bases 145 to 908.

The amino acid sequence of the lycopene cyclase gene from wild-type *B. linens* NCIMB 8546 was deduced using the TRANSLATE algorithm from the Wisconsin Genetic Computer Group's Sequence Analysis Software Package (1994). When all three reading frames (in both directions) were compared with aligned lycopene cyclase amino acid sequences from plants and bacteria (see Appendix), there was insufficient homology in conserved regions, to suggest that the region of *B. linens* genome sequenced is in fact a lycopene cyclase gene, even when acceptable substituted amino acids were considered. Values for sequence similarity range from 38%, with *Flavobacterium* ATCC21588 to 44% with *Erwinia herbicola* Eho10 when the most homologous *B. linens* amino acid sequence reading frame is compared. Despite the lack of homology with conserved amino acid regions, these values

compare closely with those obtained when the *Agrobacterium aurantiacum* lycopene cyclase amino acid sequence is compared with those of *E. uredovora* or *E. herbicola* (44% and 45% respectively), though homology in the conserved regions is considerably higher.

For the nucleotide sequences of the lycopene cyclase gene, similarity values range from 37% for *E. uredovora* to almost 42% for *Flavobacterium* ATCC21588 when compared with the *B. linens* sequence. In comparison, the nucleotide sequence similarities between the lycopene cyclase (*crt*Y) genes of *E. longus* and those of *E. herbicola*, *E. uredovora* and *Synechococcus sp.* were found to be 40.2%, 37.4% and 22.9% respectively (Matsumura, *et al.*, 1997).

In another carotenoid biosynthesis enzyme, phytoene synthase (CrtB), homology has also been observed. In the case of *Agrobacterium aurantiacum* which has similarity values of 47%, 34% and 27% compared with *E. uredovora*, *R. capsulatus* and tomato respectively, there is greater diversity amongst the similarity values when compared with the homologies between the lycopene cyclase genes of *B. linens* and the same organisms. The phytoene desaturase enzyme, CrtI of *A. aurantiacum*, which catalyses neurosporene formation from phytoene, generally displays greater amino acid sequence similarity, with values of 63%, 64% and 43% in the same order as above.

5.3 The genetic manipulation of carotenogenic organisms: Obtaining the partial sequence of a potential *B. linens* vector, pBL100

An increasing number of strategies involve the manipulation of the carotenoid biosynthesis genes, frequently in an attempt to express these genes in non-carotenogenic hosts or to increase the output of cell pigments. *E. coli* has been made to express the carotenoid biosynthesis genes of *Erwinia herbicola* and an altered lycopene cyclase gene has been expressed in both *E. coli* and *S. cereviseae* (Ausich, 1994). Manipulation of the *crtE* (GGDP synthase) gene led to increased carotenoid enzyme activity in transformed *E. coli* (Ausich, 1994). In cloning the carotenoid biosynthesis genes of *Erwinia herbicola* into *E. coli* Haycock (1996) found levels of pigment higher in transformants than the native host, probably as a result of increased copy number. In cells carrying multicopy plasmids encoding the *crtB* gene of *Thermus thermophilus* HB27, carotenoid production increased by twenty times (Hoshino *et al.*, 1994) and *T. thermophilus* in itself presents a potentially useful property in that its carotenogenic gene cluster appears to reside upon a plasmid (Tabata *et al*, 1994).

Sometimes, even novel carotenoids are synthesised as the result of introducing non-native carotenoid biosynthesis genes into carotenogenic organisms, as in the case of *R. sphaeroides*, transformed with *crt*I, *crt*Y, *crt*B (phytoene synthase) and *crt*Z (β-carotene hydroxylase) genes from *E.herbicola* (Hunter *et al.*, 1994). In an experiment to investigate the enhancement of isoprenoid biosynthesis in *E. coli*, the presence of an exogenous isopentenyl diphosphate isomerase gene led to an uprating in the β-

carotene output of cells transformed with the genes *crt*E (GGDP synthase), *crt*B, *crt*I and *crt*Y (Kajiwara *et al.*, 1997).

Whilst molecular genetic approaches have been applied to the study of carotenoid biosynthesis, other work of relevance to the transformation of *B. linens* has taken place amongst members of the Corynebacteria. In *Brevibacterium lactofermentum* a 4.3kb native plasmid pBL1 with a copy number of approximately thirty has been fused with a bifunctional *Streptomyces lividans/Eschericia coli* vector pIJ860 capable of functioning in all three organisms. Further work resulting in the fusion of a 4.4kb *B. lactofermentum* plasmid with a pBR322 derivative (Yeh *et al*, 1986) has resulted in a shuttle vector capable of replication in *E.coli*, *Corynebacterium glutamicum* and *B. lactofermentum*. A much larger plasmid of 37 kb has also been found in *B. lactofermentum* (Kaneko *et al.*, 1979) along with another of 4.45 kb which has been completely sequenced (Filpula *et al*, 1986). Another potentially useful shuttle vector exists between *E.coli* and *Corynebacterium*, one species of which, *Corynebacterium glutamicum* contains an isolated and characterised transposable element (Vertès et al, 1994) which suggests a possible use in transposon mutagenesis. The circular double stranded 14 kb bacteriophage BL1 from *B. lactofermentum* has been efficiently introduced into protoplasts of its host (Sánchez, *et al.* 1986). Though little work has been done on *B. linens*, it is also known that this species is transformable with plasmid pBL1 (Sandoval *et al*, 1985).

Whilst the largest part of all bacteriological genetic manipulation has been concerned with *E. coli*, high frequency transformation of protoplasts (Santamaria *et*

al, 1985) and electrotransformation are easily reproducible mechanisms for introducing shuttle or ordinary *Brevibacterium* vectors into many brevibacteria. Electroporation appears to have the advantage that in many species any restriction/modification systems appear to be overcome (Bonnassie *et al*, 1990), particularly since no restriction-deficient corynebacterial host strains are known (Santamaria *et al*, 1985). Indeed, recalcitrant strains of the amino acid producer *Brevibacterium lactofermentum* were made electrotransformable using an ampicillin pre-treatment but restriction-modification appears to be of no obstacle when electrotransformation was used to introduce *E. coli* DNA into *B. lactofermentum* (Bonnassie, *et al*, 1990).

Since the production of non-native carotenoids in *B. linens* requires that foreign genes be introduced, the existence of an effective vector is of potential value. It is also possible that future strains of *B. linens* may be manipulated using plasmids carrying copies of carotenoid biosynthesis genes to increase gene dosage or that carotenoid biosynthesis genes with stronger promoters may be carried on suitable vectors. Whilst it is not certain that non-native *crt* gene promoters will be recognised when transferred into *B. linens*, nor that the required transcriptional regulatory factors will be present (McClure, 1985), the successful incorporation of productive carotenoid genes from *Erwinia* into *E.coli* provides a strong indication that this modification may be possible.

As discussed in Introduction to *Brevibacterium linens*: The use of *Brevibacterium linens* for the commercial objective of lycopene production, *B. linens*

CECT75 is known to posses a 7.75kb plasmid, pBL100, a restriction map of which is known (Figure 5.3) (Sandoval *et al*, 1985). In light of its potential application in future genetic manipulation experiments involving *B. linens*, an attempt was made to obtain the sequence of pBL100 by first purifying it from cultures of *B. linens* CECT75, restriction digesting and ligating into pBluescript (see Figure 5.3(b)), cloning in *E. coli* XL1 Blue and then purifying the insert containing vector from suitable clones (see Materials and Methods Sections 2.20.2, 2.20.10, 2.20.11 and 2.20.12). Finally, parts of the plasmid were sequenced (see Materials and Methods Sections 2.20.13).

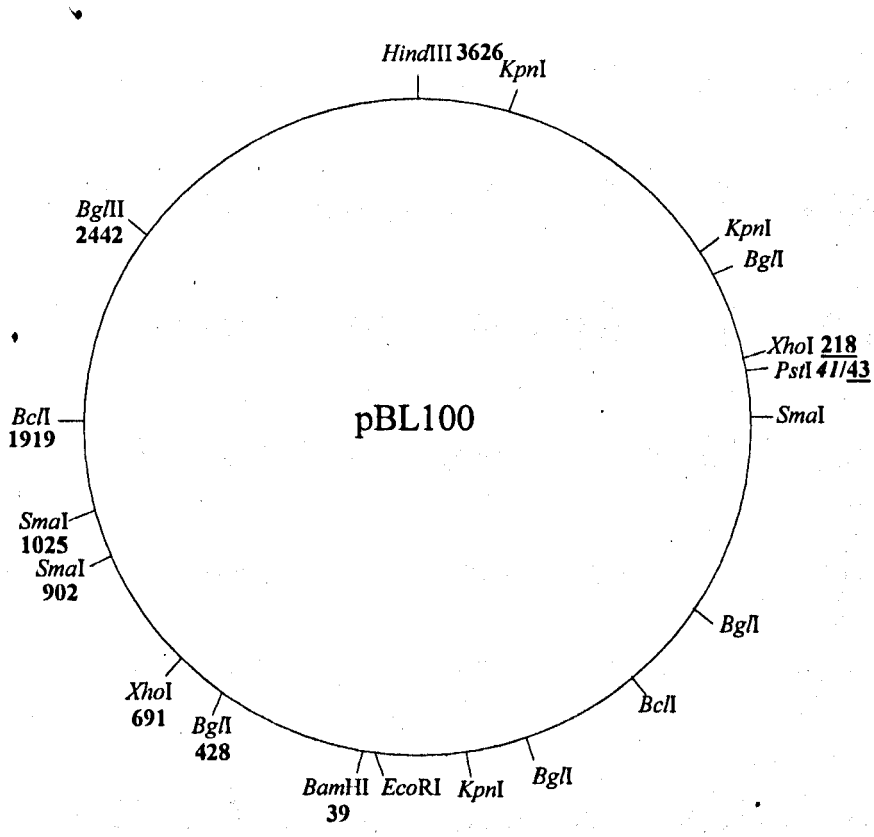


Figure 5.3 The *B. linens* CECT75 plasmid pBL100 of 7.75kb in length. Numbers correspond to nucleotide positions in the sequences given (see below). Bold numbers refer to the *Bam*HI to *Hind*III sites sequence; italic numbers refer to the clockwise sequence from the *Pst*I site; underlined numbers refer to the anticlockwise sequence from the *Pst*I site.

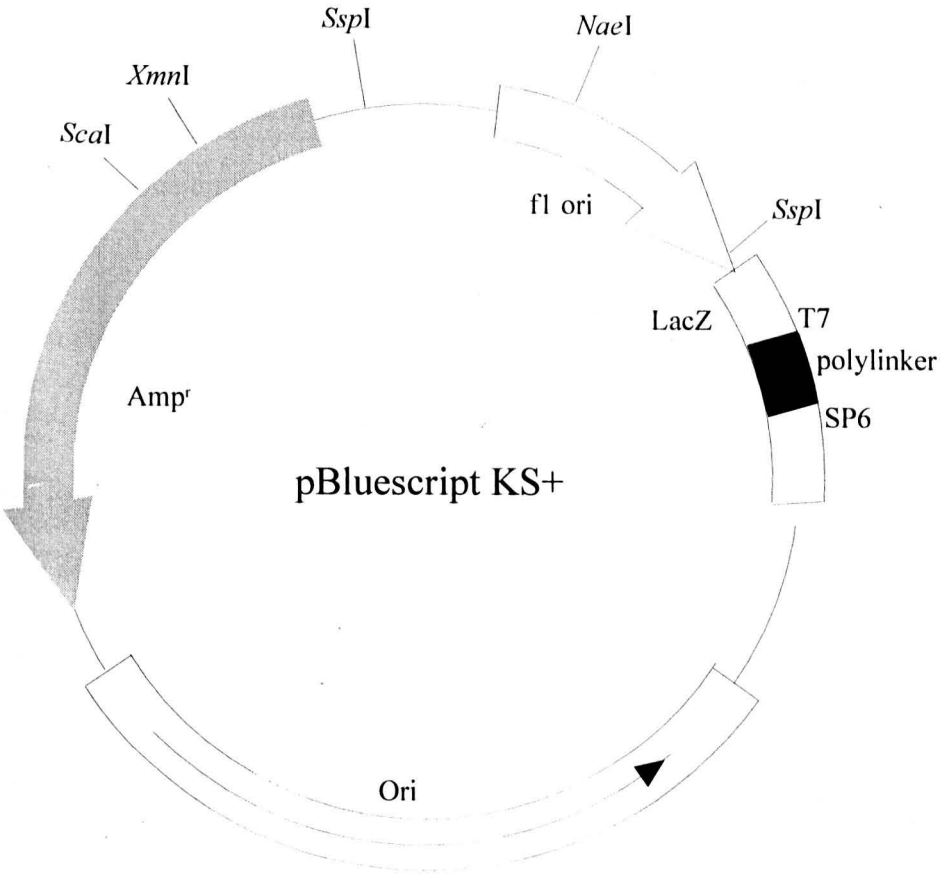


Figure 5.3(b) pBluescript vector (Stratagene). The polylinker site contained the insert.

The first sequence shown (see below) is taken from a fragment of plasmid pBL100 generated after restriction digestion with the enzymes *Hind*III and *Bam*HI. In all, eight sequencing primers were used to obtain the sequence between restriction enzyme sites *Hind*III and *Bam*HI, including the standard primers SP6 and T7, which prime from regions of the pBluescript vector which flank the insert. The insert, after excision from the vector, is shown in Plate 5.3. The other sequencing primers were designed based upon regions obtained by sequencing. The second sequences shown (see below) were taken from opposite ends of the entire plasmid, restriction digested at the *Pst*I site, the primers used were SP6 and T7. The insert, after excision from the vector, is shown in Plate 5.3(b). Restriction maps are shown, generated by the

Wisconsin Genetic Computer Group's Sequence Analysis Software Package (1994) using the MAP algorithm. Sites noted on the exisiting map of pBL100 (see Results and Discussion) are emboldened.

An attempt was also made to asign putative open reading frames (ORFs) to the sequences, and these are also shown below. Deduced amino acid sequences were obtained for all the plasmid sequences in all three reading frames and from both DNA strands. These sequences were compared with those of the 3159bp *B. linens* ATCC 19391 plasmid pBL-A8 (Leret *et al.,* 1995), the linecin encoding gene linM18 from *B. linens* M18 (Valdes-Stauber and Scherer, 1996), the putative theta replicase from plasmid pRBL1 from *B. linens* RBL1 (Ankri, *et al.,* 1995), a *Shigella sonnei* ColE5-O99 plasmid replicon region (Hiraga *et al.,* 1994) and a *Streptococcus pyogenes* pSM19035 plasmid replicon region (Sorokin *et al.,* 1990). In all cases it was not possible to find regions which suggested a notable level of homology when sequences were compared with the deduced pBL100 amino sequences individually, or in the case of the replicon regions, when multiple sequence alignenments were made and conserved regions compared with the pBL100 deduced amino acid sequences. When the full pBl100 plasmid sequence becomes available, it is feasible that homology will be found with the replicon regions listed above, as was found by Ankri *et al.,* (1995) when comparing them with the pRBL1 plasmid. Since the presence of linecins is unknown in *B. linens* CECT75, it is not possible to predict possible homologies with the linM18 gene mentioned.

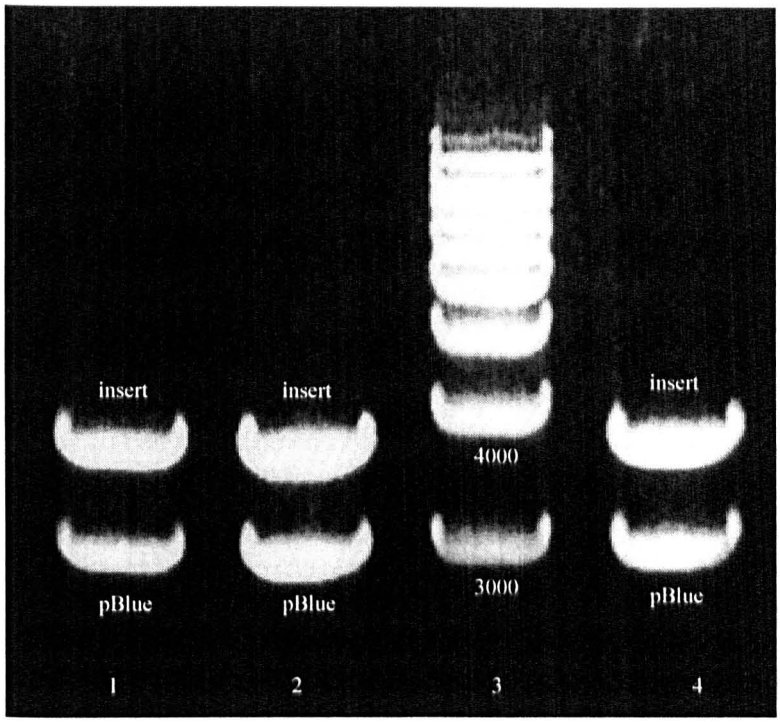


Plate 5.3 0.5%(w/v) agarose gel displaying the *Hind*III and *Bam*HI restriction digested pBluescript plasmid which contained the insert pBL100 fragment produced by insertion of a pBL100 *Hind*III and *Bam*HI digested plasmid fragment (lanes 1,2 and 4). Values show length of marker bands (bp) (lane 3) (KB Marker Ladder, Stratagene, UK).

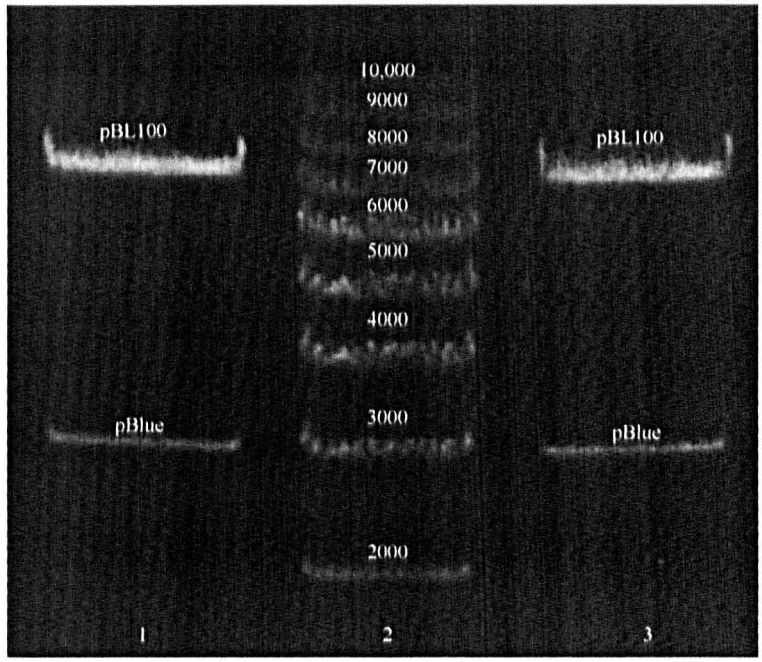
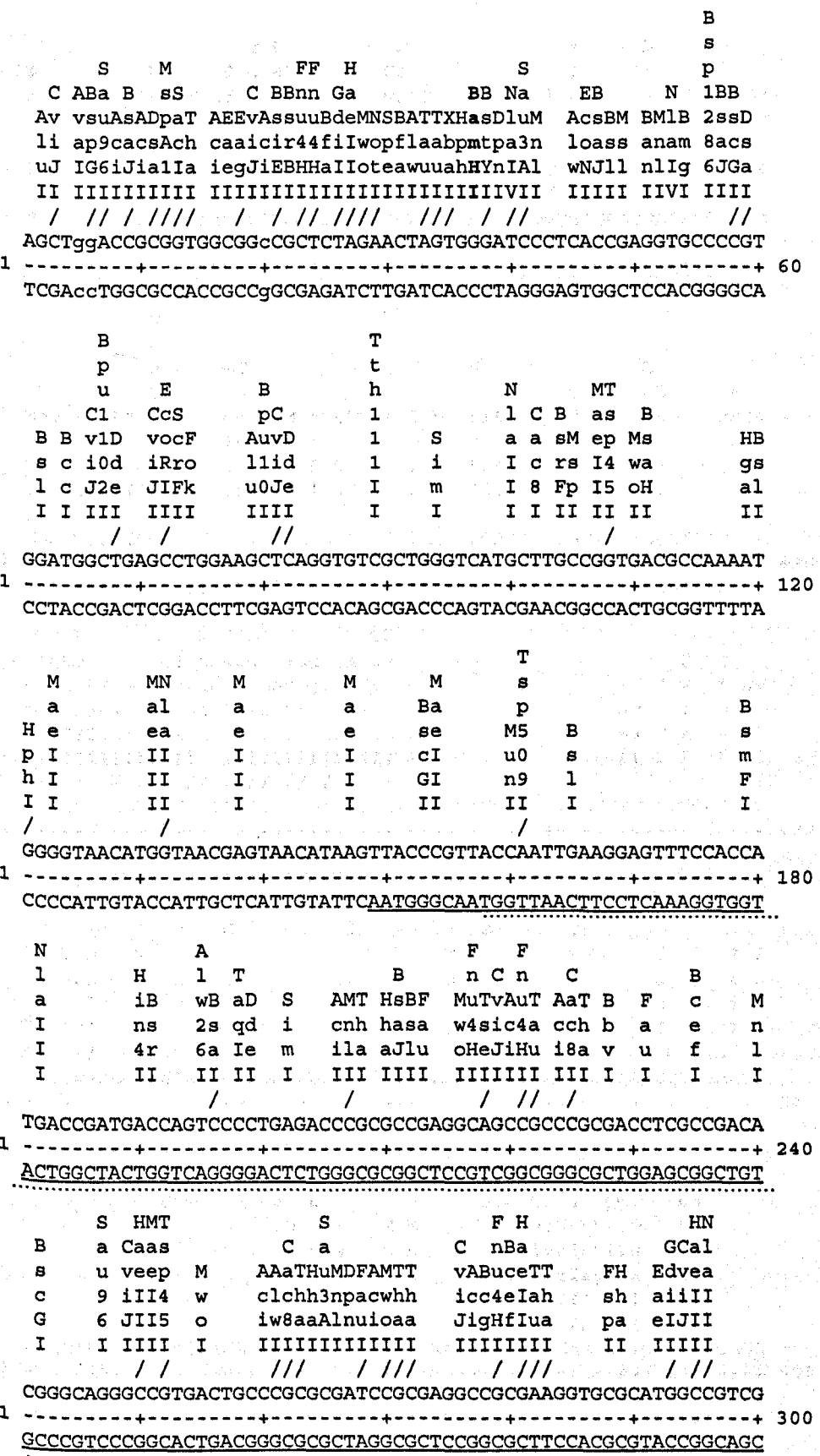
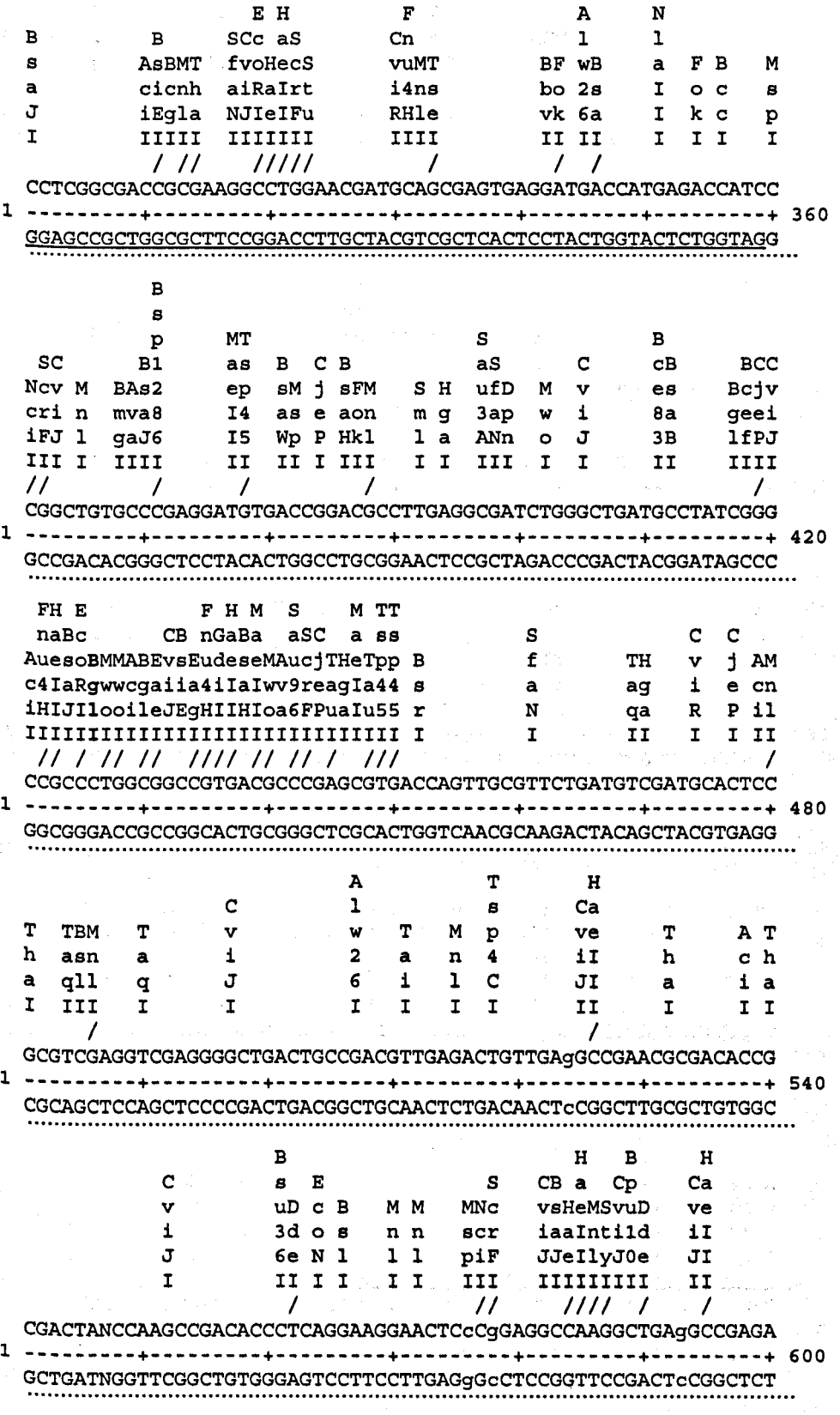


Plate 5.3(b) 0.5%(w/v) agarose gel displaying the restriction digested pBlue plasmid which contained the insert pBL100, restriction digested with *Pst*I (lanes 1 and 3). Values show length of marker bands (bp) (lane 2) (KB Marker Ladder, Stratagene, UK)

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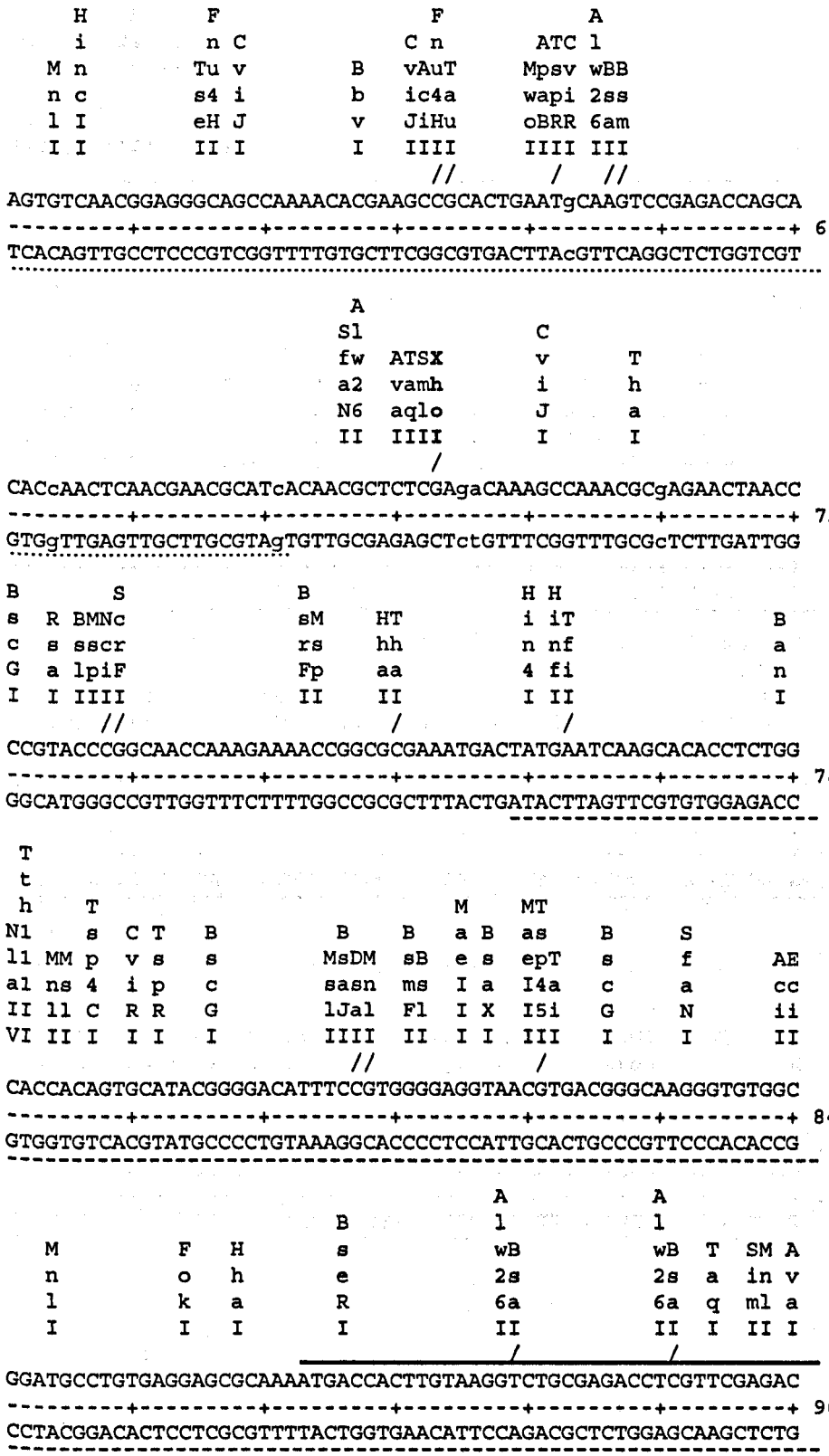
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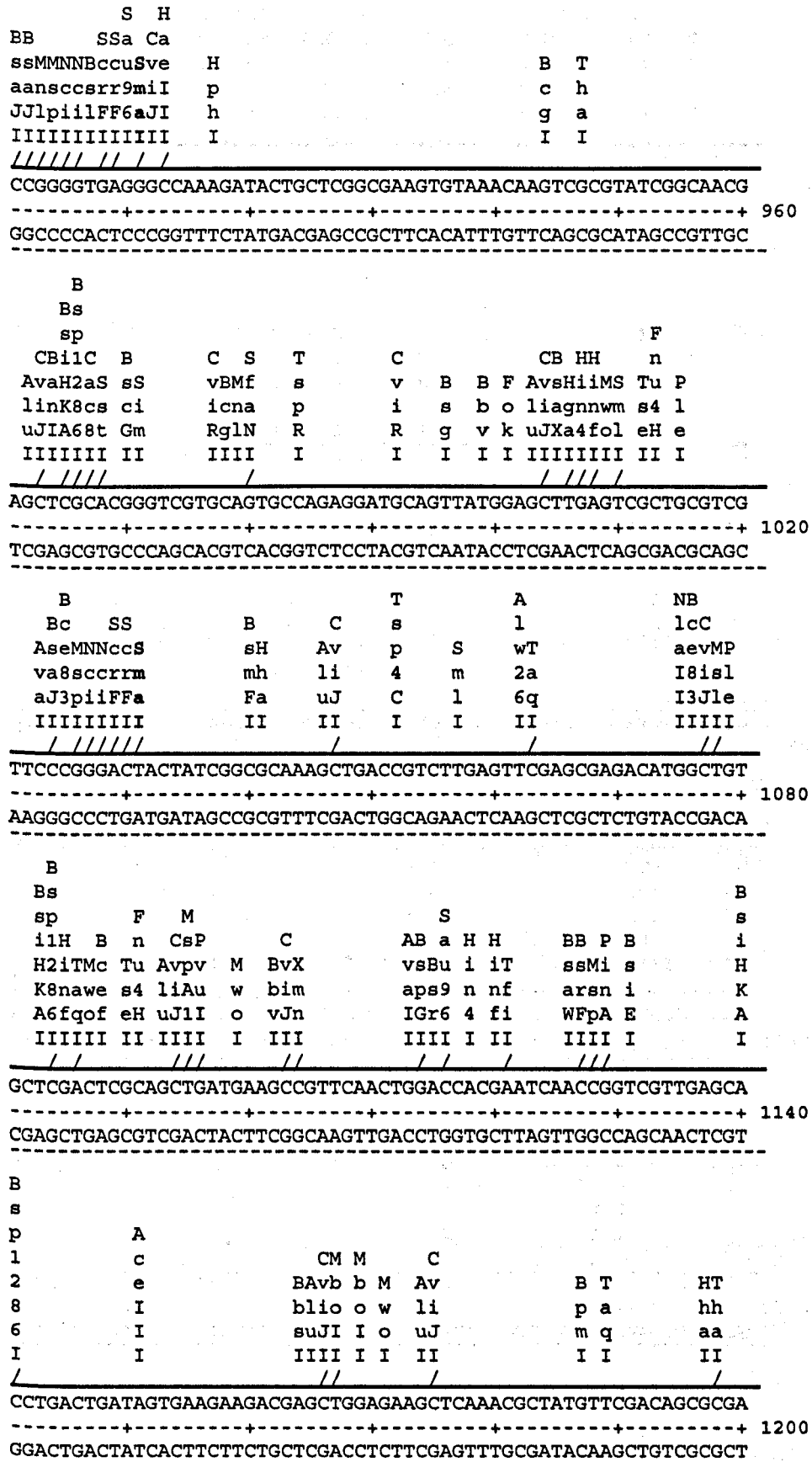
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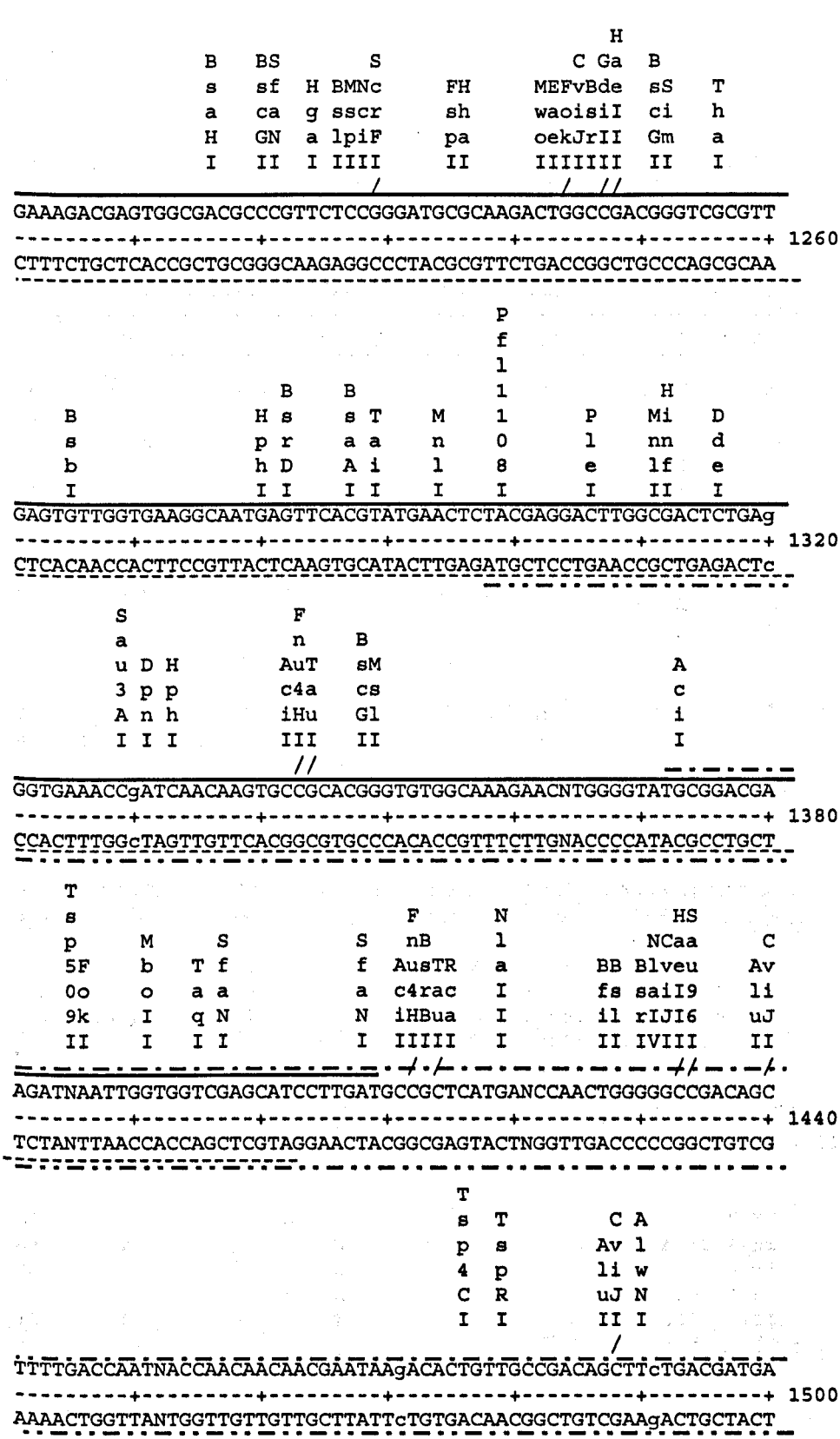
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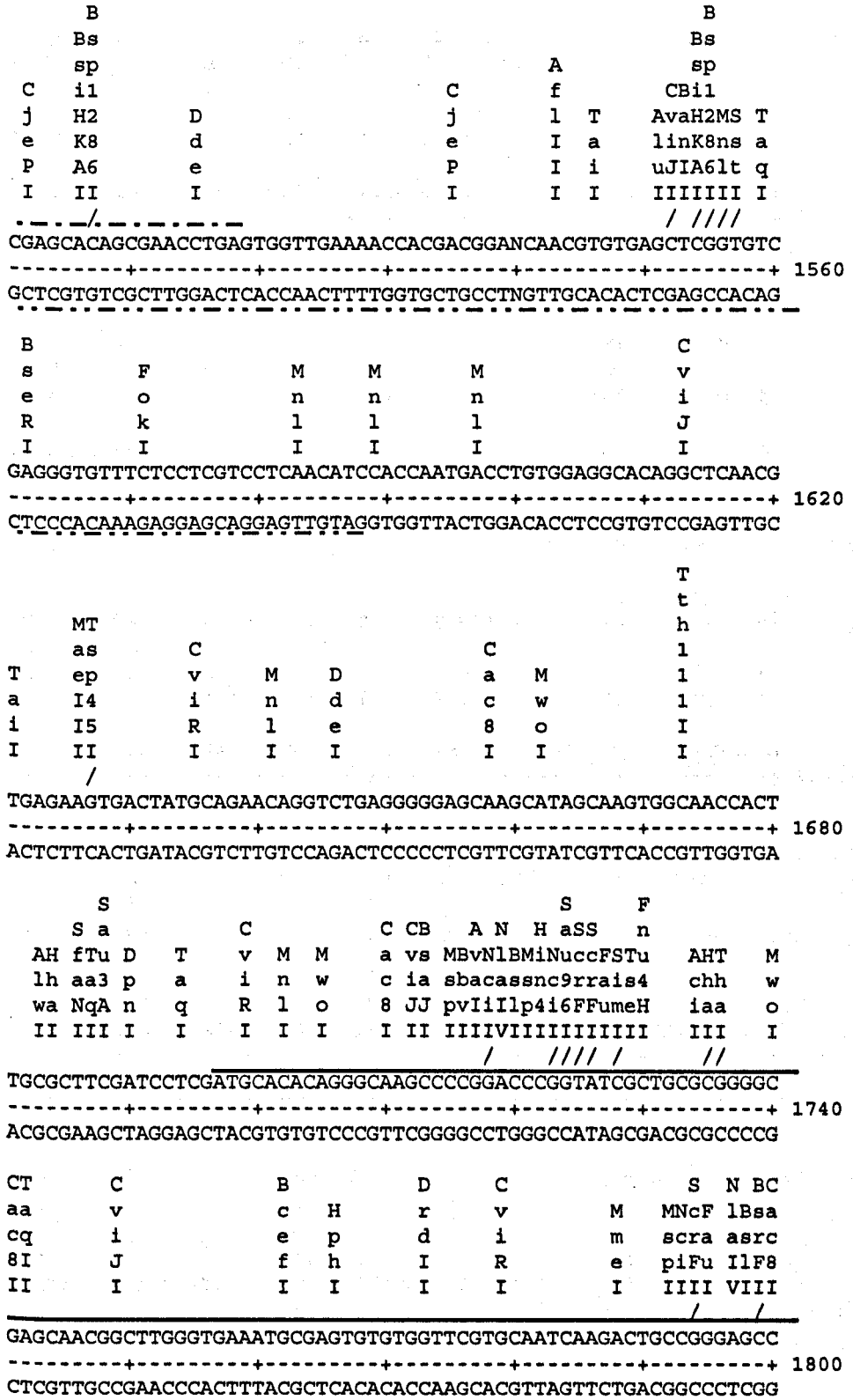
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1740
1741
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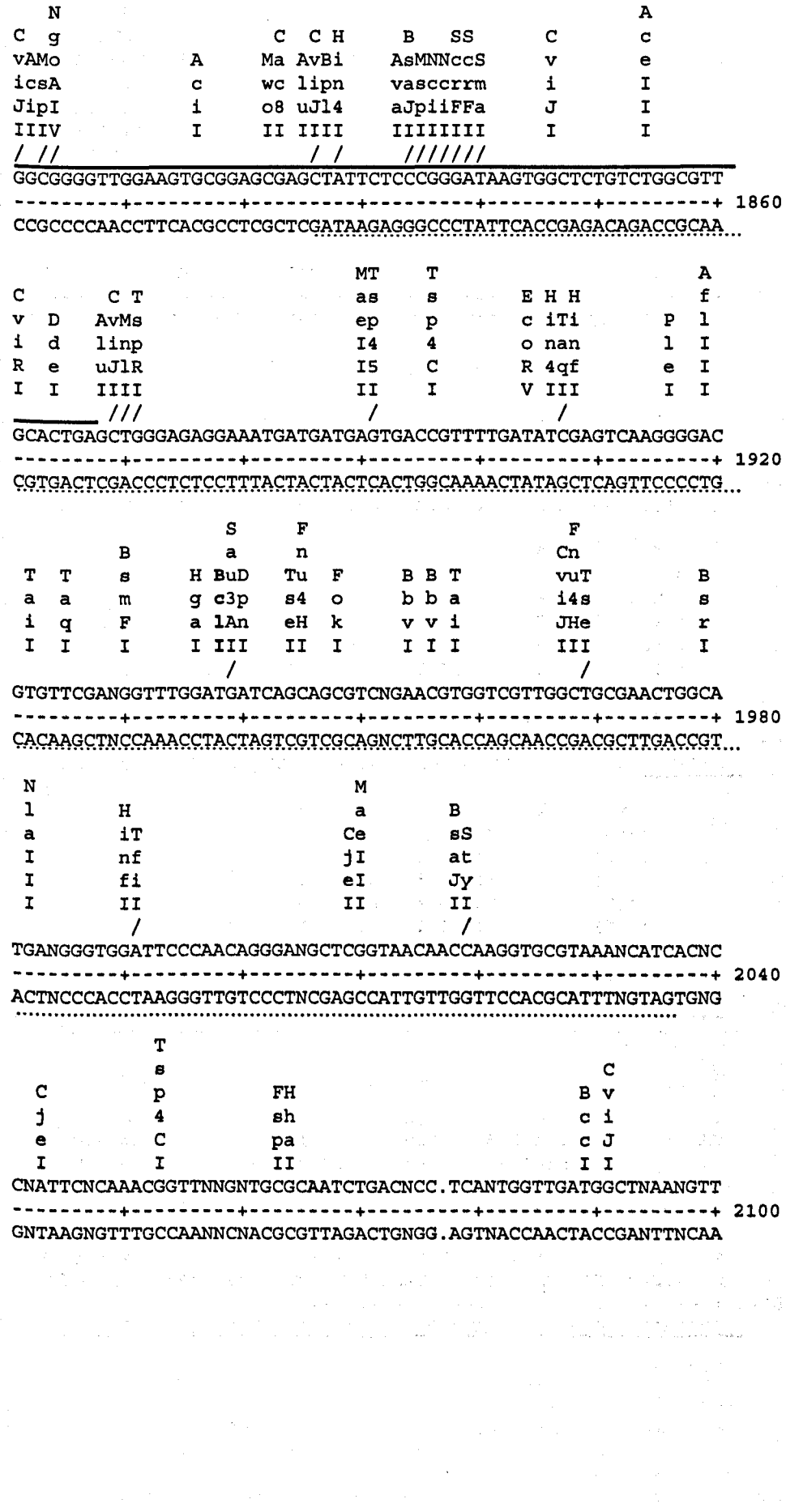
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1861 ---------+---------+---------+---------+---------+---------+ 1920
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GTGTTCGANGGTTTGGATGATCAGCAGCGTCNGAACGTGGTCGTTGGCTGCGAACTGGCA
1921 ---------+---------+---------+---------+---------+---------+ 1980
CACAAGCTNCCAAACCTACTAGTCGTCGCAGNCTTGCACCAGCAACCGACGCTTGACCGT
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1981 ---------+---------+---------+---------+---------+---------+ 2040
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CNATTCNCAAACGGTTNNGNTGCGCAATCTGACNCC.TCANTGGTTGATGGCTNAANGTT
2041 ---------+---------+---------+---------+---------+---------+ 2100
GNTAAGNGTTTGCCAANNCNACGCGTTAGACTGNGG.AGTNACCAACTACCGANTTNCAA

```
          H
        GBCa                B
       EdsveM   B           s
       airiIs   s           m
       eIFJIp   l           F
       IIIIII   I           I
        / ///
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    TATTATNGGACANATAAGGNGGTANGTGNGGNAATANCNTAAAGGGGNATCCNAACGGNN
2161 ---------+---------+---------+---------+---------+---------+ 2220
    ATAATANCCTGTNTATTCCNCCATNCACNCCNTTATNGNATTTCCCCNTAGGNTTGCCNN

                        M
                        s
                        p
                        I
    ACCTTACNCCCCNGNNAANNACCGGNTCGTNNAANCCNCATNCGNCNNCCNNCAAANTTN
2221 ---------+---------+---------+---------+---------+---------+ 2280
    TGGAATGNGGGGNCNNTTNNTGGCCNAGCANNTTNGGNGTANGCNGNNGGNNGTTTNAAN

                                                    A
                          A              BCC        c            M
          A T     FRDT    l     T        Asav       e       MDS b
          c h     asda    w     a        lmci       I       srw o
          i a     uaet    N     q        uF8J       I       eaa I
          I I     IIII    I     I        IIII       I       III I
                   / /                    / /                  /
    CANTTCCCCGCGTGTACTCAGGGACTGCTCGACAGCTCGCAAGTGAAGATATTTAAATAT
2281 ---------+---------+---------+---------+---------+---------+ 2340
    GTNAAGGGGCGCACATGAGTCCCTGACGAGCTGTCGAGCGTTCACTTCTATAAATTTATA

                                  S                        M
    M                             Ba          C            CsBP
    b B             S       A     suD   C A   Av           Avpcv
    o f             s       v     t3p   j l   li           liAeu
    I a             p       a     YAn   e w   uJ           uJ1fI
    I I             I       I     III   I I   II           IIIII
                                   /           /            // /
    GTTCTAGTTTCTTCAATATTTTCGCTCGGGATCTATGTAGCTTCCACCAGCTGTAAGTTC
2341 ---------+---------+---------+---------+---------+---------+ 2400
    CAAGATCAAAGAAGTTATAAAAGCGAGCCCTAGATACATCGAAGGTGGTCGACATTCAAG

                                                                E
                                                                c
          H MT                A          E           S S       o
    BB  GCa as                l  P       cBSS     B B aBa      AO
    ssDEdveCep          BB    wBBf       osce     gAsDusu D    v1
    acsaiiIjI4          sf    2ssl       Rarx     lltp3a3 p    a0
    JGaeIJIeI5          ri    6alM       IXFA     IwYnABA n    I9
    IIIIIIIIII          II    IIII       IIII     IIIIIII I    II
     // / / //              / /          /       ///           /
    CCCGTGGCCGTGACCTTCCCCCAGTTCTTGGAGACCAGGTAGATCTTGATCCTTTCAGGG
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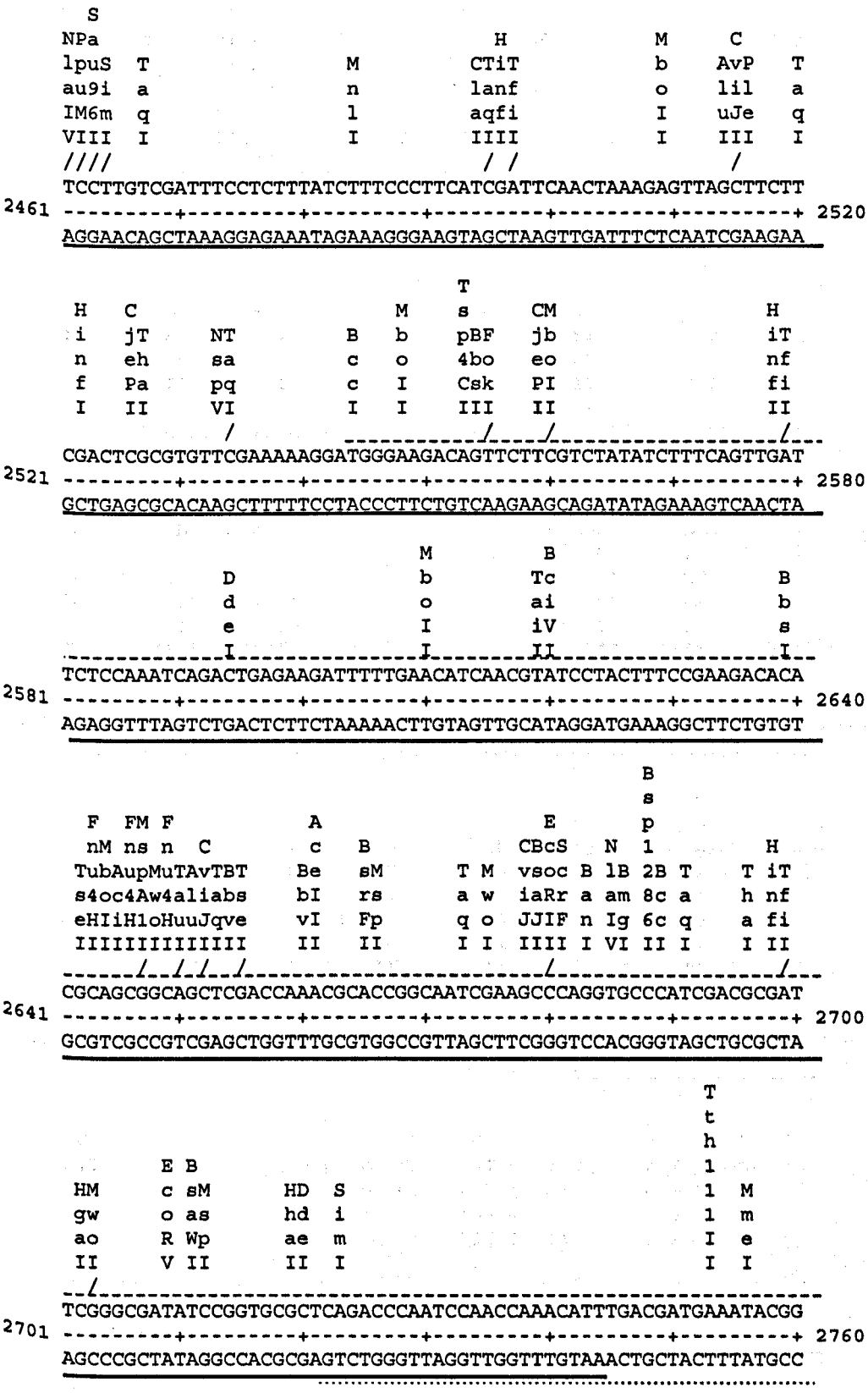
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2521
2580
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2581
2640
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2641
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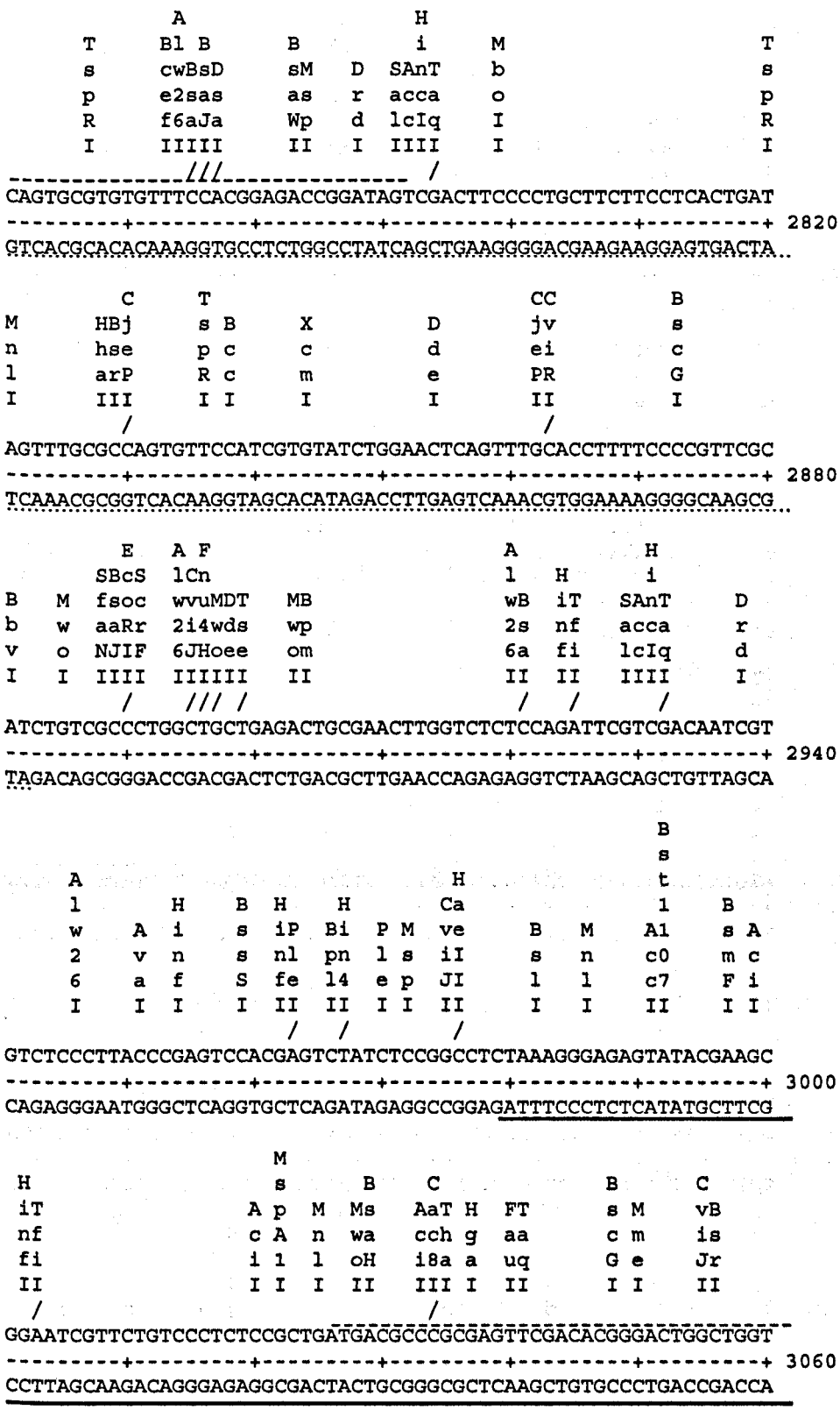
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2821 2880
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2881 2940
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GTCTCCCTTACCCGAGTCCACGAGTCTATCTCCGGCCTCTAAAGGGAGAGTATACGAAGC
2941 3000
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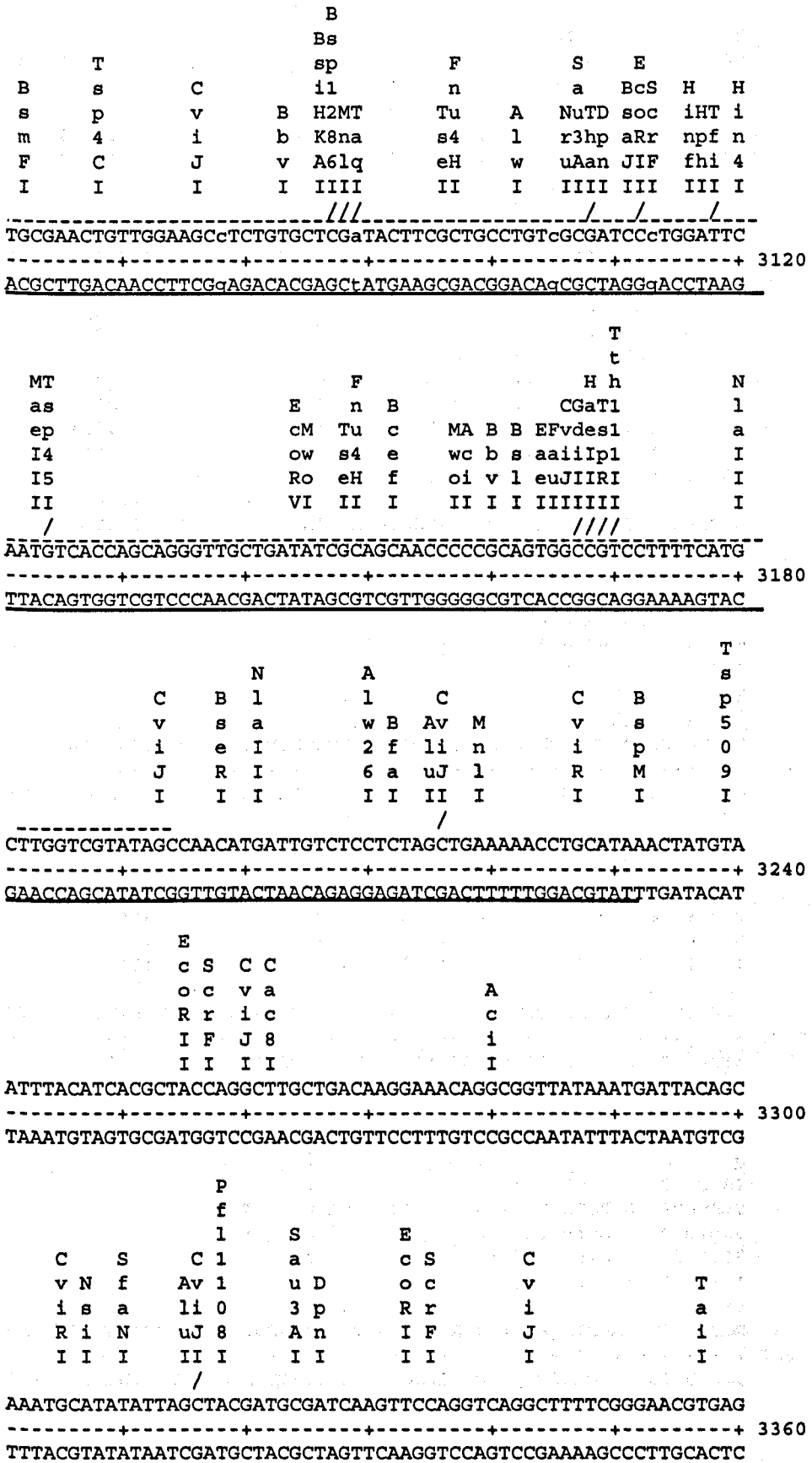
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3121 + 3180
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3181 + 3240
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3241 + 3300
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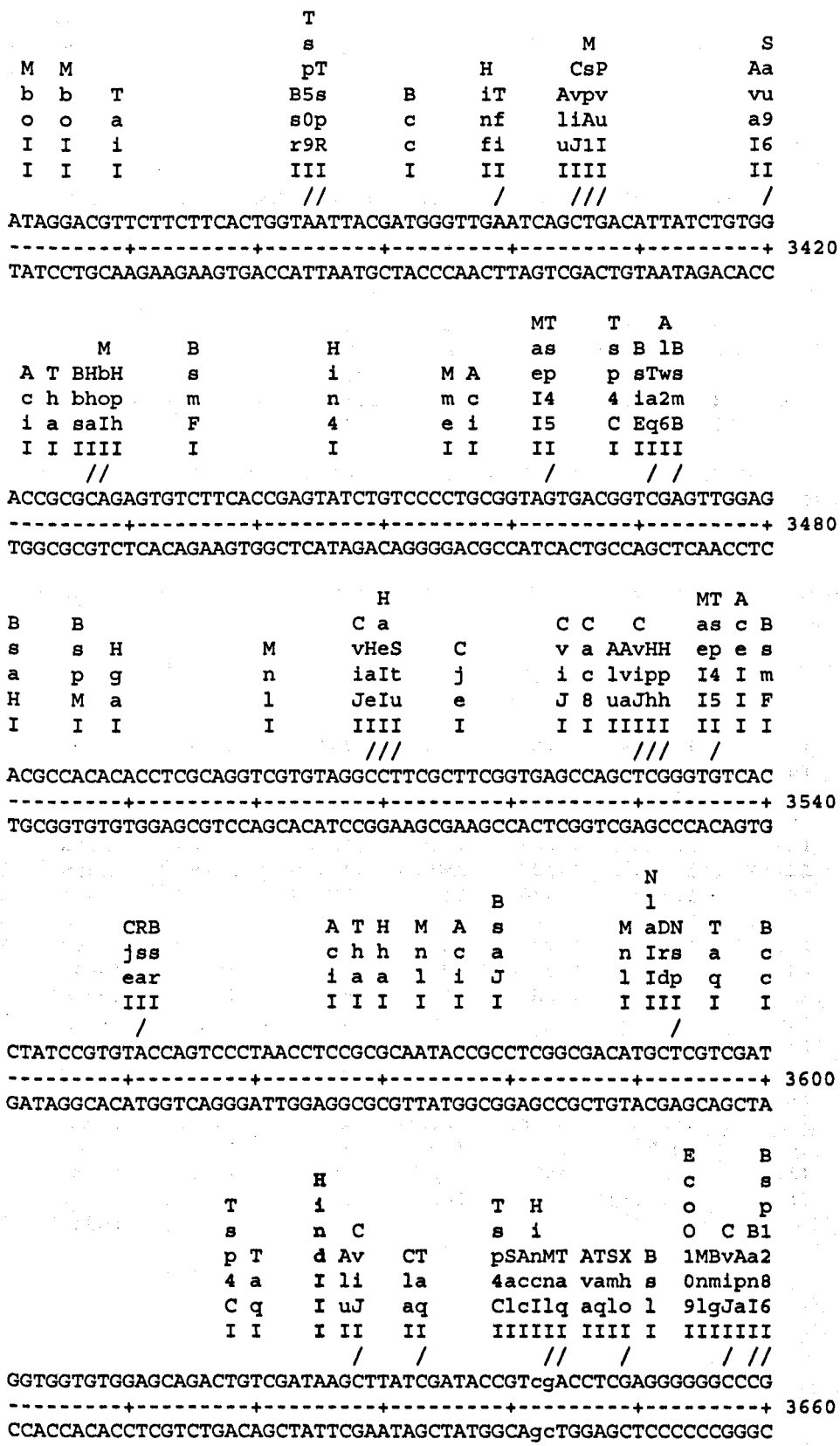
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3481 3540
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3541 3600
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3601 3660
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```
     H  SS
     aNNaa
     elluu  M
     Iaa99  s
     III66  e
     IVVII  I
     /////
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3661 ---------+---------+---------+---------+---------+---------+ 3720
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                                  H                    N
                 B                GCa      B           Rl       B
              R  c                Edve     s RT        laM      c
              s  e                aiiI     r sa        eIs      e
              a  f                eIJI     G at        AIl      f
              I  I                IIII     I II        III      I
                                   / /       /
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3721 ---------+---------+---------+---------+---------+---------+ 3780
     GGTNTCCTCATG.ACTGNA.CCTNCACCGGCACTACATGTGCCGTGTACTTCCACTCACA

     GGG
3781 --- 3783
     CCC
```

Enzymes that do cut:

AccI	AceIII	AciI	AflIII	AluI	AlwI	Alw26I	AlwNI
ApaI	ApaBI	AvaI	AvaII	BamHI	BanI	BanII	BbsI
BbvI	BccI	Bce83I	BcefI	BcgI	BciVI	BclI	BfaI
BfiI	BglI	BglII	BmgI	BplI	BpmI	Bpu10I	Bpu1102I
BsaI	BsaAI	BsaBI	BsaHI	BsaJI	BsaWI	BsaXI	BsbI
BscGI	BseRI	BsgI	BsiEI	BsiHKAI	BslI	BsmI	BsmBI
BsmFI	Bsp1286I	BspGI	BspMI	BsrI	BsrBI	BsrDI	BsrFI
BsrGI	BssSI	Bst1107I	BstYI	Bsu36I	Cac8I	CjeI	CjePI
ClaI	CviJI	CviRI	DdeI	DpnI	DraI	DrdI	DrdII
DsaI	EaeI	EagI	EciI	EcoNI	Eco0109I	EcoRII	EcoRV
FauI	Fnu4HI	FokI	FspI	GdiII	HaeI	HaeIII	HgaI
HhaI	Hin4I	HincII	HindIII	HinfI	HphI	MaeIII	MboII
MmeI	MnlI	MseI	MslI	MspI	MspA1I	MunI	MwoI
NciI	NgoAIV	NlaIII	NlaIV	NotI	NruI	NsiI	NspI
NspV	Pfl1108I	PflMI	PinAI	PleI	PpuMI	PvuII	RcaI
RleAI	RsaI	SacII	SalI	Sau96I	Sau3AI	ScrFI	SexAI
SfaNI	SimI	SmaI	SmlI	SpeI	SspI	SstI	StuI
StyI	SwaI	TaiI	TaqI	TaqII	TatI	TauI	TfiI
ThaI	TseI	Tsp45I	Tsp4CI	Tsp509I	TspRI	Tth111II	XbaI
XcmI	XhoI	XmnI					

Enzymes that do not cut:

AatII	AflII	AhdI	ApaLI	ApoI	AscI	AvrII	BaeI
BaeI	Bsp24I	Bsp24I	BspEI	BspLU11I	BssHII	BstEII	BstXI
DraIII	EarI	Eco47III	Eco57I	EcoRI	FseI	HaeII	HgiEII
HpaI	KpnI	MluI	MscI	NarI	NcoI	NdeI	NheI
PacI	PmeI	PmlI	PshAI	Psp1406I	PstI	PvuI	RsrII
SanDI	SapI	ScaI	SfcI	SfiI	SgfI	SgrAI	SnaBI
SphI	SrfI	Sse8387I	Sse8647I	SunI	Tth111I	UbaDI	VspI

The sequence above shows the position of restriction enzymes sites in the region of plasmid pBL100 sequenced between the restriction sites *Hind*III and *Bam*HI. The restriction enzymes that have recognition sites and those that do not are also listed. Solid, broken and dotted lines indicate possible open reading frames.

```
      M     S  F H                S       F                E
  B   sS   CaC nBa              BB NaB B   Cn C  SS         c S
 AsADFpaTA auvAuseTBX   SAB B asDlusAsAMNvuMvNPccSST       o c
 cacsaAchc c9ic4rIafb   plf b mtpa3avalsci4nicsrrfms       R r
 iJiau1Iai 86JiHBIuaa   ewa v HYnIAJaJwpiJH1RitFFcae       I F
 IIIIIIIII IIIIIIIII    III I IIIVIIIIIIIIIIIIIIIIII       I I
  / /////   /  / // /     /    / //  / //  / / /////
 CCGCGGTGGCGGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAGGTGGAAGTCC
1 ---------+---------+---------+---------+---------+---------+ 60
 GGCGCCACCGCCCGGCGAGATCTTGATCACCTAGGGGGCCCGACGTCCTCCACCTTCAGG

                          T
          T               s                              S
          s  H      NBBM  E p          C   H        M     a
          p  i B    lssbMAcP5     T H a   iT       b     u D
          4  n s    aapospol0     h h c   nf       o     3 p
          C  f 1    IWEIpoRe9     a a 8   fi       I     A n
          I  I I    VIIIIIIII     I I I   II       I     I I
                     /// ///              /
 TGGTTTTTTACCGTGAGTCGGTTCCGGAATTCTTCGCGCTGGCGAATCAACTCCTGCCGA
61 ---------+---------+---------+---------+---------+---------+ 120
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                ES          F
               Aca          n  B   C              C
      M    TB  vouA T       AuT sM  v    B       j       B
      n    as  a59c h       c4a pw  i    p       e       s
      l    ql  I76i a       iHu Mo  J    m       P       r
      I    II  IIII I       III II  I    I       I       I
                /           //
 TCTTCGCCCATTTCGAGGTCCGCGACCTGCCGCTTCAGCCGACGGACTTCTCGCTTACTC
121 ---------+---------+---------+---------+---------+---------+ 180
 AGAAGCGGGTAAAGCTCCAGGCGCTGGACGGCGAAGTCGGCTGCCTGAAGAGCGAATGAG

      S           N        H   S                F
      a           l    C BCa  Ba   B     H     Cn C C
      u D   TMHM  aNT   j sveM suTBBsDP   iT   BvuTv a   BM
      3 p   hshn  Isa   e riIs i3asbipv   nf   bi4si c   cw
      A n   alal  Ipq   P FJIp EAqgvEnu   fi   vJHeR 8   go
      I I   IIII  III   I IIII IIIIIIII   II   IIIII I   II
              /     /// /  /// /     /      /
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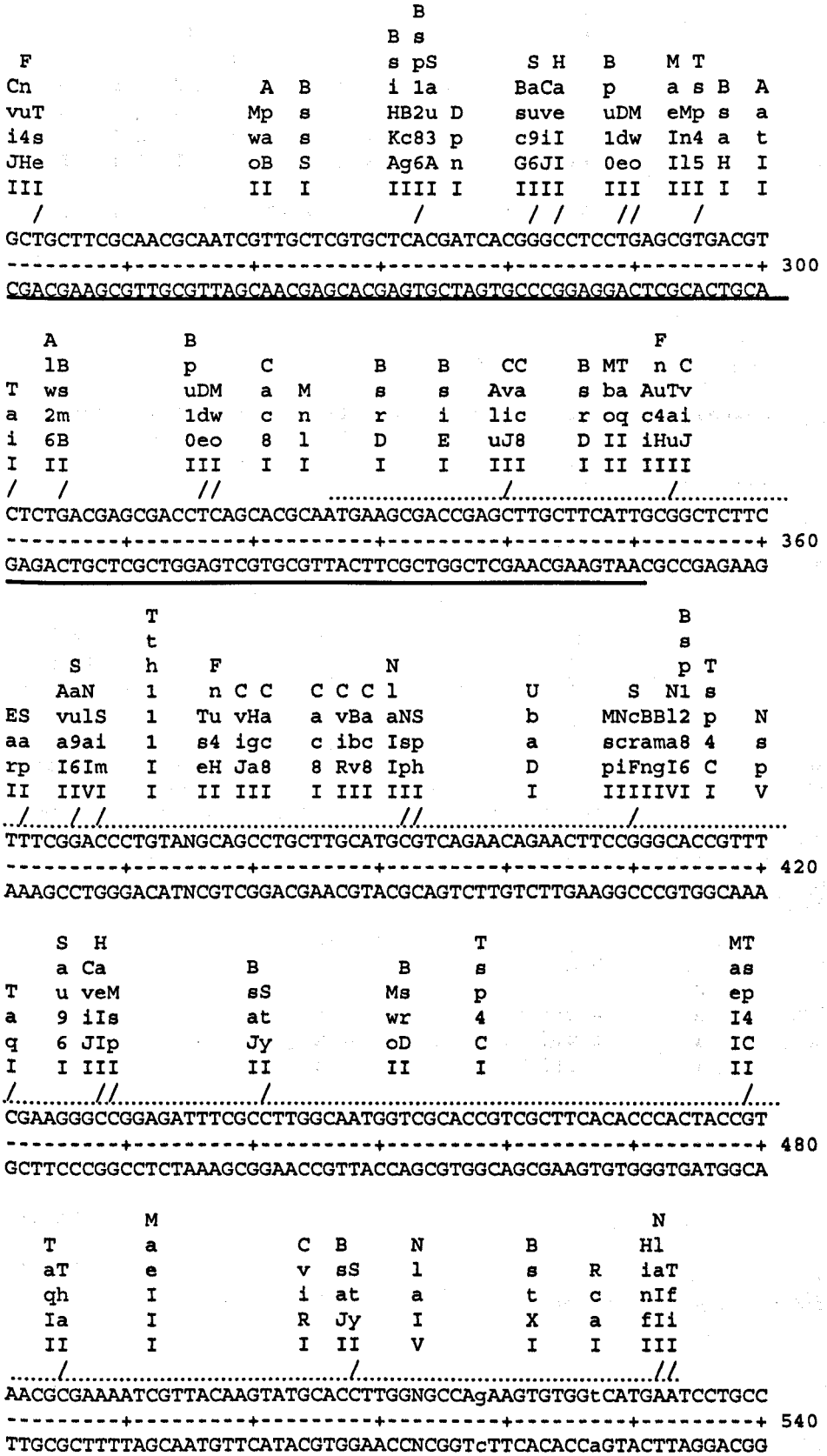
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241 ---------+---------+---------+---------+---------+---------+ 300
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301 ---------+---------+---------+---------+---------+---------+ 360
GAGACTGCTCGCTGGAGTCGTGCGTTACTTCGCTGGCTCGAACGAAGTAACGCCGAGAAG
TTTCGGACCCTGTANGCAGCCTGCTTGCATGCGTCAGAACAGAACTTCCGGGCACCGTTT
361 ---------+---------+---------+---------+---------+---------+ 420
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421 ---------+---------+---------+---------+---------+---------+ 480
GCTTCCCGGCCTCTAAAGCGGAACCGTTACCAGCGTGGCAGCGAAGTGTGGGTGATGGCA
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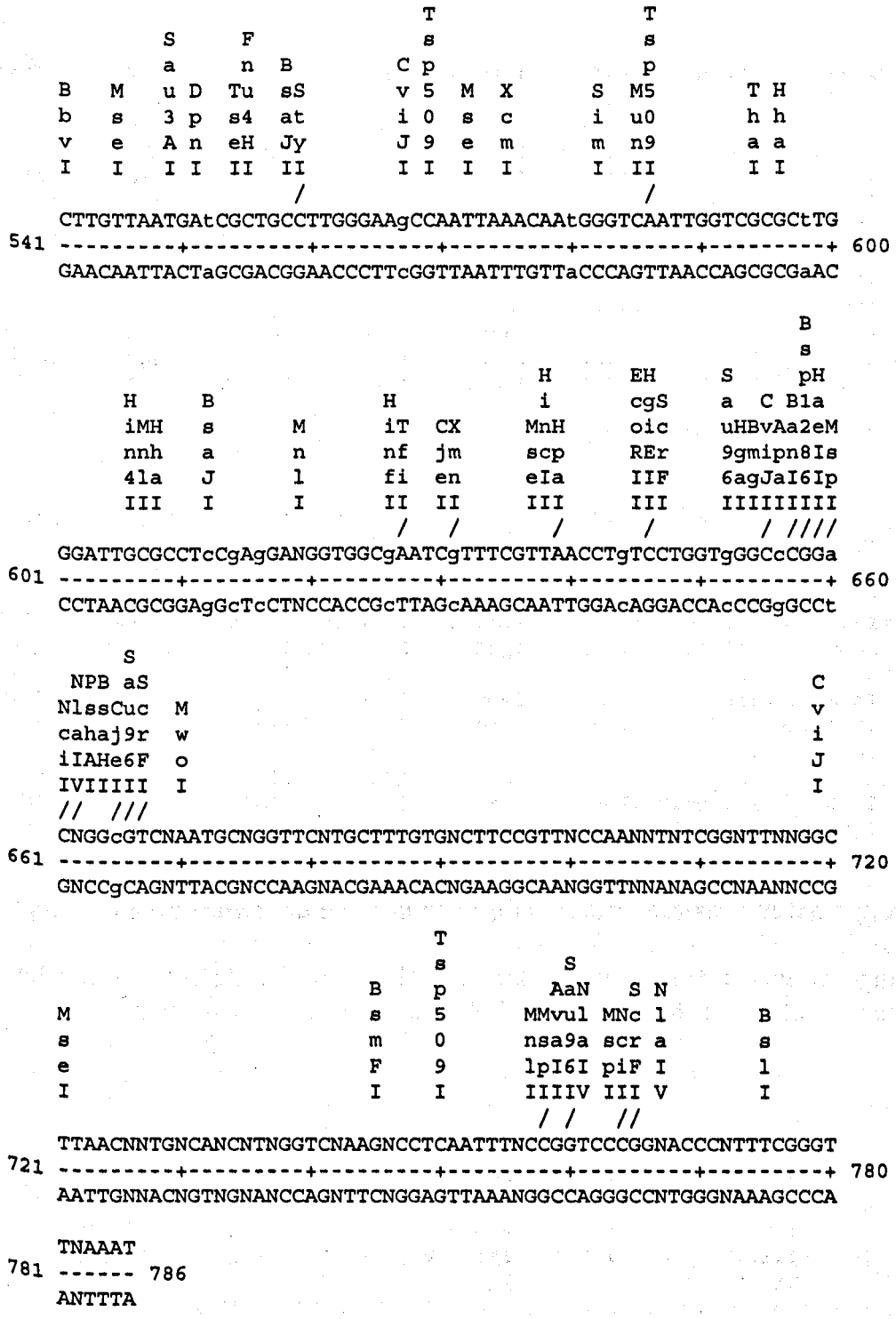
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601 660
CCTAACGCGGAgGcTcCTNCCACCGcTTAGcAAAGCAATTGGAcAGGACCAcCCGgGCCt
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661 720
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721 780
AATTGNNACNGTNGNANCCAGNTTCNGGAGTTAAANGGCCAGGGCCNTGGGNAAAGCCCA
TNAAAT
781 786
ANTTTA

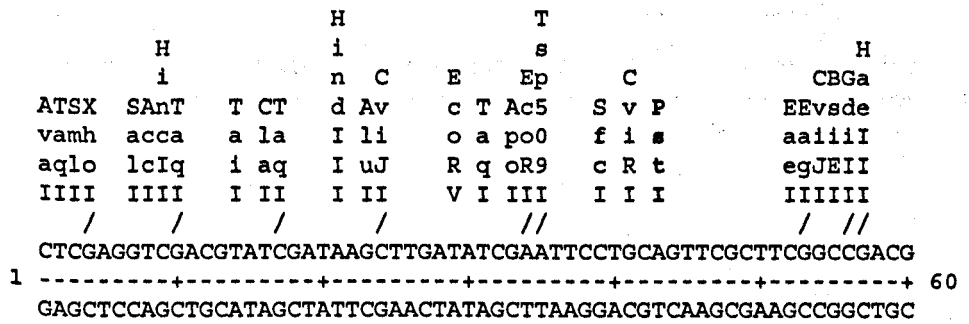
Enzymes that do cut:

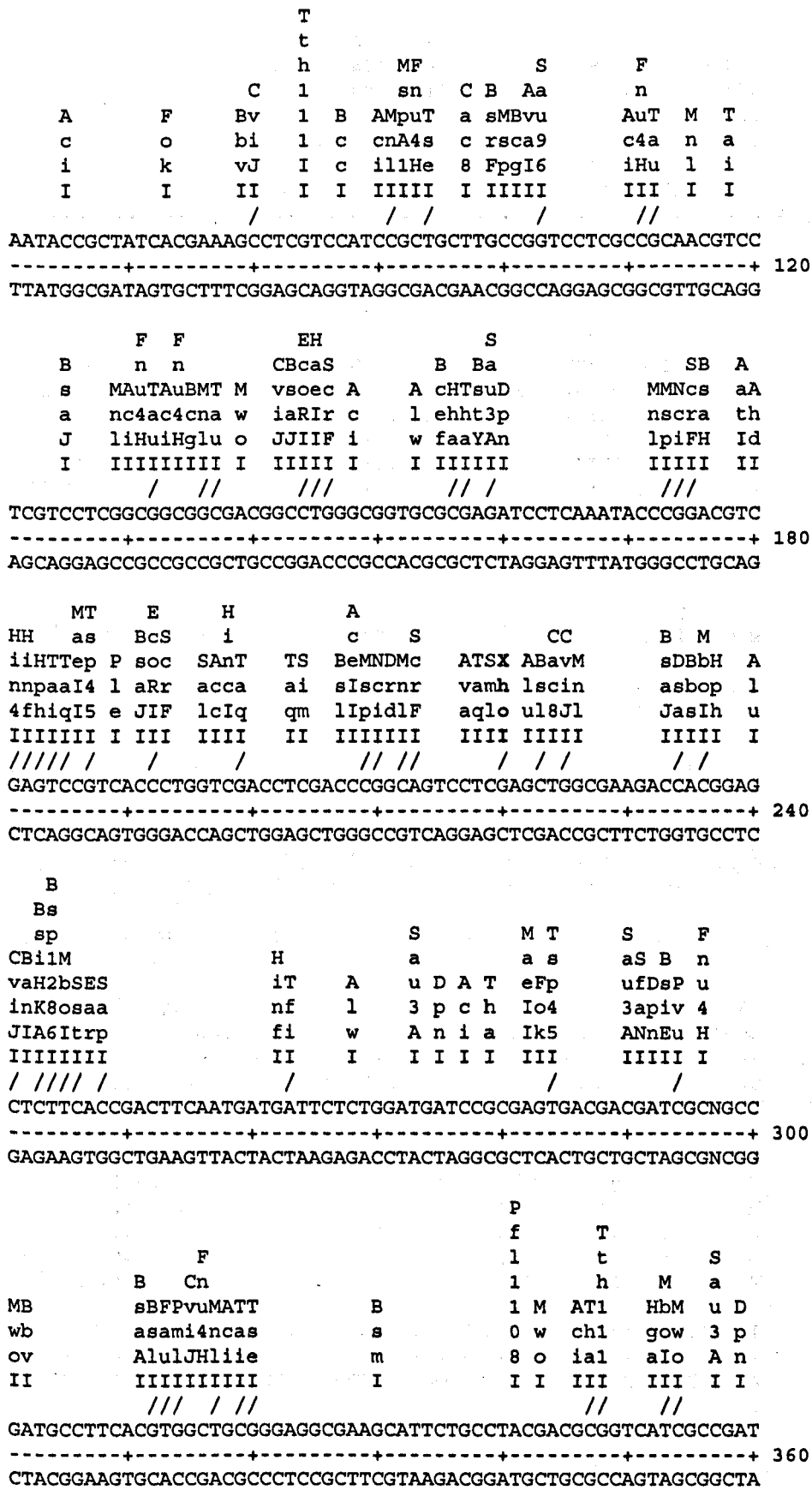
AatII	AciI	AluI	AlwI	Alw26I	ApaI	ApaBI	ApoI
AvaI	AvaII	BamHI	BanI	BanII	BbvI	BcgI	BfaI
BmgI	BpmI	Bpu10I	BsaHI	BsaJI	BsaWI	BscGI	BsgI
BsiEI	BsiHKAI	BslI	BsmBI	BsmFI	Bsp1286I	BspEI	BspMI
BsrI	BsrBI	BsrDI	BsrFI	BssSI	BstXI	BstYI	Cac8I
CjeI	CjePI	CviJI	CviRI	DdeI	DpnI	DsaI	EarI
Eco57I	EcoRI	EcoRII	FauI	Fnu4HI	HaeIII	HgaI	HgiEII
HhaI	Hin4I	HincII	HinfI	HpaI	MaeIII	MboII	MnlI
MseI	MslI	MspI	MspA1I	MunI	MwoI	NciI	NlaIII
NlaIV	NspI	NspV	PleI	PshAI	PstI	PvuI	RcaI
SacII	SapI	Sau96I	Sau3AI	ScrFI	SfcI	SimI	SmaI
SpeI	SphI	StyI	TaiI	TaqI	TaqII	TauI	TfiI
ThaI	TseI	Tsp45I	Tsp4CI	Tsp509I	Tth111II	UbaDI	XbaI
XcmI	XmnI						

Enzymes that do not cut:

AccI	AceIII	AflII	AflIII	AhdI	AlwNI	ApaLI	AscI
AvrII	BaeI	BaeI	BbsI	BccI	Bce83I	BcefI	BciVI
BclI	BfiI	BglI	BglII	BplI	Bpu1102I	BsaI	BsaAI
BsaBI	BsaXI	BsbI	BseRI	BsmI	Bsp24I	Bsp24I	BspGI
BspLU11I	BsrGI	BssHII	Bst1107I	BstEII	Bsu36I	ClaI	DraI
DraIII	DrdI	DrdII	EaeI	EagI	EciI	Eco47III	EcoNI
Eco0109I	EcoRV	FokI	FseI	FspI	GdiII	HaeI	HaeII
HindIII	HphI	KpnI	MluI	MmeI	MscI	NarI	NcoI
NdeI	NgoAIV	NheI	NotI	NruI	NsiI	PacI	Pfl1108I
PflMI	PinAI	PmeI	PmlI	PpuMI	Psp1406I	PvuII	RleAI
RsaI	RsrII	SalI	SanDI	ScaI	SexAI	SfaNI	SfiI
SgfI	SgrAI	SmlI	SnaBI	SrfI	Sse8387I	Sse8647I	SspI
SstI	StuI	SunI	SwaI	TatI	TspRI	Tth111I	VspI
XhoI							

The sequence above shows the position of restriction enzymes sites in the region of plasmid pBL100 sequenced from the restriction site *Pst*I in a clockwise direction (with reference to the plasmid map). The restriction enzymes that have recognition sites and those that do not are also listed. Solid, broken and dotted lines indicate possible open reading frames.

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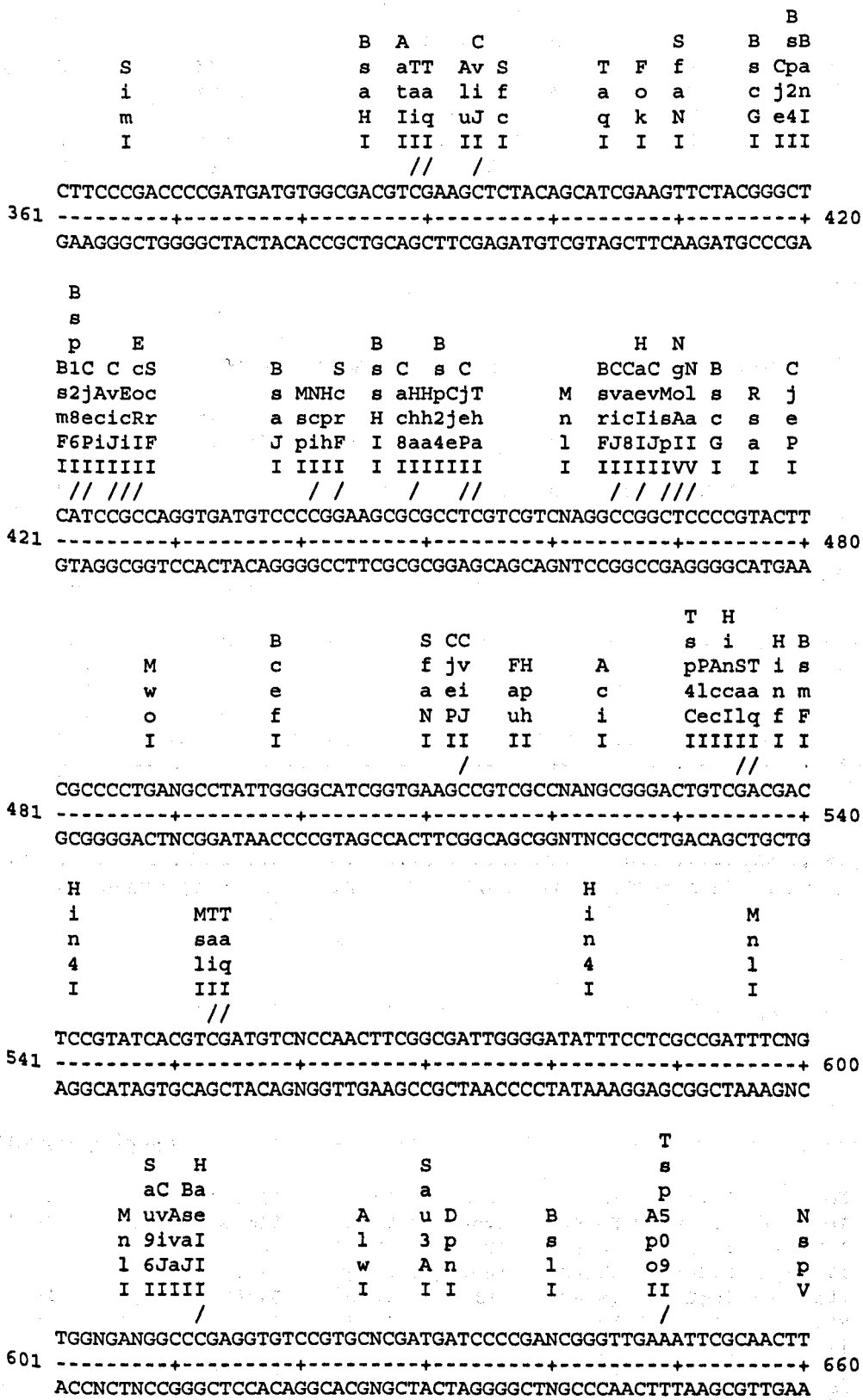
61 ---------+---------+---------+---------+---------+---------+ 120
TTATGGCGATAGTGCTTTCGGAGCAGGTAGGCGACGAACGGCCAGGAGCGGCGTTGCAGG
TCGTCCTCGGCGGCGGCGACGGCCTGGGCGGTGCGCGAGATCCTCAAATACCCGGACGTC
121 ---------+---------+---------+---------+---------+---------+ 180
AGCAGGAGCCGCCGCCGCTGCCGGACCCGCCACGCGCTCTAGGAGTTTATGGGCCTGCAG
GAGTCCGTCACCCTGGTCGACCTCGACCCGGCAGTCCTCGAGCTGGCGAAGACCACGGAG
181 ---------+---------+---------+---------+---------+---------+ 240
CTCAGGCAGTGGGACCAGCTGGAGCTGGGCCGTCAGGAGCTCGACCGCTTCTGGTGCCTC
CTCTTCACCGACTTCAATGATGATTCTCTGGATGATCCGCGAGTGACGACGATCGCNGCC
241 ---------+---------+---------+---------+---------+---------+ 300
GAGAAGTGGCTGAAGTTACTACTAAGAGACCTACTAGGCGCTCACTGCTGCTAGCGNCGG
GATGCCTTCACGTGGCTGCGGGAGGCGAAGCATTCTGCCTACGACGCGGTCATCGCCGAT
301 ---------+---------+---------+---------+---------+---------+ 360
CTACGGAAGTGCACCGACGCCCTCCGCTTCGTAAGACGGATGCTGCGCCAGTAGCGGCTA


CTTCCCGACCCCGATGATGTGGCGACGTCGAAGCTCTACAGCATCGAAGTTCTACGGGCT
361 ---------+---------+---------+---------+---------+---------+ 420
GAAGGGCTGGGGCTACTACACCGCTGCAGCTTCGAGATGTCGTAGCTTCAAGATGCCCGA
CATCCGCCAGGTGATGTCCCCGGAAGCGCGCCTCGTCGTCNAGGCCGGCTCCCCGTACTT
421 ---------+---------+---------+---------+---------+---------+ 480
GTAGGCGGTCCACTACAGGGGCCTTCGCGCGGAGCAGCAGNTCCGGCCGAGGGGCATGAA
CGCCCCTGANGCCTATTGGGGCATCGGTGAAGCCGTCGCCNANGCGGGACTGTCGACGAC
481 ---------+---------+---------+---------+---------+---------+ 540
GCGGGGACTNCGGATAACCCCGTAGCCACTTCGGCAGCGGNTNCGCCCTGACAGCTGCTG
TCCGTATCACGTCGATGTCNCCAACTTCGGCGATTGGGGATATTTCCTCGCCGATTTCNG
541 ---------+---------+---------+---------+---------+---------+ 600
AGGCATAGTGCAGCTACAGNGGTTGAAGCCGCTAACCCCTATAAAGGAGCGGCTAAAGNC
TGGNGANGGCCCGAGGTGTCCGTGCNCGATGATCCCCGANCGGGTTGAAATTCGCAACTT
601 ---------+---------+---------+---------+---------+---------+ 660
ACCNCTNCCGGGCTCCACAGGCACGNGCTACTAGGGGCTNGCCCAACTTTAAGCGTTGAA

```
                                        S T
                 SC                 B  ARa s  R
T               MNcv                sM vsu p  l            M       B
a               scri                as ar9 4  e            m       b
q               piFJ                Wp II6 C  A            e       v
I               IIII                II III I  I            I       I
/                //                   //
CGAAGGNCTNCCCGGCTNNAAGACTTCCAACCGGACCGTGATNGCTGTCTGTGGGCANNG
661 ---------+---------+---------+---------+---------+---------+ 720
GCTTCCNGANGGGCCGANNTTCTGAAGGTTGGCCTGGCACTANCGACAGACACCCGTNNC

  H
  i           F  F
  n  C        n  n C                          N
BBd Av       Tu TuFv  M  AT                   l
sbI li       s4 s4ai  w  ch                   a
gvI uJ       eH eHuR  o  ia                   I
III II       II IIII  I  II                   V
    /                    /
TCAAGCTTCANGCTGCTGCACNCGCGGGTGTCNATTAGGAACCNGNNCCTGNGTCGGTAA
721 ---------+---------+---------+---------+---------+---------+ 780
AGTTCGAAGTNCGACGACGTGNGCGCCCACAGNTAATCCTTGGNCNNGGACNCAGCCATT

 H            B C
 iT           s v
 nf           c i
 fi           G J
 II           I I
 /
TGAATCTNNGAACGGGCTTCTAAAAAGNGCTNTNAAANGGTTTCCGTTNTTNGNAAAGTN
781 ---------+---------+---------+---------+---------+---------+ 840
ACTTAGANNCTTGCCCGAAGATTTTTCNCGANANTTTNCCAAAGGCAANAANCNTTTCAN

GTTNNNNATAANTATTTNNANNTATTNTNNNNNTTNTNNNANNNCATTTTTNTANTNNTN
841 ---------+---------+---------+---------+---------+---------+ 900
CAANNNNTATTNATAAANNTNNATAANANNNNNAANANNNTNNNGTAAAAANATNANNAN

              M
              s
              e
              I
TATTNNANTGTTGTTAAGNANNGNNNANTNNANNTTNTTG
901 ---------+---------+---------+---------+ 940
ATAANNTNACAACAATTCNTNNCNNNTNANNTNNAANAAC
```

Enzymes that do cut:

AatII	AccI	AceIII	AciI	AhdI	AluI	AlwI	ApoI
AvaI	AvaII	BanII	BbsI	BbvI	BccI	BcefI	BcgI
BsaAI	BsaHI	BsaJI	BsaWI	BscGI	BsgI	BsiEI	BsiHKAI
BslI	BsmI	BsmFI	Bsp24I	Bsp1286I	BsrFI	BssHII	BstYI
Cac8I	CjeI	CjePI	ClaI	CviJI	CviRI	DpnI	DrdI
DsaI	EaeI	EagI	EarI	EciI	EcoRI	EcoRII	EcoRV
FauI	Fnu4HI	FokI	GdiII	HaeIII	HgaI	HhaI	Hin4I
HincII	HindIII	HinfI	HphI	MaeIII	MboII	MmeI	MnlI
MseI	MslI	MspI	MspA1I	MwoI	NciI	NgoAIV	NlaIV
NspV	Pfl1108I	PleI	PmlI	PstI	PvuI	RleAI	RsaI
RsrII	SalI	SapI	Sau96I	Sau3AI	ScrFI	SfaNI	SfcI
SimI	SmlI	SstI	TaiI	TaqI	TauI	TfiI	ThaI
TseI	Tsp45I	Tsp4CI	Tsp509I	Tth111I	Tth111II	XhoI	

Enzymes that do not cut:

AflII	AflIII	Alw26I	AlwNI	ApaI	ApaBI	ApaLI	AscI
AvrII	BaeI	BaeI	BamHI	BanI	Bce83I	BciVI	BclI
BfaI	BfiI	BglI	BglII	BmgI	BplI	BpmI	Bpu10I
Bpu1102I	BsaI	BsaBI	BsaXI	BsbI	BseRI	BsmBI	BspEI
BspGI	BspLU11I	BspMI	BsrI	BsrBI	BsrDI	BsrGI	BssSI
Bst1107I	BstEII	BstXI	Bsu36I	DdeI	DraI	DraIII	DrdII
Eco47III	Eco57I	EcoNI	Eco0109I	FseI	FspI	HaeI	HaeII
HgiEII	HpaI	KpnI	MluI	MscI	MunI	NarI	NcoI
NdeI	NheI	NlaIII	NotI	NruI	NsiI	NspI	PacI
PflMI	PinAI	PmeI	PpuMI	PshAI	Psp1406I	PvuII	RcaI
SacII	SanDI	ScaI	SexAI	SfiI	SgfI	SgrAI	SmaI
SnaBI	SpeI	SphI	SrfI	Sse8387I	Sse8647I	SspI	StuI
StyI	SunI	SwaI	TaqII	TaqII	TatI	TspRI	UbaDI
VspI	XbaI	XcmI	XmnI				

The sequence above shows the position of restriction enzymes sites in the region of plasmid pBL100 sequenced from the restriction site *Pst*I in an anticlockwise direction (with reference to the plasmid map). The restriction enzymes that have recognition sites and those that do not are also listed. (The length of sequence was insufficient to determine any putative ORFs).

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Appendix

Composition of Bundaberg Direct Consumption Raw Sugar (Brisbane, Australia).

The composition of Bundaberg Direct Consumption Raw Sugar is as follows:

Composition (per 100g):

Sucrose 98.5-99.7g

Calcium 7mg

Sodium 1mg

Potassium 21mg

No protein, fat or cholesterol

Composition of Oxoid Nutrient Broth No.2

Nutrient Broth No.2 (CM67) is composed as follows:

	gdm^{-3}
'Lab-Lemco' powder	10.0
Bacteriological Peptone	10.0
Sodium chloride	5.0

The compositions of 'Lab-Lemco' powder and Bacteriological Peptone are given in the table below.

Table of analysis for some of the proprietary media employed (Bridson, 1994)

Analysis	Medium					
	Tryptone	Neutralised Soya Peptone	Lab Lemco Powder	Bacteriological Peptone	Yeast Extract	Malt Extract
Moisture (%w/w)	<5	5.8	<5	<5	<5	<5
Ash (%w/w)	7.6	13.5	9.7	4.2	9.7	1.2
Amino Nitrogen (%w/w)	3.7	2.3	2.5	2.9	2.5	0.6
Total Nitrogen (%w/w)	13.3	9.1	13.3	15.2	13.3	1.1
AN:TN	28	25	19	19	19	55
pH	7.3	7.2	7.2	6.3	7.2	5.6
NaCl (%w/w)	0.3	0.4	1.1	1.0	1.1	0.1
K (%w/w)	0.4	3.3	1.9	3.6	1.9	0.5
Ca (ppm)	1350	225	140	635	140	140
Mg (ppm)	200	1530	140	265	140	703
Fe (ppm)	54	90	20	22	20	9
Cu (ppm)	2	2	2	1	2	3
Pb (ppm)	0.6	0.3	0.3	0.4	0.3	0.3
Mn (ppm)	28	1.0	0.4	3.4	0.4	1.7
Sn (ppm)	1.7	1.1	9.8	1.0	9.8	10.5
Zn (ppm)	28	12	18	9.2	18	1.2
Co (ppm)	0.1	0.2	0.5	0.1	0.5	<0.1
Alanine (%w/w)	3.12	2.57	5.85	3.92	0.91	Not determined
Arginine (%w/w)	5.53	4.64	7.10	4.99	3.31	Not determined
Aspartic acid (%w/w)	7.31	7.06	5.10	6.06	7.07	Not determined
Cystine (%w/w)	0.22	0.53	0.68	1.66	0.76	Not determined
Glutamic acid (%w/w)	17.61	14.71	10.71	9.93	13.49	Not determined
Glycine (%w/w)	1.99	2.83	10.85	7.71	5.95	Not determined
Isoleucine (%w/w)	2.51	2.51	3.17	3.81	4.81	Not determined
Leucine (%w/w)	6.88	4.31	3.15	3.79	6.04	Not determined
Lysine (%w/w)	7.17	3.77	4.78	4.38	5.40	Not determined
Methionine (%w/w)	2.08	0.62	2.61	1.58	0.80	Not determined
Phenylalanine (%w/w)	3.43	0.38	2.34	2.60	3.78	Not determined
Proline (%w/w)	7.99	3.40	7.79	5.83	0.88	Not determined
Serine (%w/w)	1.29	0.67	1.87	2.81	3.42	Not determined
Threonine (%w/w)	1.87	1.68	2.54	1.25	2.73	Not determined
Tryptophan (%w/w)	1.03	0.64	0.34	0.66	0.85	Not determined
Tyrosine (%w/w)	3.10	2.09	0.66	0.39	4.95	Not determined
Valine (%w/w)	5.47	3.65	3.06	3.33	1.00	Not determined

Primers used to obtain the sequence of the *crt*Y insert in the vector pBluescript: Standard sequencing primers SP6 and T7

T7: GTA ATA CGA CTC ACT ATA GGG C
SP6: ATT TAG GTG ACA CTA TAG AAT AC

Design of primers in an attempt to amplify the *B. linens* lycopene cyclase gene

The two sequence alignments that follow show the DNA regions upon which the PCR primers were based. Species are referenced in Chapter 5

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                                1                                                  50
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... .......... ..........
          R.capsulatuscrtI      .......... .......... .......... .......... ..........
              N.crassacrtI      cccgggggtg gtgtccgatg caaaagccca tcttggcggc catgacatcg
         crtIflavobacterium     .......... .......... .......... .......... ..........

                                51                                                 100
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... .......... ..........
          R.capsulatuscrtI      .......... .......... .......... .......... ..........
              N.crassacrtI      tgcgaacagg gcgttgatcg gttccgcagt tcggttagtt cccacttgct
         crtIflavobacterium     .......... .......... .......... .......... ..........

                                101                                                150
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... .......... ..........
          R.capsulatuscrtI      .......... .......... .......... .......... ..........
              N.crassacrtI      gatgaagacg ggacggccac cgattcacga ccctctcttc cacgggatag
         crtIflavobacterium     .......... .......... .......... .......... ..........

                                151                                                200
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... .......... ..........
          R.capsulatuscrtI      .......... .......... .......... .......... ..........
              N.crassacrtI      tagcaaggat agaaagaaac gccaaatcga gacctttttg ttttcgcatg
         crtIflavobacterium     .......... .......... .......... .......... ..........

                                201                                                250
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... .......... ..........
          R.capsulatuscrtI      .......... .......... .......... .......... ..........
              N.crassacrtI      tgatgatgcc gtctccgcta tctacaacca tcgacccacg gaacaaagca
         crtIflavobacterium     .......... .......... .......... .......... ..........

                                251                                                300
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... .......... ..........
          R.capsulatuscrtI      .......... .......... .......... .......... ..........
              N.crassacrtI      gggagtggaa ggaatgtcgg tccttaatgg caatcgaaat cccaggggg
         crtIflavobacterium     .......... .......... .......... .......... ..........
```

```
                                301                                       350
SynecoccusPhytoenedesaturase    ..........  ..........  ..........  ..........  ..........
Synecocystisphytoenedesaturase  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI2                 ..........  ..........  ..........  ..........  ..........
euredovoracrtI                  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI                  ..........  ..........  ..........  ..........  ..........
aaurantiacumcrtI                ..........  ..........  ..........  ..........  ..........
R.capsulatuscrtI                ..........  ..........  ..........  ..........  ..........
N.crassacrtI                    gggggggggg aaggagggga tagtctagat gggggatagc ttcccttgct
crtIflavobacterium              ..........  ..........  ..........  ..........  ..........

                                351                                       400
SynecoccusPhytoenedesaturase    ..........  ..........  ..........  ..........  ..........
Synecocystisphytoenedesaturase  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI2                 ..........  ..........  ..........  ..........  ..........
euredovoracrtI                  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI                  ..........  ..........  ..........  ..........  ..........
aaurantiacumcrtI                ..........  ..........  ..........  ..........  ..........
R.capsulatuscrtI                ..........  ..........  ..........  ..........  ..........
N.crassacrtI                    tattgcatgg gtcgatgcgc agcacagagc gataccacga caacaccact
crtIflavobacterium              ..........  ..........  ..........  ..........  ..........

                                401                                       450
SynecoccusPhytoenedesaturase    ..........  ..........  ..........  ..........  ..........
Synecocystisphytoenedesaturase  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI2                 ..........  ..........  ..........  ..........  ..........
euredovoracrtI                  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI                  ..........  ..........  ..........  ..........  ..........
aaurantiacumcrtI                ..........  ..........  ..........  ..........  ..........
R.capsulatuscrtI                ..........  ..........  ..........  ..........  ..........
N.crassacrtI                    agcttgtgat tcatggacgg cggtggtcgg gcggtggtcg aacggtggga
crtIflavobacterium              ..........  ..........  ..........  ..........  ..........

                                451                                       500
SynecoccusPhytoenedesaturase    ..........  ..........  ..........  ..........  ..........
Synecocystisphytoenedesaturase  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI2                 ..........  ..........  ..........  ..........  ..........
euredovoracrtI                  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI                  ..........  ..........  ..........  ..........  ..........
aaurantiacumcrtI                ..........  ..........  ..........  ..........  ..........
R.capsulatuscrtI                ..........  ..........  ..........  ..........  ..........
N.crassacrtI                    gatagatcta gatatcgact gtgcgcatca tcacttgtct taaaaagttc
crtIflavobacterium              ..........  ..........  ..........  ..........  ..........

                                501                                       550
SynecoccusPhytoenedesaturase    ..........  ..........  ..........  ..........  ..........
Synecocystisphytoenedesaturase  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI2                 ..........  ..........  ..........  ..........  ..........
euredovoracrtI                  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI                  ..........  ..........  ..........  ..........  ..........
aaurantiacumcrtI                ..........  ..........  ..........  ..........  ..........
R.capsulatuscrtI                ..........  ..........  ..........  ..........  ..........
N.crassacrtI                    agtttcggga ttgtttctct ccggaacggt ccgttcaaac cttcatatac
crtIflavobacterium              ..........  ..........  ..........  ..........  ..........

                                551                                       600
SynecoccusPhytoenedesaturase    ..........  ..........  ..........  ..........  ..........
Synecocystisphytoenedesaturase  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI2                 ..........  ..........  ..........  ..........  ..........
euredovoracrtI                  ..........  ..........  ..........  ..........  ..........
eherbicolacrtI                  ..........  ..........  ..........  ..........  ..........
aaurantiacumcrtI                ..........  ..........  ..........  ..........  ..........
R.capsulatuscrtI                ..........  ..........  ..........  ..........  ..........
N.crassacrtI                    ccgttggatg tgcttgtgaa aagctcttgc tcccaccacc accatctgcc
crtIflavobacterium              ..........  ..........  ..........  ..........  ..........
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```
                                601                                                 650
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... .......... ..........
          R.capsulatuscrtI      .......... .......... .......... .......... ..........
              N.crassacrtI      ttgactgtgt gctttctact ttgttcaaag tatcttaaag ggtaatcttg
         crtIflavobacterium     .......... .......... .......... .......... ..........

                                651                                                 700
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... .......... ..........
          R.capsulatuscrtI      .......... .......... .......... .......... ..........
              N.crassacrtI      cctacgtgga atttctccag ctttcttgga cttctgtatc tctataccct
         crtIflavobacterium     .......... .......... .......... .......... ..........

                                701                                                 750
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... .......... ..........
          R.capsulatuscrtI      .......... .......... .......... .......... ..........
              N.crassacrtI      ctagactctt cagatcttct cggctttctg tgcagagaga aggagcgacg
         crtIflavobacterium     .......... .......... .......... .......... ..........

                                751                                                 800
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... .......... ..........
          R.capsulatuscrtI      .......... .......... .......... .......... ..........
              N.crassacrtI      ataagaacga taacgacgac gacgaaagag ctaagcaaaa aaaataccag
         crtIflavobacterium     .......... .......... .......... .......... ..........

                                801                                                 850
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..gcgatcgc
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ccctggtagc
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... .......... ..........
          R.capsulatuscrtI      .......... .......... .......... .......... ..........
              N.crassacrtI      acttacagac aaaatggctg agactcagag accacgaagc gccattatcg
         crtIflavobacterium     .......... .......... .......... .......... ..........

                                851                                                 900
SynecoccusPhytoenedesaturase    actttgatca gtgttgacag cgcgatcgcg .aatcagaat tgttacagaa
Synecocystisphytoenedesaturase  atttaataca aattggctat cttggcaaag tcccccgaaa tattacgaaa
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
          aaurantiacumcrtI      .......... .......... .......... ..aacgcatg aacgcccatt
          R.capsulatuscrtI      .......... .......... .......... ...aaccatg tccaagaaca
              N.crassacrtI      ttggtatgtc tctctatttg gaatttgagc tcttcacttc agcctcagga
         crtIflavobacterium     .......... .......... .......... .......... ..........
```

```
                                  901                                                 950
SynecoccusPhytoenedesaturase      tgcaacgtat actaagagac gattcttctg cccttccagc c.........
Synecocystisphytoenedesaturase    cgtaaagtat aataacaatc aacctgtaaa ccccaaatgc cttagcgaga
eherbicolacrtI2                   ......acat gaaaccaact acggtaattg gtgcaggctt tggtggcctg
euredovoracrtI                    .......cat gaaaccaact acggtaattg gtgcaggctt cggtggcctg
eherbicolacrtI                    ...aaaggat gaaaaaaacc gttgtgattg gcgcaggctt tggtggcctg
aaurantiacumcrtI                  cgcccgcggc caagaccgcc atcgtgatcg gcgcaggctt tggcgggctg
R.capsulatuscrtI                  cagaaggtat gggtcgcgcc gttgtcatcg gtgccggcct tggcggtctt
N.crassacrtI                      tgtatgctaa cttcttcccc aaaacaacag gcgcaggagc aggcggtatc
crtIflavobacterium                .......... .......... .......... .......... ..........
                                                    AACC ATTGTAATTG GTGCAGGCTT
                                  951                                                1000
SynecoccusPhytoenedesaturase      ..aaaagcca tgcgcgtagc gatcgccggt gccggacttg ccggactctc
Synecocystisphytoenedesaturase    cagtaaccca tgcgcgttgt gatcgccgga gccggattag ccggcctagc
eherbicolacrtI2                   gcattagcaa ttcgtctgca ggcggcgggg atccctgtct tactgcttga
euredovoracrtI                    gcactggcaa ttcgtctaca agctgcgggg atccccgtct tactgcttga
eherbicolacrtI                    gcgctggcga ttcgcctgca ggcggcaggg atcccaaccg tactgctgga
aaurantiacumcrtI                  gccctggcca tccgcctgca gtccgcgggc atcgccacca ccctggtcga
R.capsulatuscrtI                  gctgcagcga tgcggctggg cgcaaaaggt tacaaggtga cggtcgtcga
N.crassacrtI                      gccgtcgcgg cccgtctggc caaagccgga gtagacgtca cagttctcga
crtIflavobacterium                .......... .......... ....gatcgg tcatgcgacg gccaggtccg

                                  1001                                               1050
SynecoccusPhytoenedesaturase      ctgtgccaag tacttggccg atgccggtca tacgcccatc gtctatgaac
Synecocystisphytoenedesaturase    ctgtgccaaa tacttagccg atgcgggctt tacccccgtc gtcttggaac
eherbicolacrtI2                   gcaacgcgac aaacccggtg gccgggctta tgtctatgaa ...gatcagg
euredovoracrtI                    acaacgtgat aaacccggcg gtcgggctta tgtctacgag ...gatcagg
eherbicolacrtI                    gcagcgggac aagcccggcg gtcgggccta cgtctggcat ...gaccagg
aaurantiacumcrtI                  ggcccgggac aagcccggcg ggcgcgccta tgtctggcac ...gatcagg
R.capsulatuscrtI                  tcgtctggat cggcccggcg ggcgtggctc ttcgatcacc ...aagggcg
N.crassacrtI                      aaagaacgac ttcacaggag gccgctgcag tctcatccac acaaaagctg
crtIflavobacterium                acagcatgac ctgcgccgtg gccttggcgc tgccaacgac acccgggatg

                                  1051                                               1100
SynecoccusPhytoenedesaturase      gtcgggacgt ccttggcggc aaggttgccg cttggaaaga tgaagacggc
Synecocystisphytoenedesaturase    gtagggatgt attaggcggg aagatcgccg cgtggaaaga tgaggacgga
eherbicolacrtI2                   gattcacttt tgatgcaggg cctaccgtta ttaccgatcc cagcgccatt
euredovoracrtI                    ggtttacctt tgatgcaggc ccgacggtta tcaccgatcc cagtgccatt
eherbicolacrtI                    gctttacctt tgacgccggg ccgacggtga tcaccgatcc taccgcgctt
aaurantiacumcrtI                  gccatctctt cgacgcgggc ccgaccgtca tcaccgaccc cgatgcgctg
R.capsulatuscrtI                  ggcatcgatt cgaccttggg ccgacgatcg tgacggtgcc cgaccggctg
N.crassacrtI                      gctaccgctt cgaccaaggt ccctcactcc tcctcctacc gggtctcttc
crtIflavobacterium                cccgcacccg gatgcgtgcc cgcccccacg atgtagaagt tcgggatcgc

                                  1101                                               1150
SynecoccusPhytoenedesaturase      gactggtacg aaactggcct acatatcttt tttggggctt accccaacat
Synecocystisphytoenedesaturase    gattggtacg aaaccggcct acacattttt tttggggcct atcccaacat
eherbicolacrtI2                   gaagaactct ttaccctggc gggaaaacag ttaa...aag attacgttga
euredovoracrtI                    gaagaactgt ttgcactggc aggaaaacag ttaa...aag agtatgtcga
eherbicolacrtI                    gaggcgctgt tcaccctggc cggcaggcgc atgg...agg attacgtcag
aaurantiacumcrtI                  aaagagctgt gggccctgac cgggcaggac atgg...cgc gcgacgtgac
R.capsulatuscrtI                  cgcgagcttt gggccgattg cgggcgcgat ttcg...aca aggacgtgag
N.crassacrtI                      cgcgagacct ttgaagattt aggcaccact ctcgagcagg aagatgtcga
crtIflavobacterium                gcggtcgcgg ttatgcgggc ggaaccaggc ggattgcgtc aggatcggct

                                  1151                                               1200
SynecoccusPhytoenedesaturase      gttgcagctc tttaaggagc tgaacattga agatcgcctg cagtggaagt
Synecocystisphytoenedesaturase    gttgcagtta tttaaggaat tggatatcga agatcgtctg caatggaaag
eherbicolacrtI2                   actgctgccg gttgcgccgt tttatcgcct gtgttgggag tcaggaaagg
euredovoracrtI                    actgctgccg gttacgccgt tttaccgcct gtgttgggag tcagggaagg
eherbicolacrtI                    gctgctgccg gtaaaaccct tctaccgact ctgctgggag tccgggaaga
aaurantiacumcrtI                  gctgatgccg gtctcgccct tctatcggct gatgtggccg ggcgggaagg
R.capsulatuscrtI                  ccttgtgccg atggagccct tctacaccat cgatttcccc gatggcgaga
N.crassacrtI                      gctcctccaa tgtttccccа actacaacat ctggttctcc gacggcaagc
crtIflavobacterium                cgaccgagaa ggcgctgccg tgatgggccg acagttcggt gctgaaatcg
```

```
                                1201                                                1250
SynecoccusPhytoenedesaturase    cccactcgat gatcttcaac caacccacaa agccgggcac ctattcgcgc
Synecocystisphytoenedesaturase  agcacagcat gatcttcaac caaccagaga aaccaggtac ctactctcgg
          eherbicolacrtI2       ttttcaatta cgataacgat caggcacagc ttgaggcgca gattcagcag
           euredovoracrtI       tctttaatta cgataacgat caaacccggc tcgaagcgca gattcagcag
           eherbicolacrtI       ccctcgacta tgctaacgac agcgccgagc ttgaggcgca gattacccag
         aaurantiacumcrtI       tcttcgatta cgtgaacgag gccgatcagc tggaacgcca gatcgcccag
         R.capsulatuscrtI       aatacaccgc ttacggcgat gacgccaagg tcaaggccga ggtggcgcgg
              N.crassacrtI      gcttctcgcc caccaccgac aacgccacca tgaaggtcga gatcgaaaag
       crtIflavobacterium       gcggggctga agatgcggct gacggtcagg tgcttgcgca ggtcggggat

                                1251                                                1300
SynecoccusPhytoenedesaturase    ttcgacttcc cagacattcc agcgccaatc aacggtgttg cagcaatcct
Synecocystisphytoenedesaturase  ttcgattttc cggatattcc ggcccccatc aatggtttgg tagccattct
          eherbicolacrtI2       tttaa...tc cacgcgatgt tgaaggctat cgtcagtttc tggactattc
           euredovoracrtI       tttaa...tc cccgcgatgt cgaaggttat cgtcagtttc tggactattc
           eherbicolacrtI       ttcaa...cc cccgcgacgt cgagggctac cggcgctttc tggcttactc
         aaurantiacumcrtI       ttcaa...cc cggacgacct ggagggatac cgccgcttcc gtgattacgc
         R.capsulatuscrtI       atcag...cc ccggcgatgt cgagggcttc cgccatttca tgtgggacgc
              N.crassacrtI      tgggaaggcc ccgacggctt ccgccgctac ctctcgtggc tcgccgaggg
       crtIflavobacterium       ggcgcggcgc tccagttcct cgaagatgcg ctcggcatag cccggggcct

                                1301                                                1350
SynecoccusPhytoenedesaturase    cagcaacaac gacatgttga cctgggaaga aaaaatcaag tttggcttgg
Synecocystisphytoenedesaturase  tcgcaacaac gatatgctta cctggccgga gaaaattcgc tttggcttgg
          eherbicolacrtI2       acgtgcggtg tttaaagaag gct.......  ..atctgaag cttggcaccg
           euredovoracrtI       acgcgcggtg tttaaagaag gct.......  ..atctaaag ctcggtactg
           eherbicolacrtI       ccaggcggta ttccaggagg gat.......  ..atttgcgc ctcggcagcg
         aaurantiacumcrtI       tgaggaggtg taccaggagg gct.......  ..acgtcaag ctgggcaccg
         R.capsulatuscrtI       caaggcccgt tatgaattcg gct.......  ..atgaaaac ctcggccgca
              N.crassacrtI      ccaccaacac tacgagacca gcttgcgaca cgttctgcac cgcaacttca
       crtIflavobacterium       cggcttccca atcgacatcg gcgcggccca gatgcggaac gggcgcaagg

                                1351                                                1400
SynecoccusPhytoenedesaturase    gcttgttgcc agcgatgatt cgcggccagt cctacgtcga agagatggat
Synecocystisphytoenedesaturase  gactcttgcc ggccattgtc cagggccaga gctatgtgga agaaatggat
          eherbicolacrtI2       tgcctttctt gtcgttcagg gatatgcttc gcgccgcgcc ccaactggcg
           euredovoracrtI       tccctttttt atcgttcaga gacatgcttc gcgccgcacc tcaactggcg
           eherbicolacrtI       tgccgttcct ctcttttcgc gacatgctgc gcgccgggcc gcagctgctt
         aaurantiacumcrtI       tgcccttcct caagctgggc cagatgctca aggccgcgcc cgcgctgatg
         R.capsulatuscrtI       agccgatgag caagctgtgg gacctgatca aggttctgcc gactttcggc
              N.crassacrtI      agtccatcct cgagctggcg gacccccgcc ttgtcgtcac gttgctcatg
       crtIflavobacterium       acgtaatgcg tggacatccc ctcgggggcc aggctgggat cggtcacgca

                                1401                                                1450
SynecoccusPhytoenedesaturase    caatactcat ggacggagtg gctgcgcaaa caaaatattc cagagcgggt
Synecocystisphytoenedesaturase  aaatacactt ggtcagagtg gatggccaaa caaaatattc ccccccgcat
          eherbicolacrtI2       aaac...tgc aggcatggcg aaccgtttac agtaaagttg ccagctacat
           euredovoracrtI       aaac...tgc aggcatggag aagcgtttac agtaaggttg ccagttacat
           eherbicolacrtI       aagc...tcc aggcgtggca gagcgtctac cagtcggttt cgcgctttat
         aaurantiacumcrtI       aagt...tgg aggcctacaa gtcggtccat gccaaggtcg cgaccttcat
         R.capsulatuscrtI       tggc...tgc gcgccgaccg ctcggtctat ggccatgcca agaagatggt
              N.crassacrtI      gctc...ttc acccctcga gagcatctgg caccgcgccg ggcgttactt
       crtIflavobacterium       gggcgaatgc agatacatcg agaaatcgtc cggcaggcgt ggcccgttga

                                1451                                                1500
SynecoccusPhytoenedesaturase    caacgatgaa gtcttcatcg ccatggctaa agcgctcaac tttattgacc
Synecocystisphytoenedesaturase  cgaaaaagaa gttttcattg ccatgagtaa ggcgttgaac tttattgatc
          eherbicolacrtI2       tgaggatgaa cat.......  ..cttcgtca ggcgttttct tttcactcgc
           euredovoracrtI       cgaagatgaa cat.......  ..ctgcgcca ggcgttttct ttccactcgc
           eherbicolacrtI       tgaggatgag cat.......  ..ctgcggca ggccttctcg ttccactccc
         aaurantiacumcrtI       caaggacccc tat.......  ..ctgcggca ggcgttttcg tatcacacgc
         R.capsulatuscrtI       gaaggacgac cac.......  ..ctgcgctt cgcgctgtcg ttccatccgc
              N.crassacrtI      caagacggat cgc.......  ..atgcagcg cgtctttact tttgcgacca
       crtIflavobacterium       agatctcgtt cac.......  ..cagccccc tgtagcgcgg gccgaagatg
```

```
                                1501                                                1550
SynecoccusPhytoenedesaturase    cggacgaaat ttccgccacg gtcgtcctaa cggcactcaa ccgcttcttg
Synecocystisphytoenedesaturase  ccgatgaaat ttccgccacc attttactta ctgccctcaa tcgcttttta
eherbicolacrtI2                 tgttagtggg cggcaatccc ttcgccacct cctccattta tacgctaata
euredovoracrtI                  tgttggtggg cggcaatccc ttcgccacct catccattta tacgttgata
eherbicolacrtI                  tgctggtagg cggcaacccc ttcaccacct cgtccatcta caccctgatc
aaurantiacumcrtI                tgctggtggg cgggaatccc ttctcgacca gctcgatcta tgcgctgaac
R.capsulatuscrtI                ttttcatcgg cggcgacccc ttccatgtga cgtcgatgta tatcctcgtc
N.crassacrtI                    tgtacatggg catgagcccg ttcgatgcgc cggcgacgta cagtctgctt
crtIflavobacterium              acgctgtggt gggccaggtt ctcggggcgc ttggacaggc cgaaatgcag

                                1551                                                1600
SynecoccusPhytoenedesaturase    caagagaaga aaggttcaat gatggccttt ttggatggtg cgccgcccga
Synecocystisphytoenedesaturase  caggaaaaaa atggctctaa gatggcattc ctggatgggg caccaccgga
eherbicolacrtI2                 catgcactcg aacgcgaatg gggcgtctgg tttccacgcg gtggtaccgg
euredovoracrtI                  cacgcgctgg agcgtgagtg gggcgtctgg tttccgcgtg gcggcaccgg
eherbicolacrtI                  cacgcccttg agcgggagtg gggggtctgg ttccctgagg gcggcaccgg
aaurantiacumcrtI                cacgcgctgg agcggcgcgg cggggtctgg ttcgccaagg gcggcaccaa
R.capsulatuscrtI                agccagctcg aaaagaaatt cggcgtgcat tacgcgatcg gcggcgtgca
N.crassacrtI                    caatactcgg agttggccga gggtatctgg tatccccgcg gaggcttcca
crtIflavobacterium              cacgaacagc gacatcgacc agcgctgccg gttcaggatc gcggccttgg

                                1601                                                1650
SynecoccusPhytoenedesaturase    gcgtctttgc cagccgatcg tcgaacatgt ccaagctcgc ggtggtgatg
Synecocystisphytoenedesaturase  gcgtctttgc caacctttgg tcgactatat tacggaacgg ggaggggaag
eherbicolacrtI2                 ggcattagtg aaagggatga taaagctgtt tcaggatctg ggtggcgaag
euredovoracrtI                  cgcattagtt caggggatga taaagctgtt tcaggatctg ggtggcgaag
eherbicolacrtI                  ggcgctggtg aacggcatgg tgaagctgtt taccgatctg ggcggggaga
aaurantiacumcrtI                ccagctggtt gcgggcatgg tcgcgctgtt cgaacggctt ggcgggcaga
R.capsulatuscrtI                ggcgattgcc gatgcgatgg ccaaggtgat caccgatcag ggcggcgaga
N.crassacrtI                    caaggtgttg gacgctttgg tcaaaattgg agagaggatg ggcgtcaagt
crtIflavobacterium              tgcgcccgcg gcgggtatgg cccagcaggt cgc..... ....gatagc

                                1651                                                1700
SynecoccusPhytoenedesaturase    tgctgctgaa tgcgcctctg aaagagttcg tgctcaatga cgacagtagc
Synecocystisphytoenedesaturase  tacacattaa taaacctctc aaagaaattt tgcttaatga agatggttcc
eherbicolacrtI2                 tggtactgaa tgcgaaggtc agccaca... tggaaaccac gggggatacc
euredovoracrtI                  tcgtgttaaa cgccagagtc agccata... tggaaacgac aggaaacaag
eherbicolacrtI                  tcgaactcaa cgcccgggtc gaagagc... tggtggtggc cgataaccgc
aaurantiacumcrtI                tgctgctaaa cgccaaggtc gcgcgga... tcgacacgga cgggccgcgc
R.capsulatuscrtI                tgcgcctgaa caccgaggtc gacgagatcc tggtctcgcg tgacggcaag
N.crassacrtI                    acagactcaa cacgggcgtg tcccaggttc tcacggacgg aggcaagaac
crtIflavobacterium              tgtgcatcac gtcgccgttg ctggccaccg tatccgcgcg caactgccgc

                                1701                                                1750
SynecoccusPhytoenedesaturase    gtccaagctt ttcggattgc tggcatcaaa ggtcaagaag aacaactcat
Synecocystisphytoenedesaturase  gttaagggtt acttaatccg gggcctagat ggagcccccg acgaagtgat
eherbicolacrtI2                 .......... .....attga agccgtgcat ttagaggacg gacgcagatt
euredovoracrtI                  .......... .....attga agccgtgcat ttagaggacg gtcgcaggtt
eherbicolacrtI                  .......... .....gtaag ccaggtccgg ctggcggatg gtcggatctt
aaurantiacumcrtI                .......... .....gcgac cggcgtgacc ctggccgacg ggcgcgcctt
R.capsulatuscrtI                .......... .....gccac gggcatccgg ctgatggacg gcaccgagct
N.crassacrtI                    ggaaagaagc caaaggctac gggtgtccag cttgagaacg gcgaggtgct
crtIflavobacterium              ccgtccagca gcgtgacgcc cgtggcgcga tcgccctcgg tgtcgatccg

                                1751                                                1800
SynecoccusPhytoenedesaturase    tgaggcagat gcctacgttt cggcactgcc ggttgatccg ctcaagctac
Synecocystisphytoenedesaturase  cacagcggat ttatatgtgt ctgccatgcc ggtggatccc ctgaaaacca
eherbicolacrtI2                 cccgacccgg gctgtcgctt ccaatgcgga tgtggttcac acctatcgcg
euredovoracrtI                  cctgacgcaa gccgtcgcgt caaatgcaga tgtggttcat acctatcgcg
eherbicolacrtI                  tgacaccgac gccgtagcct cgaacgctga cgtggtgaac acctataaaa
aaurantiacumcrtI                gaccgccgac atggtcgcca gcaacggcga cgtgatgcac aactatcgcg
R.capsulatuscrtI                tccggcgcag gttgtcgtct cgaacgccga tgcgggccac acctacaagc
N.crassacrtI                    gaacgccgat ctggtggtgg ttaacgccga cttggtatat acgtacaaca
crtIflavobacterium              cgtgacgcgg gcattcagca gcagcgtgcc gccaagacgc tcgaacaggg
```

```
                                1801                                                1850
SynecoccusPhytoenedesaturase    tgttgccgga tgcatggaaa gccatgccct acttccagca actcgatggt
Synecocystisphytoenedesaturase  tggtgccagc gccctggaga gaatatcctg agtttaagca aatccaaggt
          eherbicolacrtI2       acctgttaag tcagcatcct gccgccgtga agcagtcgaa aaaactgcaa
           euredovoracrtI       acctgttaag ccagcaccct gccgcggtta agcagtccaa caaactgcag
           eherbicolacrtI       agctgctcgg ccaccatccg gtggggcaga agcgggcggc agcgctggag
         aaurantiacumcrtI       acctgctggg ccataccgcc cgcgggcaga gccgggcgaa atcgctgaac
         R.capsulatuscrtI       gtctcttgcg caaccgcgac cgctggcgct ggaccgacga gaagctcgac
              N.crassacrtI      acctcctgcc gaaggagatc gggggcatca agaagtatgc gaacaaactc
        crtIflavobacterium      cgaccatgcc cgcgaccagc tggttggtgc cgcccttggc gaaccagacg

                                1851                                                1900
SynecoccusPhytoenedesaturase    ctgcagggcg tgccggtcat caacattcac ctctggttcg atcgcaagct
Synecocystisphytoenedesaturase  ttggaaggag tcccggtcat taacctccac ctgtggtttg accgtaagtt
          eherbicolacrtI2       actaagcgca tgagcaactc attgtttgtt ctctattttg gtctgaa...
           euredovoracrtI       actaagcgca tgagtaactc tctgtttgtg ctctattttg gtttgaa...
           eherbicolacrtI       cgcaagagca tgagcaactc gctgtttgtg ctctacttcg gcctgaa...
         aaurantiacumcrtI       gcgaagcgct ggtccatgtc gctcttcgtg ctgcatttcg gcctgcg...
         R.capsulatuscrtI       aagaagcgct ggtcgatggg gcttttcgtc tggtatttcg gcaccaaggg
              N.crassacrtI      aacaaccgca aggcgtcgtg cagttctatt tctttttact g......gag
        crtIflavobacterium      ccgccgcgcc gttccagcgc atggatcagc gcatagatcg agctg.....

                                1901                                                1950
SynecoccusPhytoenedesaturase    gaccgatatc gatcacctgc tgttctcgcg atcgcccctg ctcagtgtct
Synecocystisphytoenedesaturase  aaccgacatt gatcatttgt tattctcccg atcgccgttg ttgagtgttt
          eherbicolacrtI2       ......tcac catcacgatc agcttgccca ccatacggtt tgtttcggcc
           euredovoracrtI       ......tcac catcatgatc agctcgcgca tcacacggtt tgtttcggcc
           eherbicolacrtI       ......ccag cctcattccc agctggcgca ccataccatc tgttttggtc
         aaurantiacumcrtI       ......cgag gcgcccaagg acgtggcgca tcacaccatc ctgttcggcc
         R.capsulatuscrtI       tacggccaag atgtggaagg atgtgggtca ccacaccgtc gtcgtcgggc
              N.crassacrtI      tttgtcgggt atggccaaag agttggagac gcacaatatc tttttggcgg
        crtIflavobacterium      .......... .gtcgaaaac gggttcccgc cgaccagcag cgtgtggaac

                                1951                                                2000
SynecoccusPhytoenedesaturase    atgccgacat gagtaacacc tgtcgcgagt acgaagatcc cgatcgctca
Synecocystisphytoenedesaturase  acgccgacat gagcaacacc tgccgagaat acagtgatcc agacaaatcc
          eherbicolacrtI2       ctcgctaccg tgagctgatt catgaaattt tcaatcatga cggactcgca
           euredovoracrtI       cgcgttaccg cgagctgatt gacgaaattt ttaatcatga tggcctcgca
           eherbicolacrtI       cccgctaccg ggagctgatc gacgagatct ttaccggcag cgcgctggcg
         aaurantiacumcrtI       cccgctacaa ggagctggtc aacgagatct tcaagggccc gaagctggcc
         R.capsulatuscrtI       cgcgctacaa ggaacatgtg caggacatct tcatcaaggg cgagctggcc
              N.crassacrtI      aggagtacaa ggagtccttt gacgctatct ttgagaggca ggccctgcct
        crtIflavobacterium      gagaaggcct gccgcagatg cgggtcctgg atgaagcgcg ccaccatgct

                                2001                                                2050
SynecoccusPhytoenedesaturase    atgctagagc tggtcttcgc ccccgccaaa gactggattg gccgctccga
Synecocystisphytoenedesaturase  atgttggaat tggtgctggc tccggcccag gattggatcg gcaaatccga
          eherbicolacrtI2       gacgatttct cactt..... .......... .......... ..........
           euredovoracrtI       gaggacttct cactt..... .......... .......... ..........
           eherbicolacrtI       gatgacttct cgctc..... .......... .......... ..........
         aaurantiacumcrtI       gaggatttct cgctc..... .......... .......... ..........
         R.capsulatuscrtI       gaggacatga gcctt..... .......... .......... ..........
              N.crassacrtI      gatgatccca gcttcgtaag ttttaccctc attttggttt ttttcctctc
        crtIflavobacterium      gtggaccgag cggtatgcct gcaggcgcat cagcgccggc gcggcgttca

                                2051                                                2100
SynecoccusPhytoenedesaturase    cgaagacatc ttggctgcca ccatggccga gattgaaaag ctattcccac
Synecocystisphytoenedesaturase  cgaagagatt gtggcggcca ccatggcgga gatcaagcaa ctctttcccc
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
         aaurantiacumcrtI       .......... .......... .......... .......... ..........
         R.capsulatuscrtI       .......... .......... .......... .......... ..........
              N.crassacrtI      cctctctctc tctctctccc tctaatagtc gtccgaccat cctccaacta
        crtIflavobacterium      gcatctggcc cagcttcagg aagggcgtgg tccccagctt cagataccc
```

```
                                2101                                                2150
SynecoccusPhytoenedesaturase    agcatttcag cggtgagaat ccggcacgtc tgcgcaaata caaaattgtc
Synecocystisphytoenedesaturase  aacacttcaa cggggataat ccagcccgac tgcttaaatc ccacgtggtc
eherbicolacrtI2                 .......... .......... ...tacctgc acgcaccttg tgtcacggat
euredovoracrtI                  .......... .......... ...tatctgc acgcgccctg tgtcacggat
eherbicolacrtI                  .......... .......... ...tacctgc actcgccctg cgtgaccgat
aaurantiacumcrtI                .......... ..........'...tatctgc attcgccctg cacgaccgac
R.capsulatuscrtI                .......... .......... ...tatgtcc accgtccctc ggtcactgat
N.crassacrtI                    acttcacctc acctcttgaa cagtacatcc acgtcccctc ccgcgttgac
crtIflavobacterium              tcgcgataga cctcctcggc gtaatcgtgg aagcggcgat agccatcgac

                                2151                                                2200
SynecoccusPhytoenedesaturase    aaaacgcccc tgtcggtcta caaagccacg ccgggccgtc aacaatatcg
Synecocystisphytoenedesaturase  aaaaccccca gctcagtcta caaagctacc cccggaaggc aggcttgtcg
eherbicolacrtI2                 tcgtcactgg caccggaagg ttgcggcagt tactatgtgt tagctcccgt
euredovoracrtI                  tcgtcactgg cgcctgaagg ttgcggcagt tactatgtgt tggcgccggt
eherbicolacrtI                  ccctcgctcg cgcctcccgg ctgcgccagc ttctacgtgc tggccccggt
aaurantiacumcrtI                ccggagatgg cgcctccggg catgtccacg cattacgtcc tggccccggt
R.capsulatuscrtI                ccgaccgcgg cgccgaaagg cgacgacacc ttctacgtgc tttcgccggt
N.crassacrtI                    ccctcggccg cccctcccga ccgcgacgcc gtcatcgccc tcgtccccgt
crtIflavobacterium              atc...ggcg ggattgaagg aggcgacctg gcggatcagc tcgtcgtcgt

                                2201                                                2250
SynecoccusPhytoenedesaturase    ccccgatcaa gctagcccga tcgctaattt cttcctgacc ggcgactaca
Synecocystisphytoenedesaturase  ccccgatcaa cggacatcgg tgcccaactt ttacctagca ggggacttca
eherbicolacrtI2                 gccacattt. .....aggca cagcgaa... cctcgactgg acggttgaag
euredovoracrtI                  gccgcattt. .....aggca ccgcgaa... cctcgactgg acggttgagg
eherbicolacrtI                  gccgcatct. .....tggca acgcgcc... gctggactgg gcgcaggagg
aaurantiacumcrtI                gccgcatct. .....gggcc gcgcgga... cattgattgg gcggtcgagg
R.capsulatuscrtI                gccgaacct. .....cggct tcgacaatgg cgtggactgg tcggtcgagg
N.crassacrtI                    tggccacctt ctccaaaacg gccaaccaga gctcgactgg cctactctcg
crtIflavobacterium              cgttcacgta ttcgaagctg cggccgtccg cccatgtcag ccggtagaag

                                2251                                                2300
SynecoccusPhytoenedesaturase    ccatgcagcg ctacctcgcc agtatggaag gggcggtcct atctggtaag
Synecocystisphytoenedesaturase  ccatgcaaaa atacttgggc agtatggaag gggcggtgct ttccggcaaa
eherbicolacrtI2                 ggccgcgact gcgcgatcgt atttttgagt accttgagca acattacatg
euredovoracrtI                  ggccaaaact acgcgaccgt atttttgcgt accttgagca gcattacatg
eherbicolacrtI                  ggccgaagct gcgcgaccgc atctttgact accttgaaga gcgctatatg
aaurantiacumcrtI                ggccgcgcta tgccgaccgc atcctggcca gcctggagga gcggctgatc
R.capsulatuscrtI                ccgagaaata caaggccaag gtgctgaaag tgatcgagga acggctgctt
N.crassacrtI                    tctccaaagc ccgtgccggc gttctggcca ccatccaagc ccgtaccggc
crtIflavobacterium              ggcgagaccg gcagcagcgt cacgtcacgc tccatcggtt ggccgctgag

                                2301                                                2350
SynecoccusPhytoenedesaturase    ctgacagcgc aagccatcat tgctcgccaa gatgagttgc aacgtcgcag
Synecocystisphytoenedesaturase  caatgcgccc aggcgatcgc cgccgatttc aacccccaaa ccgttccccc
eherbicolacrtI2                 cctggcttgc gtagccagtt ggttacgcag cgaatgttta cgccgttcga
euredovoracrtI                  cctggcttac ggagtcagct ggtcacgcac cggatgttta cgccgtttga
eherbicolacrtI                  cccggcctgc gtagccagct ggtgacccag cggatcttta ccccggcaga
aaurantiacumcrtI                ccgaacctgc gcgccaacct gaccacgacg cgcatcttca ccccgtccga
R.capsulatuscrtI                ccgggggttg ccgaaaagat caccgaggaa gtggtcttca cgccggaaac
N.crassacrtI                    ctgtccctgt ccccccttat caccgaagaa atcgtcaaca ccccttacac
crtIflavobacterium              ggcccacagc tctcgcaggc tgtcggggtc ggtcacgacc gtcgggcctg

                                2351                                                2400
SynecoccusPhytoenedesaturase    cagcggacga ccgctggccg cgagtcaggc ......atag acactgttac
Synecocystisphytoenedesaturase  caccagggaa atagtcaccg tgggttaagc cgcctggact ccctggtaat
eherbicolacrtI2                 ttttcgcgac cagctcaatg cctatcaagg ctcagccttt tctgttgagc
euredovoracrtI                  ttttcgcgac cagcttaatg cctatcatgg ctcagccttt tctgtggagc
eherbicolacrtI                  cttccacgac acgctggatg cgcatctggg atcggccttc tccatcgagc
aaurantiacumcrtI                tttcgccagc gaactgaacg cccatcatgg cagcgccttc tcggtcgagc
R.capsulatuscrtI                cttccgcgac cgttatctct cgccgctggg cgcgggcttc tcgctggaac
N.crassacrtI                    ctgggagacc aagttcaacc tcagcaaggg cgccatcctc ggtttggccc
crtIflavobacterium              catcgaagac gtggccctga tcgttccaga cataggcgcg gccgccgggc
```

```
                                2401                                                2450
SynecoccusPhytoenedesaturase    ccatgttgca gttgctagct ctgaagcttc actggtcacc ctctcacccc
Synecocystisphytoenedesaturase  cttcctgaca aatggcaacc ctaatgcgac aatgctaaat ggctaacggt
          eherbicolacrtI2       ccgttctcac acagagcgcc tggttccgac c..gcacaac cgcgataaaa
           euredovoracrtI       ccgttcttac ccagagcgcc tggtttcggc c..gcataac cgcgataaaa
           eherbicolacrtI       cgctgctgac ccaaagcgcc tggttccgcc c..gcacaac cgcgacagcg
         aaurantiacumcrtI       cgatcctgac gcaatccgcg tggttccggc c..gcacaac cgcgacaaga
         R.capsulatuscrtI       cgcggatcct gcaatcggcc tggttccgcc c..gcataac gcctcggaag
              N.crassacrtI      acgacttctt caacgtgctg gccttccgcc c..gcgcacc aaagcccaag
        crtIflavobacterium      ttgtcgcggg cctcgacgat ggtggtcgcg a..tgccggc cgattgcagg

                                2451                                                2500
SynecoccusPhytoenedesaturase    cgactcgcac cgtctgtctg atgctgcaga tgataccggc acggccttca
Synecocystisphytoenedesaturase  caaatttctc c.ccagcgtg cagttaccaa accccaatcc tggtggctga
          eherbicolacrtI2       ccatcaataa tctttacctg gtaggtgcag gcacccatcc cggcgcgggc
           euredovoracrtI       ccattactaa tctctacctg gtcggcgcag gcacgcatcc cggcgcaggc
           eherbicolacrtI       acattgccaa cctctacctg gtgggcgcag gtactcaccc tggggcgggc
         aaurantiacumcrtI       cgatccgcaa cttctatctg gtcggcgcgg gcacccatcc gggcgcgggg
         R.capsulatuscrtI       aggtggacgg gctttatctg gtcggcgcgg gcacccatcc gggcgccggt
              N.crassacrtI      gcatggataa cgcctacttt gtcggcgcta gcacccatcc gggaaccggc
        crtIflavobacterium      cggatggcaa gcgcaagccc gccgaaacct gcgccgatga cgatggcgga

                                2501                                                2550
SynecoccusPhytoenedesaturase    cgggcgctcg cctctctgtc ggaggcctac gaggagtgcc gacaaattac
Synecocystisphytoenedesaturase  cttccgaacc ccgtccgtcc ttaatgttac ...aactgcc caaaccgtct
          eherbicolacrtI2       attcccggcg tgatcggctc ggctaaggca actgcaggtt taatgctgga
           euredovoracrtI       attcctggcg tcatcggctc ggcaaaagcg acagcaggtt tgatgctgga
           eherbicolacrtI       attcctggcg tagtggcctc ggcgaaagcc accgccagcc tgatgattg.
         aaurantiacumcrtI       attccgggcg tcgtgggctc ggccaaggcc acggcccagg tgatgctgtc
         R.capsulatuscrtI       gtgccctcgg tgatcggctc gggcgagctt gtcgcgcaga tgatcccgga
              N.crassacrtI      gtgccgattg tccttgcagg tgccaagatc actgccgagc agattcttga
        crtIflavobacterium      actcatgctc tctcc..... .......... .......... ..........

                                2551                                                2600
SynecoccusPhytoenedesaturase    cgctccgcta cgctaagacc ttttacctcg ggacgctttt gatgcccgag
Synecocystisphytoenedesaturase  ccatctgcaa agccctgtgc ttctgttga. .......... ..........
          eherbicolacrtI2       ggacctgatt tgaataatcc gt........ .......... ..........
           euredovoracrtI       ggatctgatt tga..... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
         aaurantiacumcrtI       cgacctggcg agcgcatgag cgatctgg.. .......... ..........
         R.capsulatuscrtI       tgcgccgaag cccgagaccc ccgcggcggc tgcgcccaag gcccggacgc
              N.crassacrtI      ggagacgttt cctaagaaca caaaggtgcc gtggacgacg aacgaggaga
        crtIflavobacterium      .......... .......... .......... .......... ..........

                                2601                                                2650
SynecoccusPhytoenedesaturase    gccaagcgtc ag........ .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
         aaurantiacumcrtI       .......... .......... .......... .......... ..........
         R.capsulatuscrtI       cccgggccaa ggcggcgcaa tgat...... .......... ..........
              N.crassacrtI      ggaacagtga gcggatgagg aaggagatgg atgagaagat tacggaggag
        crtIflavobacterium      .......... .......... .......... .......... ..........

                                2651                                                2700
SynecoccusPhytoenedesaturase    .......... .......... .......... .......... ..........
Synecocystisphytoenedesaturase  .......... .......... .......... .......... ..........
          eherbicolacrtI2       .......... .......... .......... .......... ..........
           euredovoracrtI       .......... .......... .......... .......... ..........
           eherbicolacrtI       .......... .......... .......... .......... ..........
         aaurantiacumcrtI       .......... .......... .......... .......... ..........
         R.capsulatuscrtI       .......... .......... .......... .......... ..........
              N.crassacrtI      gggattatta tgaggagtaa cagcagtaag ccgggcagga gggggagtga
        crtIflavobacterium      .......... .......... .......... .......... ..........
```

```
                          2701                                          2750
SynecoccusPhytoenedesaturase  ..... ..... ..... ..... .....
Synecocystisphytoenedesaturase ..... ..... ..... ..... .....
eherbicolacrtI2               ..... ..... ..... ..... .....
euredovoracrtI                ..... ..... ..... ..... .....
eherbicolacrtI                ..... ..... ..... ..... .....
aaurantiacumcrtI              ..... ..... ..... ..... .....
R.capsulatuscrtI              ..... ..... ..... ..... .....
N.crassacrtI                  tgcttttgag ggcgccatgg aggtggttaa tctcttgtcg cagagggcgt
crtIflavobacterium            ..... ..... ..... ..... .....

                          2751                                          2800
SynecoccusPhytoenedesaturase  ..... ..... ..... ..... .....
Synecocystisphytoenedesaturase ..... ..... ..... ..... .....
eherbicolacrtI2               ..... ..... ..... ..... .....
euredovoracrtI                ..... ..... ..... ..... .....
eherbicolacrtI                ..... ..... ..... ..... .....
aaurantiacumcrtI              ..... ..... ..... ..... .....
R.capsulatuscrtI              ..... ..... ..... ..... .....
N.crassacrtI                  tccctttgtt ggtggcgttg atgggggtgc tgtatttctt gctatttgtg
crtIflavobacterium            ..... ..... ..... ..... .....

                          2801                                          2850
SynecoccusPhytoenedesaturase  ..... ..... ..... ..... .....
Synecocystisphytoenedesaturase ..... ..... ..... ..... .....
eherbicolacrtI2               ..... ..... ..... ..... .....
euredovoracrtI                ..... ..... ..... ..... .....
eherbicolacrtI                ..... ..... ..... ..... .....
aaurantiacumcrtI              ..... ..... ..... ..... .....
R.capsulatuscrtI              ..... ..... ..... ..... .....
N.crassacrtI                  aggtagggtt ctgttgggtt gacgggtttc acttaatgcg gagcgggcga
crtIflavobacterium            ..... ..... ..... ..... .....

                          2851                                          2900
SynecoccusPhytoenedesaturase  ..... ..... ..... ..... .....
Synecocystisphytoenedesaturase ..... ..... ..... ..... .....
eherbicolacrtI2               ..... ..... ..... ..... .....
euredovoracrtI                ..... ..... ..... ..... .....
eherbicolacrtI                ..... ..... ..... ..... .....
aaurantiacumcrtI              ..... ..... ..... ..... .....
R.capsulatuscrtI              ..... ..... ..... ..... .....
N.crassacrtI                  ttcatgtttc tttaagtctt ggttctagtc tagatgattt ccttgagtag
crtIflavobacterium            ..... ..... ..... ..... .....

                          2901                                          2950
SynecoccusPhytoenedesaturase  ..... ..... ..... ..... .....
Synecocystisphytoenedesaturase ..... ..... ..... ..... .....
eherbicolacrtI2               ..... ..... ..... ..... .....
euredovoracrtI                ..... ..... ..... ..... .....
eherbicolacrtI                ..... ..... ..... ..... .....
aaurantiacumcrtI              ..... ..... ..... ..... .....
R.capsulatuscrtI              ..... ..... ..... ..... .....
N.crassacrtI                  gtatagttcg attgggtata aacgatttcg tagataaagt cttgggaata
crtIflavobacterium            ..... ..... ..... ..... .....

                          2951                                          3000
SynecoccusPhytoenedesaturase  ..... ..... ..... ..... .....
Synecocystisphytoenedesaturase ..... ..... ..... ..... .....
eherbicolacrtI2               ..... ..... ..... ..... .....
euredovoracrtI                ..... ..... ..... ..... .....
eherbicolacrtI                ..... ..... ..... ..... .....
aaurantiacumcrtI              ..... ..... ..... ..... .....
R.capsulatuscrtI              ..... ..... ..... ..... .....
N.crassacrtI                  tatagctgtt ttgtttttat gtcgaagaca atggggttca tcatggctcg
crtIflavobacterium            ..... ..... ..... ..... .....
```

```
                          3001                                                3050
SynecoccusPhytoenedesaturase ..... ..... ..... ..... .....
Synecocystisphytoenedesaturase ..... ..... ..... ..... .....
          eherbicolacrtI2 ..... ..... ..... ..... .....
           euredovoracrtI ..... ..... ..... ..... .....
           eherbicolacrtI ..... ..... ..... ..... .....
         aaurantiacumcrtI ..... ..... ..... ..... .....
         R.capsulatuscrtI ..... ..... ..... ..... .....
             N.crassacrtI gaaagtatcc ttcttctcac cgatatgtgc ttagttaccc ccatatgcct
        crtIflavobacterium ..... ..... ..... ..... .....

                          3051                                                3100
SynecoccusPhytoenedesaturase ..... ..... ..... ..... .....
Synecocystisphytoenedesaturase ..... ..... ..... ..... .....
          eherbicolacrtI2 ..... ..... ..... ..... .....
           euredovoracrtI ..... ..... ..... ..... .....
           eherbicolacrtI ..... ..... ..... ..... .....
         aaurantiacumcrtI ..... ..... ..... ..... .....
         R.capsulatuscrtI ..... ..... ..... ..... .....
             N.crassacrtI agatgccgtc ttgccactgg gttctgcagt ccttttcttt ctctttttt
        crtIflavobacterium ..... ..... ..... ..... .....

                          3101                3126
SynecoccusPhytoenedesaturase ..... ..... .....
Synecocystisphytoenedesaturase ..... ..... .....
          eherbicolacrtI2 ..... ..... .....
           euredovoracrtI ..... ..... .....
           eherbicolacrtI ..... ..... .....
         aaurantiacumcrtI ..... ..... .....
         R.capsulatuscrtI ..... ..... .....
             N.crassacrtI ttttcttttg gaagaggtta gtaccg
        crtIflavobacterium ..... ..... .....
```

The above alignement is based upon the nucleotide sequences of phytoene desaturase genes from the organisms listed above. The consensus primer CRTIRH is emboldened. The primer used was the reverse complement of that shown. Sequences were aligned using the PILEUP algorithm of the Wisconsin Genetic Computer Group's Sequence Analysis Software Package (1994).

```
                    1                                                   50
               arab2 ..... ..... ..... ..... .....
             arabcrtY ..... ..... ..... ..... .....
          cannuummcrtL cttaattata gaaatactta agatatatca ttgcccttta atcatttatt
lycopersiconcrtL1 ..... ..... ..... ..... .....
          NtabacumcrtL ..... ..... ..... ..... .....
synechoccuscrtY ..... ..... ..... ..... .....
             eherb2crtY ..... ..... ..... ..... .....
             eured.crtY ..... ..... ..... ..... .....
aaurantiacumcrtY ..... ..... ..... ..... .....
             flavocrtY ..... ..... ..... ..... .....
          sgriseuscrtY ..... ..... ..... ..... .....
```

```
                51                                                  100
          arab2 .......... .......... .......... .......... ..........
        arabcrtY .......... .......... .......... .......... ..taattgtc
     cannuummcrtL tttaactctt ttaagtgttt aaagattgat tctttgtaca tgttctgctt
lycopersiconcrtL1 .......... .......... .......... .......... ..........
     NtabacumcrtL .......... .......... .......... .......... ..........
   synechoccuscrtY .......... .......... .......... .......... ..........
       eherb2crtY .......... .......... .......... .......... ..........
       eured.crtY .......... .......... .......... .......... ..........
 aaurantiacumcrtY .......... .......... .......... .......... ..........
        flavocrtY .......... .......... .......... .......... ..........
     sgriseuscrtY .......... .......... .......... .......... ..........

                101                                                 150
          arab2 .......... .......... .......... .......... ..........
        arabcrtY tccatctcca tgaagctact gcttctgggt aagttttgtg gtcttcgttc
     cannuummcrtL catttgtgtt gaaaattgag ttgttttctt gaattttgca agaatatagg
lycopersiconcrtL1 .......... .......... .......... .......... .........g
     NtabacumcrtL .......... .......... .......... .......... ..........
   synechoccuscrtY .......... .......... .......... .......... ..........
       eherb2crtY .......... .......... .......... .......... ..........
       eured.crtY .......... .......... .......... .......... ..........
 aaurantiacumcrtY .......... .......... .......... .......... ..........
        flavocrtY .......... .......... .......... .......... ..........
     sgriseuscrtY .......... .......... .......... .......... ..........

                151                                                 200
          arab2 .......... .......... .......... .......... ..........
        arabcrtY atcttttctc tagcaattta gtattccatt ttctcaatcc ctctggttag
     cannuummcrtL ggaccccatt tgtgttgaaa attgagcagc tttctttgtg ttttgttcga
lycopersiconcrtL1 gcacgaggaa acttttctct cttcactagc tgtttacatg ctt.....ga
     NtabacumcrtL .......... .......... .......... .......... ..........
   synechoccuscrtY .......... .......... .......... .......... ..........
       eherb2crtY .......... .......... .......... .......... ..........
       eured.crtY .......... .......... .......... .......... ..........
 aaurantiacumcrtY .......... .......... .......... .......... ..........
        flavocrtY .......... .......... .......... .......... ..........
     sgriseuscrtY .......... .......... .......... .......... ..........

                201                                                 250
          arab2 .......... .......... .......... .......... ..........
        arabcrtY aaatcgtgtc cggtgatttt tgaattatat ccttttggtg ttttcttcga
     cannuummcrtL tttttcaaga atataggacc ccatttttctg ttttcttgag ataaattgca
lycopersiconcrtL1 aatttcaaga ttttaggacc ccatttgaag ttttcttgaa acaaatatta
     NtabacumcrtL .....ggaac tttcttgaaa tcctgtttgt agttttcaaa aaaaattgaa
   synechoccuscrtY .......... .......... .......... .......... ..........
       eherb2crtY .......... .......... .......... .......... ..........
       eured.crtY .......... .......... .......... .......... ..........
 aaurantiacumcrtY .......... .......... .......... .......... ..........
        flavocrtY .......... .......... .......... .......... ..........
     sgriseuscrtY .......... .......... .......... .......... ..........
```

```
                 251                                                 300
          arab2  .......... ....gatgga tactctgttg aaaacaccca acaagctcga
        arabcrtY ttttgggggga attcgatgga tactctgttg aaaacaccca acaagctcga
     cannuummcrtL ccttgttggg aaaatatgga tacgctcttg agaacccccaa acaatcttga
lycopersiconcrtL1 ccctgttgga aaaagatgga tactttgttg aaaacccccaa ataaccttga
     NtabacumcrtL cccctgttgg aagatatgga tacattgttg aaaacccccaa ataagcttga
   synechoccuscrtY .......... .......... .......... .......... ..........
        eherb2crtY .......... .......... .......... .......... ..........
        eured.crtY .......... .......... .......... .......... ..........
  aaurantiacumcrtY .......... .......... .......... .......... ..........
        flavocrtY .......... .......... .......... .......... ..........
     sgriseuscrtY .......... .......... .......... .......... ..........

                 301                                                 350
          arab2  ttttttcatc cctcagtttc atgggtttga gagattatgc agtaacaatc
        arabcrtY ttttttcatc cctcagtttc atgggtttga gagattatgc agtaacaatc
     cannuummcrtL atttctg... .........c atggatttgg tgttaaagtt agtgccttta
lycopersiconcrtL1 atttctgaac ccac...atc atggttttgc tgttaaagct agtaccttta
     NtabacumcrtL gtttctgcac ccag...ttc atggattttc tgttaaagct agctccttta
   synechoccuscrtY .......... .......... .......... .......... ..........
        eherb2crtY .......... .......... .......... .......... ..........
        eured.crtY .......... .......... .......... .......... ..........
  aaurantiacumcrtY .......... .......... .......... .......... ..........
        flavocrtY .......... .......... .......... .......... ..........
     sgriseuscrtY .......... .......... .......... .......... ..........

                 351                                                 400
          arab2  catacccttc aagggttagg cttggtgtga agaaaagggc tatcaaaatt
        arabcrtY cataccattc aagggttagg cttggtgtga agaaaagggc tatcaaaatt
     cannuummcrtL gctctgtgaa gtctcagaag tttggtgcta agaagttttg tgaaggtttg
lycopersiconcrtL1 gatctgagaa gcatcataat tttggttcta ggaagttttg tgaaactttg
     NtabacumcrtL actctgtaaa gccccataag tttggttcta ggaaaatttg tgaaaattgg
   synechoccuscrtY .......... .......... .......... .......... ....acctga
        eherb2crtY .......... .......... .......... .......... ..........
        eured.crtY .......... .......... .......... .......... ..........
  aaurantiacumcrtY .......... .......... .......... .......... ..........
        flavocrtY .......... .......... .......... .......... ..........
     sgriseuscrtY .......... .......... .......... .......... ..........

                 401                                                 450
          arab2  gtctcta... ......gtgt agtgagtggt agcgctgctc ttttggatct
        arabcrtY gtctcta... ......gtgt agtgagtggt agcgctgctc ttttggatct
     cannuummcrtL gggagtagaa gtgtctgtgt gaaggctagt agtagtgctc ttttggagct
lycopersiconcrtL1 g...gtagaa gtgtttgtgt taagggtagt agtagtgctc ttttagagct
     NtabacumcrtL g...gtaaag gggtttgtgt taaggctaag agtagtgccc ttttggagct
   synechoccuscrtY cgcactacaa tagcctcagt cagctcgatc acctcagccg ctaggggcga
        eherb2crtY .......... .......... .......... .......... ..........
        eured.crtY .......... .......... .......... .......... ..........
  aaurantiacumcrtY .......... .......... .......... .......... ..........
        flavocrtY .......... .......... .......... .......... ..........
     sgriseuscrtY .......... .......... .......... .......... ..........
```

```
                451                                                 500
          arab2 tgttcctgaa actaagaagg agaatcttga ctttgagctt cctttgtacg
        arabcrtY tgttcctgaa actaagaagg agaatcttga ctttgagctt cctttgtacg
     cannuummcrtL tgtacctgag acaaaaaagg aaaatcttga ttttgagctt cctatgtatg
lycopersiconcrtL1 tgtacctgag accaaaaagg agaatcttga ttttgagctt cctatgtatg
     NtabacumcrtL tgtacctgag accaaaaagg aaaatcttga ttttgagctt cctatgtatg
   synechoccuscrtY ttgccccctc agggctagg. ..atcgcaag cggtgatagt agcgcaagca
        eherb2crtY .......... .......... .......... .......... ..........
         eured.crtY .......... .......... .......... .......... ..........
  aaurantiacumcrtY .......... .......... .......... .......... ..........
        flavocrtY .......... .......... .......... .......... ..........
     sgriseuscrtY .......... .......... .......... .......cag cgcgtcaccg

                501                                                 550
          arab2 acacttccaa gagtcaagtt gttgatttgg ctattgttgg tggtggtcct
        arabcrtY acacttccaa gagtcaagtt gttgatttgg ctattgttgg tggtggtcct
     cannuummcrtL acccttcaaa aggggttgtt gtggatcttg ctgtggtcgg tggtggtcct
lycopersiconcrtL1 acccttcaaa aggggttgtt gtggatcttg ctgtggttgg tggtggccct
     NtabacumcrtL acccttcaaa aggtcttgtt gtagatctag ctgtggttgg tggtggaccc
   synechoccuscrtY acagggagag cgatcgcgtg ttcgatgcct tagtgatcgg gtcggggcca
        eherb2crtY .......... .......... .......... .......... ..........
         eured.crtY .......... .......... .......... .......... ..........
  aaurantiacumcrtY .......... .......... .......... .......... ..........
        flavocrtY ........gc ggaactcatg ctctctcctg cagcaggggg cgttcgggca
     sgriseuscrtY cgcaacaggg tcatggggtg ctcccttcgg gggcggtcgg tgcgggggaa

                551                                                 600
          arab2 gctggtttag ccgtggctca gcaggtttct gaagctggac tctctgtttg
        arabcrtY gctggtttag ccgtggctca gcaggtttct gaagctggac tctctgtttg
     cannuummcrtL gcaggtcttg ctgttgcaca gcaagtttct gaagcaggac tttctgtttg
lycopersiconcrtL1 gcaggacttg ctgttgcaca gcaagtttct gaagcaggac tctctgtttg
     NtabacumcrtL gctggacttg cagttgcaca gcaggtttcg gaggctggac tatcggttgt
   synechoccuscrtY gccggactgg cgatcgcggc agagctggca cagcgcggct tgaaagtcca
        eherb2crtY .......... .......... .......... ..ctatgcga ccgcattatg
         eured.crtY .......... .......... .......... ...tatgcaa ccgcattatg
  aaurantiacumcrtY .......... .......... .......... ..gagcgcac gtgacccatg
        flavocrtY ..ggcagcgc acggcctgc. gacagcggaa tgggcgggcg tccggtgacg
     sgriseuscrtY acggagccgc cgggcgtgca cgccgggttc tgaagggaag ttcgaagacg
                                                                    ATG
                601                                                 650
          arab2 ttccattgat cc...ttctc ctaagctcat atggcctaac aattatggag
        arabcrtY ttccattgat cc...ttctc ctaagctcat atggcctaac aattatggag
     cannuummcrtL ttcgattgat cc...gaatc ctaaattgat atggcctaat aactatggtg
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     NtabacumcrtL ttcaatcgat cc...atcgc cgaaattgat atggcccaat aactatggtg
   synechoccuscrtY aggactatcc cccgtcgacc cattccatcc ttgggaaaat acctacggca
        eherb2crtY atctgatttt agtgggcgcc gggctggcta atggcctcat tgccctgcgc
         eured.crtY atctgattct cgtgggggct ggactcgcga atggccttat cgccctgcgt
  aaurantiacumcrtY acgtgctgct ggcaggggcg ggccttgcca acgggctgat cgccctggcg
        flavocrtY atgcgaagcc ggtcggccaa tgtcaggcgc ccggcataga agcgctcgat
     sgriseuscrtY gtccgcagca tggggaccat cggtgtccgc aggccgatga gcaggtcctc
                ATGTGATGCT GGTGGGCGCT
```

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                651                                                 700
          arab2 tttgggttga tg.agtttga ggctatggat ttactagact gcctggatac
        arabcrtY tttgggttga tg.agtttga ggctatggat ttactagact gcctggatac
    cannuummcrtL tttgggtgga tg.aatttga ggctatggac ttgttagatt gtcttgatgc
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    NtabacumcrtL tttgggtgga tg.aatttga ggccatggat ttgttggatt gcctcgacgc
  synechoccuscrtY tctggggacc cg.agctgga tagtcttggc ctcgagcatc tctttgggca
       eherb2crtY cttcagcagc agcaacctga catgcgtatt ctgcttatcg aagcggcccc
       eured.crtY cttcagcagc agcaacctga tatgcgtatt ttgcttatcg acgccgcacc
 aaurantiacumcrtY ctgcgcgcgg cgcggcccga cctgcgcgtg ctgctgctgg accatgccgc
        flavocrtY cagcggctgc ggcaggcggt agaaccgctg cagcaggcga tagcgacggt
     sgriseuscrtY gtgcagttgc gaacggccgt ccatgaagct cagcagccgc tcgccgggaa

                701                                                 750
          arab2 cacatggtct ggtgctgttg tctatgtcga tgaaggtgtc aagaaggatt
        arabcrtY cacatggtct ggtgctgttg tctatgtcga tgaaggtgtc aagaaggatt
    cannuummcrtL tacttggtct ggtgcagcgg tgtacattga tgataaaaca actaaagatc
lycopersiconcrtL1 tacctggtct ggtgcagcag tgtacattga tgataatacg gctaaagatc
    NtabacumcrtL cacatggtca ggtactgttg tttatattga tgacaataca actaaagatc
  synechoccuscrtY tcgctggtcg aactgcgtta gctacttcgg tgaggcgccg gttcagcacc
       eherb2crtY ccaggcgggc ggtaatcata cctggtcctt tcacgacgct gatttaaccg
       eured.crtY ccaggcgggc gggaatcata cgtggtcatt tcaccacgat gatttgactg
 aaurantiacumcrtY aggaccgtca gacggccaca cctggtcctg ccacgacccc gacctgtcgc
        flavocrtY cgggcgggca gccgcggaac agcatccggt tcagcagccg ..caggaagc
     sgriseuscrtY tgtggcggaa gaggcggtgg aagaagtccg cgccgtccac ..cctgccgc

                751                                                 800
          arab2 tgagccggcc ttatgggaga gttaaccgga aacagctcaa atccaaaatg
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    cannuummcrtL ttaatagacc ttatggaagg gttaaccgaa agcagttgaa atcgaaaatg
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  synechoccuscrtY aataca...a ctacgggctg tttgatcgcg cccaactaca acagcactgg
       eherb2crtY agagccaaca tcgctgggta gcaccgctgg tggtatatca ctggcccgac
       eured.crtY agagccaaca tcgttggata gctccgctgg tggttcatca ctggcccgac
 aaurantiacumcrtY cggactggct ggcgcggctg aagcccctgc gccgcgccaa ctggcccgac
        flavocrtY ggtcgcgatc cgcgcgatcg atggcccagc cgcgcaccgc gcgacgggcg
     sgriseuscrtY tgtccagggc ccgcagcagc acggcgtcca tcagccgcgc ccgacgcccg

                801                                                 850
          arab2 cttcagaaat gtattaccaa cggtgttaaa tttcatcagt ctaaggtcac
        arabcrtY cttcagaaat gtattaccaa cggtgttaaa tttcatcagt ctaaggtcac
    cannuummcrtL atgcagaaat gtatactgaa tggtgttaaa ttccatcaag ccaaagttat
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  synechoccuscrtY ttgcggcaat gtgagcaagg cggcctgcaa tggcaactcg gcaaagcagc
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        flavocrtY gacgcggtcg tca..... .ggtcgcgcg ccgcgatggc atccgcgacc
     sgriseuscrtY tacggggcgg gcacacgcag ggggcgtccg gagcgcaact ggtcggcgac
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                851                                                 900
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        arabcrtY taatgtggtt cacgaggagg caaactccac tgtggtctgc agtgacggtg
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     NtabacumcrtL aaaggtaatt cacgaggaag ctaaatctat gctgatttgc aatgatggtg
   synechoccuscrtY tgcgatcgcc catgactccc accattcctg cgttacgaca gcagcagggc
        eherb2crtY tttctgtgac ttcgcagcgt tttgccgagg ttttacaaca gaagtttggt
        eured.crtY tttgtattac ttctcagcgt ttcgctgagg ttttacagcg acagtttggc
  aaurantiacumcrtY ggtcgctgga cggggcggcg ctggcggatg cggtggtccg ...gtcgggc
        flavocrtY tgcgcggcat agggcagcga atatccggtg acggggtgga acagccctgc
     sgriseuscrtY ggcccggctc tgtcgctgca cggccgcgaa ggtgtagccg gtagacgggc

                901                                                 950
          arab2 taaagattca ggcttccgtg gttcttgatg ccactgggtt ttcccgatgc
        arabcrtY taaagattca ggcttccgtg gttcttgatg ccactgggtt ttcccgatgc
     cannuummcrtL ttactattca ggcgacagtg gtgctcgatg caactggctt ctctagatct
lycopersiconcrtL1 ttactattca ggcaacggtg gtgctcgatg caactggctt ctctagatct
     NtabacumcrtL taactattca ggcaacggtg gtgcttgatg caactggctt ctcaagatgt
   synechoccuscrtY aggagttaca ggcgcggctg gttgtcgata cgactgggca ccaagcggct
        eherb2crtY cagcacttat ggattagccg cgcggtggca gaggttcacg ccgacgctgt
        eured.crtY ccgcacttgt ggatggatac cgcggtcgca gaggttaatg cggaatctgt
  aaurantiacumcrtY gccgagatcc gctgggacag cgacatcgcc ctgctggatg cgcaggggc
        flavocrtY ccccagccca accggcaccg ccccctgcgc gtggtcgcgc cagaagccta
     sgriseuscrtY gggtcgcgcc tccggcggtg .ccgatgcga tacaccgaac gccccgcctt

                951                                                1000
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        arabcrtY ttggttcagt atgacaaacc ttacaaccct gggtaccaag tagcttacgg
     cannuummcrtL cttgttcagt atgataagcc ttataacccc gggtatcaag tagcttatgg
lycopersiconcrtL1 cttgttcagt atgataagcc ttataacccc gggtatcaag ttgcttatgg
     NtabacumcrtL cttgttcagt atgataagcc atataaacct ggatatcaag tagcttatgg
   synechoccuscrtY tttatccagc gacctcattc agacgcgatc gcctaccaag cggcctacgg
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  aaurantiacumcrtY gacgctgtcc tgcggcaccc ggatcgaggc gggcgcggtc ctggacgggc
        flavocrtY tggcgtcatg ggccag..cg cgat.gggca ggatgccccc ttcgcgccgc
     sgriseuscrtY gtgcgggaag cggccgtccg tcat.gggga tgacaccgtg ctcctgcgcc

                1001                                               1050
          arab2 gattatagct g......... .......aag ttgatggtca cccattcgat
        arabcrtY gattgtagct g......... .......aag ttgatggtca cccattcgat
     cannuummcrtL cattttggct g......... .......aag ttgaagagca ccccttttgat
lycopersiconcrtL1 cattttggct g......... .......aag tggaagagca ccccttttgat
     NtabacumcrtL catattggca g......... .......aag tggaggaaca tcccttttgat
   synechoccuscrtY catcattggc c......... .......agt tttcgcagcc gccgatcgag
        eherb2crtY gaggttatac gccaaattca gcgctgaacg tgggattcca ggcgtttatc
        eured.crtY ggggttatgc ggcaaattca gcactgagcg tgggcttcca ggcgtttatt
  aaurantiacumcrtY ggggcgcgca gccgtcgcgg catctgaccg tgggtttcca gaaattcgtg
        flavocrtY atctcctgcc cggtccagcc ccgcctggcg gcatagtcca gcgacgcctg
     sgriseuscrtY gtcacctgga gcggcccgag ccgcaacacg tcgtgggtgt agtggcgcag

                1051                                               1100
          arab2 gtagacaaaa tggtgttcat ggattggaga gacaaacatc tggactcata
        arabcrtY gtagacaaaa tggtgttcat ggattggaga gacaaacatc tggactcata
     cannuummcrtL gtaaacaaga tggttttcat ggattggcgc gactctcatt tgaagaacaa
lycopersiconcrtL1 gtaaacaaga tggttttcat ggattggcga gattctcatt tgaagaacaa
     NtabacumcrtL acaagtaaga tggttctcat ggattggcga gattcgcatc ttggtaataa
   synechoccuscrtY ccccatcagt ttgtgctgat ggactaccgc agcgaccatc t.........
        eherb2crtY ggacaggaat ggcgactcag taaaccgcac ggtttatctt cacccattat
        eured.crtY ggccaggaat ggcgattgag ccacccgcat ggtttatcgt ctcccattat
  aaurantiacumcrtY ggtgtcgaga tcgagaccga ccgcccccac ggcgtgcccc gcccgatgat
        flavocrtY cgccagcgcg ccatcgtcca gatcgccgcc gtcgctgtag cgc...gtat
     sgriseuscrtY ggctcgccgg tagccgtcgg tgtccagcgg ggcaggtgag aactcggtgt
```

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                1101                                                1150
          arab2 tcctgagctg aaagaacgga acagcaagat cccaacgttc ttgtacgcta
        arabcrtY tcctgagctg aaagaacgga acagcaagat cccaacgttc ttgtacgcta
    cannuummcrtL cgttgagctc aaggagagaa atagtagaat accaactttc ctttatgcca
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    NtabacumcrtL tatggagctg aaggagagaa atagaaaagt tccaactttt ttgtatgcca
  synechoccuscrtY ..ctcacctg aagaacgcca actgcca... .ccgaccttt ctctacgcga
       eherb2crtY tatggatgcc acggtcgatc agcaaaatgg ttatcgcttt gtttacagcc
       eured.crtY catggatgcc acggtcgatc agcaaaatgg ttatcgcttc gtgtacagcc
 aaurantiacumcrtY catggacgcg accgtcaccc agcaggacgg gtaccgcttc atctatctgc
       flavocrtY cctcgatcag gatgcgggtg ggactgaagg gcagcagata gatgaagcgg
    sgriseuscrtY actcgaccag cgcggtgtgc gggtccagcg gcaggacgta accgaacgag

                1151                                                1200
          arab2 tgccattttc ttccaaccga atatttcttg aagaaacttc tttagttgct
        arabcrtY tgccattttc ttccaaccga atatttcttg aagaaacttc tttagttgct
    cannuummcrtL tgccattttc atccaacagg atatttcttg aagaaacctc acttgttgct
lycopersiconcrtL1 tgccattttc atccaacagg atatttcttg aagaaacatc actcgtagct
    NtabacumcrtL tgccattttc atcaaataaa atatttcttg aagaaacctc acttgttgct
  synechoccuscrtY tggatctcgg gaacgacgtc tactttgtag aggaaacatc gctggcggct
       eherb2crtY tgccgctatc tgcgacagaa ctgttaatcg aagataccca ctatatcgat
       eured.crtY tgccgctctc gccgaccaga ttgttaattg aagacacgca ctatattgat
 aaurantiacumcrtY tgcccttctc tccgacgcgc atcctgatcg aggacacgcg ctattccgat
       flavocrtY taccc..... ..........  ..........  .....gtcca tctgcggaac
    sgriseuscrtY agccc..... .gcgagcggg ctgcggcgta cggaagtcca tcaggtccgc

                1201                                                1250
          arab2 agacctggtc tgagaatgga agatatccaa gaaagaatgg ctgctagact
        arabcrtY agacctggtc tgagaatgga agatatccaa gaaagaatgg ctgctagact
    cannuummcrtL cgtcctggtt tgggtatgga tgatattcaa gaacgaatgg tggctcgttt
lycopersiconcrtL1 cgtcctggct tgcgtataga tgatattcaa gaacgaatgg tggctcgttt
    NtabacumcrtL cgtcctggat tacgtatgga cgatattcaa gaaagaatgg tggctcgttt
  synechoccuscrtY tgcccggcta ttccctacga tcgcctcaaa caacggctct atcaacgctt
       eherb2crtY aacgcgacac tggaacctga acgcgcgcga caaaatattc gcgattatgc
       eured.crtY aatgcgacat tagatcctga atgcgcgcgg caaaatattt gcgactatgc
 aaurantiacumcrtY ggcggcgatc tggacgacga cgcgctggcg gcggcgtccc acgactatgc
       flavocrtY ggtcgcgtcc atgatcatcg ggcgctcgac gccatggggg gcgtcggtct
    sgriseuscrtY ggtcccggga tcgaacacgg gacgctcggt gcggacgaac cagccggtga

                1251                                                1300
          arab2 gaaacatctg gggatcaatg tgaagaggat tgaggaagac gagcgttgtg
        arabcrtY gaaacatctg gggatcaatg tgaagaggat tgaggaagac gagcgttgtg
    cannuummcrtL aagtcacttg gggataaaag ttaagagcat tgaagaggat gaacattgtg
lycopersiconcrtL1 aaaccatttg gggataaaag tgaagagcat tgaagaagat gaacattgtc
    NtabacumcrtL aaatcacttg ggtataaaag ttaagagcat tgaagaggac gagcattgtg
  synechoccuscrtY agccactcgc ggtgtgacgg tgcaagtgat tcagcacgag gaatattgcc
       eherb2crtY tgcccagcag gattggcagc ttcagactct gcttcgtgag gaacagggcg
       eured.crtY cgcgcaacag ggttggcagc ttcagacact gctgcgagaa gaacagggcg
 aaurantiacumcrtY ccgccagcag ggctg...ga ccggggccga ggtccggcgc gaacgcggca
       flavocrtY cgatctcgac gcccacgaat ttctggaaac ccacggtcag gtgcggggtc
    sgriseuscrtY agtgctgaa. .gcagcgtcg tgcgggcggg gggcaggcgg tgggacggcc
```

```
                1301                                                1350
          arab2 tgatcccgat gggcggtcct ttaccagtct tacctcaacg ggttgtgggg
        arabcrtY tgatcccgat gggcggtcct ttaccagtct tacctcaacg ggttgtgggg
    cannuummcrtL taataccaat gggtggtcct cttccagtat tacctcagag agttgttgga
lycopersiconcrtL1 taataccaat gggtggtcca cttccagtat tacctcagag agtcgttgga
    NtabacumcrtL taattccgat gggaggctcc cttcctgtaa tacctcagag agttgttgga
  synechoccuscrtY tgtttccgat gaatttgccg ctgcccgatc tcactcagtc agtggttggc
       eherb2crtY cattgccaat aaccttaacg ggcgatagca ccgcgttttg gcagcaacag
        eured.crtY ccttacccat tactctgtcg ggcaatgccg acgcattctg gcagcagcgc
 aaurantiacumcrtY tccttcccat cgcgctggcc catgatgcgg cgggcttctg ggccgatcac
       flavocrtY tcgacggcac cacgggcgtc gatcacgcag gcagcctcga tccgcgagcc
    sgriseuscrtY gggagtcgaa gacgagacgg ccgcggacaa ggatccgttc accgcacgcc

                1351                                                1400
          arab2 attggtggga cagcaggaat ggttcatcct tcaactggtt acatggttgc
        arabcrtY attggtggga cagcaggaat ggttcatcct tcaactggtt acatggttgc
    cannuummcrtL attggtggca cagccggtat ggttcatcca tccaccggtt atatggtagc
lycopersiconcrtL1 atcggtggta cagctggcat ggttcatcca tccaccggtt atatggtggc
    NtabacumcrtL actggtggta cagctggtct ggttcatccc tcaacaggtt atatggtagc
  synechoccuscrtY tttgggggggg cggccagtat ggtgcatccc gcttcgggct acatggtcgg
       eherb2crtY cctttagcct gcagt..... .ggactgcgt gccggactgt ttcaccccac
        eured.crtY cccctggcct gtagt..... .ggattacgt gccggtctgt tccatcctac
 aaurantiacumcrtY gcggcggggc ctgttcccgt gggactgcgc gcggggttct ttcatccggt
       flavocrtY gtccg....t cagcgtcgcg ccggtatcgt ccagcgtcgc gacatgcgta
    sgriseuscrtY gttcgggtca ggacctcgcc gccgacgccg gacgggtcgt cgcgtacgga

                1401                                                1450
          arab2 taggactctt gcagctgcac caatagttgc aaatgccatt gtgagatacc
        arabcrtY taggactctt gcagctgcac caatagttgc aaatgccatt gtgagatacc
    cannuummcrtL aaggacacta gctgcagctc ctgtcgttgc caatgccata attcagtacc
lycopersiconcrtL1 aaggacacta gctgcggctc ctgttgttgc caatgccata attcaatacc
    NtabacumcrtL aaggacccta gctgcagctc cggtcgtcgc taatgcaata attcactacc
  synechoccuscrtY ggcgctactg cggcgcgctc ctgatctggc gaatgcgatc gcggctggac
       eherb2crtY aaccggttat tcactccccc ttgcggttgc gctggccgat cgcctgagtg
        eured.crtY caccggctat tcactgccgc tggcggttgc cgtggccgac cgcctgagtg
 aaurantiacumcrtY caccggctat tcgctgccct atgcggcaca ggtggcggac gtggtggcgg
       flavocrtY ttccaccgca gatcgacacc ctgcagcagc ccgatcagcg cgcccgcctc
    sgriseuscrtY actcgccgtc g.cctccatc cggcacaagt ccggtgcacg ggagaaccgt

                1451                                                1500
          arab2 tcggttcacc aagtagtaat agcctgagag gagatcaact ctctgctgag
        arabcrtY tcggttcacc aagtagtaat agcctgagag gagatcaact ctctgctgag
    cannuummcrtL tcagttctga aagaagtcat ......tcgg gtgatgagtt atccgcagct
lycopersiconcrtL1 tcggttctga aagaagtcat ......tcgg gtaatgaatt atccacagct
    NtabacumcrtL ttggttctga gaaagacctt ......ttag gtaatgagtt atctgcagct
  synechoccuscrtY tgaatgc... ...cagttcc agtctgacca cggcagaact tgctacccaa
       eherb2crtY cgcttgatgt ctttac...a tcgtcctcaa ttcatcaggc gattacccac
        eured.crtY cacttgatgt ctttac...g tcggcctcaa ttcaccatgc cattacgcat
 aaurantiacumcrtY gtctgtccgg gccgcccggc accgacgcgc tgcgcggcgc catccgcgat
       flavocrtY gatcgagcca tagcctgtcg tcaggcggcg cgaatggtcg ggaaacgcga
    sgriseuscrtY tgctcgacca gtgcctcgaa ggcgtccgag cgcagcatct tgtagcgcag

                1501                                                1550
          arab2 gtttggagag acttgtggcc tatcgaacgg cgtagacaga gggagttctt
        arabcrtY gtttggagag acttgtggcc tatcgaacgg cgtagacaga gggagttctt
    cannuummcrtL gtttggaagg atttgtggcc gatagagagg aggcgtcaaa gagagttctt
lycopersiconcrtL1 gtttggaaag atttgtggcc tatagagagg agacgtcaaa gagagttctt
    NtabacumcrtL gtttggaaag atttgtggcc catagaaagg agacgtcaac gagagttctt
  synechoccuscrtY gcctggcgag gactctggcc aaccgaaaaa attcgcaagc actacatcta
       eherb2crtY tttgcccacg agcgctggca gcagcaacgc ttttccgca tgttgaatcg
        eured.crtY tttgcccgcg agcgctggca gcagcagggc ttttccgca tgctgaatcg
 aaurantiacumcrtY tacgcgatcg accgggcgcg ccgcgaccgc tttctgcgcc ttttgaaccg
       flavocrtY cctcctgatc cg........ .......tcc attcgccgcg acgaatgggc
    sgriseuscrtY ccgcgggagc tgggccacgg tggaggcccc gtcggccgcc cggacccgca
```

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                1551                                                1600
          arab2 ctgttttgga atggatattc tgctgaaact cgatttagac gctactagaa
        arabcrtY ctgttttgga atggatattc tgctgaaact cgatttagac gctactagaa
    cannuummcrtL ctgcttcggt atggacattc ttctgaagct tgacttaccg gctacaagga
lycopersiconcrtL1 ctgcttcggt atggatattc ttctgaagct tgatttacct gctacaagaa
    NtabacumcrtL ttgtttcggt atggatattc ttctgaagct tgatttaccc gctacaagaa
  synechoccuscrtY ccaattcggc ctcgaaaagc tgatgcgctt ttccgaagcc cagctcaatc
       eherb2crtY catgctgttt ttagcgggac ccgccgattc acgctggcgt gttatgcagc
        eured.crtY catgctgttt ttagccggac ccgccgattc acgctggcgg gttatgcagc
 aaurantiacumcrtY gatgctgttc cgcggctgcg cgcccgaccg gcgctatacc ctgctgcagc
        flavocrtY gacaggcgcg ccagccattc ggg..... .......... ...cgaaaga
    sgriseuscrtY gccggggcca ggaagcggcc aggacgggat cgtagggccc gcccggcggc

                1601                                                1650
          arab2 ggttctttga tgcattcttt gatctgcaac ctcattactg gcacggattc
        arabcrtY ggttctttga tgcattcttt gatctgcaac ctcattactg gcacggattc
    cannuummcrtL ggttctttga tgcattcttc gacttagaac ctcgttattg gcatggcttc
lycopersiconcrtL1 ggttctttga tgcattcttt gacttagaac ctcgttattg gcatggcttc
    NtabacumcrtL ggtttttcga tgccttttt gatctagaac ctcgttattg gcatggcttc
  synechoccuscrtY atcacttcca gaccttcttt ggcctgccga aggagcagtg gtacggcttt
       eherb2crtY gcttctatgg tttacctgaa gatttaatct cccgttttta tgcgggcaaa
        eured.crtY gtttttatgg tttacctgaa gatttaattg cccgttttta tgcgggaaaa
 aaurantiacumcrtY ggttctaccg catgccgcat ggactgatcg aacggttcta tgccggccgg
        flavocrtY tccgtgtcgt ggcaggacca ggtgtgctgg tccgaggggc cggaccgcgc
    sgriseuscrtY tcccagaagc accaggtccg cgggggcgct ctcaaaggcc ccggcggggc

                1651                                                1700
          arab2 ttgtcttcca ggctgtttct cccggaactg ttggtcttcg ggttgtcgct
        arabcrtY ttgtcttcca ggctgtttct cccggaactg ttggtcttcg ggttgtcgct
    cannuummcrtL ttgtcatcca ggttgtttct acctgaactc atagtttttg ggctctcact
lycopersiconcrtL1 ttatcgtctc gattgtttct acctgaactc atagtttttg ggctgtctct
    NtabacumcrtL ttgtcatctc gcctgtatct tcctgagctt atatttttcg ggctgtccct
  synechoccuscrtY ttgaccaata cgctgtcgct accggagctg attcaagcga tgctcagatt
       eherb2crtY ctcacgctga ccgatcggct acgtattctg agcggcaagc cgcctgttcc
        eured.crtY ctcacgctga ccgatcggct acgtattctg agcggcaagc cgcctgttcc
 aaurantiacumcrtY ctgagcgtgg cggatcagct gcgcatcgtg accggcaagc ctcccattcc
        flavocrtY gtcgagcatc acgatgcgcg catccggtct gcggtcgcga acggcaag..
    sgriseuscrtY gtccacgagc gcgaccgaca aaggtacgtc gctgtccggt gcgcagaggt

                1701                                                1750
          arab2 cttctcacac gcttccaata cctcaagatt ggagatcatg acaaagggga
        arabcrtY cttctcacac gcttccaata cctcaagatt ggagatcatg acaaagggga
    cannuummcrtL tttctctcat gcttcaaata cttctagatt agagataatg acaaagggaa
lycopersiconcrtL1 attctctcat gcttcaaata cttctagatt tgagataatg acaaagggaa
    NtabacumcrtL tttctctcgc gcttcaaata cttctagaat agagattatg acaaagggaa
  synechoccuscrtY atttgcccaa gcgccgaatg atgtgcgctg gggtctgatg gaacaacaag
       eherb2crtY ggtattagcg gcatt..gca ggcaattatg acgactcatc gttaaagagc
        eured.crtY ggtattagca gcatt..gca agccattatg acgactcatc gttaa.....
 aaurantiacumcrtY ccttggcacg gccatccgct gcctgcccga acgtcccctg ctgaaggaaa
        flavocrtY ....cgcgat cagcgcaccg gacagccccg cgcccgcgat cagcagatca
    sgriseuscrtY ggtacgccag gctcatcccg gccgcacccg cgcccacgat cacgacgtcg
```

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                1751                                                1800
          arab2 ctgttcctct tgcta.agat gatcaacaat ttggtacaag atagagacta
        arabcrtY ctgttcctct tgcta.agat gatcaacaat ttggtacaag atagagacta
    cannuummcrtL ctcttccatt agtac.atat gatcaacaat ttgttacagg ataaagaatg
lycopersiconcrtL1 ctgttccatt agtaa.atat gatcaacaat ttgttacagg ataaagaatg
    NtabacumcrtL ctcttccttt ggtaa.atat gatcaacaat ttgttacagg atacagaat.
  synechoccuscrtY gtcgcgaatt acaactcttt tggcaagcga tcgcggcccg ctagctgcta
       eherb2crtY gaca...... .......... .......... .......... ..........
        eured.crtY .......... .......... .......... .......... ..........
 aaurantiacumcrtY acgcatgaa. .......... .......... .......... ..........
       flavocrtY tggctcatgt at........ .......... .......... ..........
    sgriseuscrtY aagtcagtgg gcacggcatt .......... .......... ..........

                1801                                                1850
          arab2 aggaccagaa acttagacat ataagtatat ctgttctttg gttcttgacc
        arabcrtY aggaccagaa acttagacat ataagtacat ctgttctttg gttcttgacc
    cannuummcrtL aattcgactt atctgggatc ttgt...... .......... ..........
lycopersiconcrtL1 aatccgagta attcggaatc ttgtccaatc tcgtgcc... ..........
    NtabacumcrtL .....gactt accaggaatc ttgttcaata ttacatagca tgtgttaata
  synechoccuscrtY aaaactagcc gcccttgc.. .......... .......... ..........
       eherb2crtY .......... .......... .......... .......... ..........
        eured.crtY .......... .......... .......... .......... ..........
 aaurantiacumcrtY .......... .......... .......... .......... ..........
       flavocrtY .......... .......... .......... .......... ..........
    sgriseuscrtY .......... .......... .......... .......... ..........

                1851                                                1900
          arab2 agtagtatat ccgcattgca agtcgttgga taattgtgta taaaccacag
        arabcrtY agtagtatat ccgcattgca agtcgttgga taattgtgta taaaccacag
    cannuummcrtL .......... .......... .......... .......... ..........
lycopersiconcrtL1 .......... .......... .......... .......... ..........
    NtabacumcrtL cactgctc.. .......... .......... .......... ..........
  synechoccuscrtY .......... .......... .......... .......... ..........
       eherb2crtY .......... .......... .......... .......... ..........
        eured.crtY .......... .......... .......... .......... ..........
 aaurantiacumcrtY .......... .......... .......... .......... ..........
       flavocrtY .......... .......... .......... .......... ..........
    sgriseuscrtY .......... .......... .......... .......... ..........

                1901                                                1950
          arab2 atccataacc tgaatccttg tgaaatcaaa ttgttactac tagttcatta
        arabcrtY at.cataacc tgaatccttg tgaaatcaaa ttgttactac tagttcatta
    cannuummcrtL .......... .......... .......... .......... ..........
lycopersiconcrtL1 .......... .......... .......... .......... ..........
    NtabacumcrtL .......... .......... .......... .......... ..........
  synechoccuscrtY .......... .......... .......... .......... ..........
       eherb2crtY .......... .......... .......... .......... ..........
        eured.crtY .......... .......... .......... .......... ..........
 aaurantiacumcrtY .......... .......... .......... .......... ..........
       flavocrtY .......... .......... .......... .......... ..........
    sgriseuscrtY .......... .......... .......... .......... ..........

                1951                                                2000
          arab2 aaacc..... .......... .......... .......... ..........
        arabcrtY aaattaatac tttgtgctgc attgtgtttc accaactctt gtaaatccaa
    cannuummcrtL .......... .......... .......... .......... ..........
lycopersiconcrtL1 .......... .......... .......... .......... ..........
    NtabacumcrtL .......... .......... .......... .......... ..........
  synechoccuscrtY .......... .......... .......... .......... ..........
       eherb2crtY .......... .......... .......... .......... ..........
        eured.crtY .......... .......... .......... .......... ..........
 aaurantiacumcrtY .......... .......... .......... .......... ..........
       flavocrtY .......... .......... .......... .......... ..........
    sgriseuscrtY .......... .......... .......... .......... ..........
```

```
                2001                                                2050
        arab2   .......... .......... .......... .......... ..........
      arabcrtY  aactagaggc aaaatgtaat aagataaaag agtggatttt gaacaccaaa
   cannuummcrtL .......... .......... .......... .......... ..........
lycopersiconcrtL1 .......... .......... .......... .......... ..........
   NtabacumcrtL .......... .......... .......... .......... ..........
 synechoccuscrtY .......... .......... .......... .......... ..........
     eherb2crtY .......... .......... .......... .......... ..........
     eured.crtY .......... .......... .......... .......... ..........
aaurantiacumcrtY .......... .......... .......... .......... ..........
      flavocrtY .......... .......... .......... .......... ..........
   sgriseuscrtY .......... .......... .......... .......... ..........

                2051       2069
        arab2   .......... .........
      arabcrtY  aaaaaaaaaa aaaaaaaaa
   cannuummcrtL .......... .........
lycopersiconcrtL1 .......... .........
   NtabacumcrtL .......... .........
 synechoccuscrtY .......... .........
     eherb2crtY .......... .........
     eured.crtY .......... .........
aaurantiacumcrtY .......... .........
      flavocrtY .......... .........
   sgriseuscrtY .......... .........
```

The above alignement is based upon the nucleotide sequences of lycopene cyclase genes from the organisms listed above. The consensus primer CRTYLH is emboldened. Again the Wisconsin Genetic Computer Group's Sequence Analysis Software Package (1994) PILEUP algorithm was used.

The estimated product size of the amplified region of *B. linens* genome based upon the lycopene cyclase gene in *Erwinia herbicola*

The below nucleotide sequence is taken from the carotenoid biosynthesis gene cluster of *E. herbicola* (Armstrong *et al.*, 1993). The estimated product size is 1178bp (bases 3136-4314). Primer sites are emboldened. Primers used are from the multiple sequence alignements shown above, so are not exact matches to those shown.

```
2881 atttgatcag cccggcgtgg cctcacgtat tgtttctcat gggataggaa aacgtgcgtc
2941 ccgttttacc accagccatg cgctggcgcg ccacatgcgt gcattgctga ccaacgtcga
3001 ctacccgcaa cgcatgacga aaatccagac cgcccttcgt ttagccggcg gcacaatggc
3061 cgctgcagat atcgttgagc aggccatgcg caccggccag cccgttttga aggggagcgg
3121 ctatgcgacc gcattatgat ctgattttag tgggcgccgg gctggctaat ggcctcattg
3181 ccctgcgcct tcagcagcag caacctgaca tgcgtattct gcttatcgaa gcggcccccc
3241 aggcgggcgg taatcatacc tggtcctttc acgacgctga tttaaccgag agccaacatc
3301 gctgggtagc accgctggtg gtatatcact ggcccgacta tcaggtccgc tttcccacgc
3361 ggcgtcgtaa gctgaacagc ggctattttt ctgtgacttc gcagcgtttt gccgaggttt
3421 tacaacagaa gtttggtcag cacttatgga ttagccgcgc ggtggcagag gttcacgccg
3481 acgctgttcg actgaataat ggtcaggtca tcagtgccag cgcggtgatt gatgggcgag
3541 gttatacgcc aaattcagcg ctgaacgtgg gattccaggc gtttatcgga caggaatggc
3601 gactcagtaa accgcacggt ttatcttcac ccattattat ggatgccacg gtcgatcagc
3661 aaaatggtta tcgctttgtt tacagcctgc cgctatctgc gacagaactg ttaatcgaag
3721 atacccacta tatcgataac gcgacactgg aacctgaacg cgcgcgacaa aatattcgcg
3781 attatgctgc ccagcaggat tggcagcttc agactctgct tcgtgaggaa cagggcgcat
3841 tgccaataac cttaacgggc gatagcaccg cgttttggca gcaacagcct ttagcctgca
3901 gtggactgcg tgccggactg tttcacccca caaccggtta ttcactcccc cttgcggttg
3961 cgctggccga tcgcctgagt gcgcttgatg tctttacatc gtcctcaatt catcaggcga
4021 ttacccactt tgcccacgag cgctggcagc agcaacgctt tttccgcatg ttgaatcgca
4081 tgctgttttt agcgggaccc gccgattcac gctggcgtgt tatgcagcgc ttctatggtt
4141 tacctgaaga tttaatctcc cgtttttatg cgggcaaact cacgctgacc gatcggctac
4201 gtattctgag cggcaagccg cctgttccgg tattagcggc attgcaggca attatgacga
4261 ctcatcgtta aagagcgaca acatgaaacc aactacggta attggtgcag gctttggtgg
4321 cctggcatta gcaattcgtc tgcaggcggc ggggatccct gtcttactgc ttgagcaacg
4381 cgacaaaccc ggtggccggg cttatgtcta tgaagatcag ggattcactt ttgatgcagg
4441 gcctaccgtt attaccgatc ccagcgccat tgaagaactc tttaccctgg cgggaaaaca
4501 gttaaaagat tacgttgaac tgctgccggt tgcgccgttt tatcgcctgt gttgggagtc
```

Conserved regions in the deduced amino acid sequences of lycopene cyclase enzymes from plants, cyanobacteria and bacteria

```
1                                                                  50
  arabep  MECVGARNFA AMAVSTFPSW SCRRKFPVVK RYSYRNIRFG LCSVRASGGG
  escuep  MECVGVQNVG AMAVLTRPRL N...RWSGGE LCQEKSIFLA YEQYESKCNS
     cap  .......... .....MDTLL RTPNNLEFL. ...HGFGVK. ...VSAFSSV
    escu  .......... .....MDTLL KTPNNLEFLN PH.HGFAVK. ...ASTFRSE
    arab  .......... .....MDTLL KTPNKLDFFI PQFHGFERL. ...CSNNPYP
    narc  .......... .....MDTLL RTHNRLELLY P.LHELAKRH FLSPSPNPQN
    syne  .......... .......... .......... .......... ..........
agrocrty  .......... .......... .......... .......... ..........
   flavo  .......... .......... .......... .......... ..........
eho10(1)  .......... .......... .......... .......... ..........
eho10(2)  .......... .......... .......... .......... ..........
   eho13  .......... .......... .......... .......... ..........
    ured  .......... .......... .......... .......... ..........
  longus  .......... .......... .......... .......... ..........
sgris(1)  .......... .......... .......... .......... ..........
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          51                                                        100
  arabep  SSGSESCVAV REDFADEEDF VKAGGSEIL. FVQMQQNKDM DEQSKLVDKL
  escuep  SSGSDSCVVD KEDFADEEDY IKAGGSQLV. FVQMQQKKDM DQQSKLSDEL
     cap  KSQKFGAKKF CEGLGSRSVC VKASSSALLE LVPETKKENL DFELPMYDPS
    escu  KHHNFGSRKF CETLG.RSVC VKGSSSALLE LVPETKKENL DFELPMYDPS
    arab  SRVRLGVKKR AIKIVS...S VVSGSAALLD LVPETKKENL DFELPLYDTS
    narc  PNFKFFSRKP YQK.KCRNGY IGVSSNQLLD LVPEIKKEHL EFDLPLYDPS
    syne  .......... .......... .......... .......... ..........
agrocrty  .......... .......... .......... .......... ..........
   flavo  .......... .......... .......... .......... ..........
eho10(1)  .......... .......... .......... .......... ..........
eho10(2)  .......... .......... .......... .......... ..........
   eho13  .......... .......... .......... .......... ..........
    ured  .......... .......... .......... .......... ..........
  longus  .......... .......... .......... .......... .....MSDSE
sgris(1)  .......... .......... .......... .......... ..........

                    β βββ ββ  αα α αα ααααα ααα  α loop   βββ  β ββ Secondary structure
                    pssssGGGxG Gsxxsxxsxx xx..   xxsx  ssa FAD-binding Motif
                          A  A                            S
          101                                                       150
  arabep  PPISIGDGAL DHVVIGCGPA ....GLALAA ESAKLGLKVG LIG...PDLP
  escuep  RQISAGQTVL DLVVIGCGPA ....GLALAA ESAKLGLNVG LVG...PDLP
     cap  KGV.....VV DLAVVGGGPA ....GLAVAQ QVSEAGLSVC SIDP.NPKLI
    escu  KGV.....VV DLAVVGGGPA ....GLAVAQ QVSEAGLSVC SIDP.NPKLI
    arab  KSQ.....VV DLAIVGGGPA ....GLAVAQ QVSEAGLSVC SIDP.SPKLI
    narc  KAL.....TL DLAVVGGGPL ....ARSCST SLG.GGLSVV SIDP.NPKLI
    syne  .........MF DALVIGSGPA ....GLAIAA ELAQRGLKVQ GLSPVDPFHP
agrocrty  .......MTH DVLLAGAGLA NGLIALALRA ARPD..LRVL LLDHAAGP..
   flavo  .......MSH DLLIAGAGLS GALIALAVRD RRPD..ARIV MLDARSGP..
eho10(1)  .....MR... DLILVGGGLA NGLIAWRLRQ RYPQ..LNLL LIEAGEQP..
eho10(2)  .....MR... DLILVGGGLA NGLIAWRLRQ RYPQ..LNLL LIEAGEQP..
   eho13  .....MRPHY DLILVGAGLA NGLIALRLQQ QQPD..MRIL LIEAAPQA..
    ured  .....MQPHY DLILVGAGLA NGLIALRLQQ QQPD..MRIL LIDAAPQA..
  longus  IDSVPNDDSC DCAIVGGGLA GGLIALALQR ARPE..FRIR VIEAGRTI..
sgris(1)  .....MPTDF DVVIVGAGAA GMSLAYHLCA PDSDVPLSVA LVDAPPGPLR

          151                                                       200
  arabep  FTNN.YGVWE DEFNDLG... LQKCIEHVWR ETIVYLDDDK PITIGRAYGR
  escuep  FTNN.YGVWE DEFKDLG... LQACIEHVWR DTIVYLDDDE PILIGRAYGR
     cap  WPNN.YGVWV DEFEAMD... LLDCLDATWS GAAVYIDDKT TKDLNRPYGR
    escu  WPNN.YGVWV DEFEAMD... LLDCLDATWS GAAVYIDDNT AKDLHRPYGR
    arab  WPNN.YGVWV DEFEAMD... LLDCLDTTWS GAVVYVDEGV KKDLSRPYGR
    narc  WPNN.YGVWV DEFEDMD... LLDCLDATWS GAIVYVDDRS TKNLSRPYAR
    syne  WENT.YGIWG PELDSLG... LEHLFGHRWS NCVSYFGEAP VQHQYN.YGL
agrocrty  SDGHTWSCHD PDLSPDWLAR LKPLRRANW. PDQEVRFPRH ARRLATGYGS
   flavo  SDQHTWSCHD TDLSPEWLAR LSPIRRGEW. TDQEVAFPDH SRRLTTGYGS
eho10(1)  GGNHTWSFHE DDLTPGQHAW LAPLVAHAW. PGYEVQFPDL RRRLARGYYS
eho10(2)  GGNHTWSFHE DDLTPGQHAW LAPLVAHAW. PGYEVQFPDL RRRLARGYYS
   eho13  GGNHTWSFHD ADLTESQHRW VAPLVVYHW. PDYQVRFPTR RRKLNSGYFS
    ured  GGNHTWSFHH DDLTESQHRW IAPLVVHHW. PDYQVRFPTR RRKLNSGYFC
  longus  GGNHRWSWFD SDLSDAGRAL LADFRQTDWE GGYEVRFPKY RRKLKTAYRS
sgris(1)  APPRTWCFWE PPGGPYDPVL AASWPRLRVR AADGASTVAQ LPRLR..YKM
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          201                                                      250
  arabep  VSRRLLHEEL LRRCVESGVS YLSSKVDSIT EASDGLRLVA CDDNNVIPCR
  escuep  VSRHFLHEEL LKRCVEAGVL YLNSKVDRIV EATNGQSLVE CEGDVVIPCR
     cap  VNRKQLKSKM MQKCILNGVK FHQAKVIKVI HE.ESKSMLI CNDGITIQAT
    escu  VNRKQLKSKM MQKCIMNGVK FHQAKVIKVI HE.ESKSMLI CNDGITIQAT
    arab  VNRKQLKSKM LQKCITNGVK FHQSKVTNVV HE.EANSTVV CSDGVKIQAS
    narc  VNRKNLKSKM MKKCVSNGVR FHQATVVKAM HE.EEKSYLI CSDGVTIDAR
    syne  FDRAQLQQHW LRQCEQGGLQ WQLGKAAAIA HD.SHHSCVT TAAGQELQAR
agrocrty  LDGAALADAV VRS...GAE. IRWDSDIALL DAQ.....GA TLSCGT....
   flavo  IEAGALIGLL ..Q...GVD. LRWNTHVATL DDT.....GA TLTDGS....
eho10(1)  ITSERFAEAL HQAL..GEN. IWLNCSVSEV LPN.....SV RLANGE....
eho10(2)  ITSERFAEAL HQAL..GEN. IWLNCSVSEV LPN.....SV RLANGE....
   eho13  VTSQRFAEVL QQKF..GQH. LWISRAVAEV HAD.....AV RLNNGQ....
    ured  ITSQRFAEVL QRQF..GPH .LWMDTAVAEV NAE.....SV RLKKGQ....
  longus  MASTDFHEGL LRAL..PEGS VILGRKAVGL DAR.....GV DLAPSQYGPA
sgris(1)  LRSDAFEALV EQRFSRAPDL CRMEATASSV RDDPSGVGGE VLTRTACGER

          251                                                      300
  arabep  LATVASGAAS GKLLQYEVGG PRVCVQTAYG VEVEVENSPY DPDQMVFMDY
  escuep  FVTVASGAAS GKFLQYELGS PRVSVQTAYG VEVEVDNNPF DPSLMVFMDY
     cap  VVLDATGFSR S.LVQYDKPY NPGY.QVAYG ILAEVEEHPF DVNKMVFMDW
    escu  VVLDATGFSR S.LVQYDKPY NPGY.QVAYG ILAEVEEHPF DVNKMVFMDW
    arab  VVLDATGFSR C.LVQYDKPY NPGY.QVAYG IIAEVDGHPF DVDKMVFMDW
    narc  VVLDATGFSR C.LVQYDKPY NPGY.QVAYG ILAEVEEHPF DVDKMVFMDW
    syne  LVVDTTGHQA A.FIQRPHSD AIAY.QAAYG IIGQFSQPPI EPHQFVLMDY
agrocrty  .RIEAGAVLD GR.GAQPSRH LTVGFQKFVG VEIETDRP.H GVPRPMIMDA
   flavo  .RIEAACVID AR.GAVETPH LTVGFQKFVG VEIETDAP.H GVERPMIMDA
eho10(1)  .ALLAGAVID GR.GVTASSA MQTGYQLFLG QQWRLTQP.H GLTVPILMDA
eho10(2)  .ALLAGAVID GR.GVTASSA MQTGYQLFLG QQWRLTQP.H GLTVPILMDA
   eho13  .VISASAVID GR.GYTPNSA LNVGFQAFIG QEWRLSKP.H GLSSPIIMDA
    ured  .VIGARAVID GR.GYAANSA LSVGFQAFIG QEWRLSHP.H GLSSPIIMDA
  longus  TRINARSVID CR.SFKPSAH LKGGWQVFLG RHMRLQEP.H GVENPVIMDA
sgris(1)  ILVRGRLVFD SRPSHRLPPA RTTLLQHFTG WFVRTERPVF DPGTADLMDF

          (cunningham et al., 1994) motif1
301                                                                350
  arabep  RD...YTNEK VRSLEAEYPT FLYAMPMTKS RLFFEETCLA SKDVMPFDLL
  escuep  RD...YLRHD AQSLEAKYPT FLYAMPMSPT RVFFEETCLA SKDAMPFDLL
     cap  RDSHLKNNVE LKERNSRIPT FLYAMPFSSN RIFLEETSLV ARPGLGMDDI
    escu  RDSHLKNNTD LKERNSRIPT FLYAMPFSSN RIFLEETSLV ARPGLRIDDI
    arab  RDKHLDSYPE LKERNSKIPT FLYAMPFSSN RIFLEETSLV ARPGLRMEDI
    narc  RDSHLNGKAE LNERNAKIPT FLYAMPFSSN RIFLEETSLV ARPGLKMEDI
    syne  RSDHLSP... ..EERQLPPT FLYAMDLGND VYFVEETSLA ACPAIPYDRL
agrocrty  TVTQQ..... .DGYR..... FIYLLPFSPT RILIEDTRYS DGGDLDDDAL
   flavo  TVPQM..... .DGYR..... FIYLLPFSPT RILIEDTRYS DGGDLDDGAL
eho10(1)  TVAQQ..... .QGYR..... FVYTLPLSAD TLLIEDTRYA NVPQRDDNAL
eho10(2)  TVAQQ..... .QGYR..... FVYTLPLSAD TLLIEDTRYA NVPQRDDNAL
   eho13  TVDQQ..... .NGYR..... FVYSLPLSAT ELLIEDTHYI DNATLEPERA
    ured  TVDQQ..... .NGYR..... FVYSLPLSPT RLLIEDTHYI DNATLDPECA
  longus  TVDQLAPHGN GGSYR..... FVYVLPLGSH DVFIEDTYYA DDPLLDRNAL
sgris(1)  RTPQPA.... .RGLS..... FGYVLPLDPH TALVEYTEFS PAP.LDTDGY
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            351                                                     400
  arabep    KTKLMLRLDT LGIRIL.KTY EEEWSYIPVG GSLPNTEQK. ......NLAF
  escuep    KKKLMLRLNT LGVRIK.EIY EEEWSYIPVG GSLPNTEQK. ......TLAF
     cap    QERMVARLSH LGIKVK.SIE EDEHCVIPMG GPLPVLPQR. ......VVGI
    escu    QERMVARLNH LGIKVK.SIE EDEHCLIPMG GPLPVLPQR. ......VVGI
    arab    QERMAARLKH LGINVK.RIE EDERCVIPMG GPLPVLPQR. ......VVGI
    narc    QERMVARLNH LGIRIK.SIE EDERCVIPMG GPLPVIPQR. ......VVGI
    syne    KQRLYQRLAT RGVTVQ.VIQ HEEYCLFPMN LPLPDLTQS. ......VVGF
agrocrty    AAASHDYARQ QGWTGA.EV. RRERGILPIA LAHDAAGFWA DHAAGPVPV.
   flavo    AQASLDYAAR RGWTGQ.EM. RRERGILPIA LAHDAIGFWR DHAQGAVPV.
eho10(1)    RQTVTDYAHS KGWQLA.QLE REETGCLPIT LAGDIQALWA DAPGVPRS..
eho10(2)    RQTVTDYAHS KGWQLA.QLE REETGCLPIT LAGDIQALWA DAPGVPRS..
   eho13    RQNIRDYAAQ QDWQLQ.TLL REEQGALPIT LTGDSTAFWQ QQP.LACS..
    ured    RQNICDYAAQ QGWQLQ.TLL REEQGALPIT LSGNADAFWQ QRP.LACS..
  longus    SGRIDQYARA NGWENG.TPV HHEAGVLPVL TGGDFSAYQD EVRIPGVAIA
sgris(1)    RRALRHYTHD VLRLGPLQVT AQEHGVIPMT .....DGRFP HKAGRSVYRI

                    motif2          Predicted transmembrane helix
                      (Cunningham et al., 1994)
            401                                                     450
  arabep    GA..AASMVH PATGYSVVRS LSEAPKYASV IAEILREETT KQI.....NS
  escuep    GA..AASMVH PATGYSVVRS LSEAPKCASV LANILRQHYS KNMLTSSSIP
     cap    GG..TAGMVH PSTGYMVART LAAAPVVANA IIQYLSSERS H....S..GD
    escu    GG..TAGMVH PSTGYMVART LAAAPVVANA IIQYLGSERS H....S..GN
    arab    GG..TAGMVH PSTGYMVART LAAAPIVANA IVRYLGSPSS N....SLRGD
    narc    GG..TAGMVH PSTGYMVART LAAAPIVANS IVQYLVSDSG L....S..GN
    syne    GG..AASMVH PASGYMVGAL LRRAPDLANA IAAGLNA..S S....SLTTA
agrocrty    GL..RAGFFH PVTGY....S LPYAAQVADV VAGLSGPPGT DALRGAIRDY
   flavo    GL..GAGLFH PVTGY....S LPYAAQVADA IAA..RDLTT ASARRAVRGW
eho10(1)    GM..RAGLFH PTTGY....S LPLAVALADA IADSPR.LGS VPLYQLTRQF
eho10(2)    GM..RAGLFH PTTGY....S LPLAVALADA IADSPR.LGS VPLYQLTRQF
   eho13    GL..RAGLFH PTTGY....S LPLAVALADR LSALDV.FTS SSIHQAITHF
    ured    GL..RAGLFH PTTGY....S LPLAVAVADR LSALDV.FTS ASIHHAITHF
  longus    GA..RGGFTH PLTSY....T MCVAVENALA MAE.QPDLSG EQLAAFFDSR
sgris(1)    GT..AGGATR PSTGY....T FAAVQRQSRA VADQLRSGRP LRVPAPYGRR

            451                                                     500
  arabep    NISRQAWDTL WPPERKRQRA FFLFGLALIV QFDTEGIRSF FRTFFRLPKW
  escuep    SISTQAWNTL WPQERKRQRS FFLFGLALIL QLDIEGIRSF FRAFFRVPKW
     cap    ELSAAVWKDL WPIERRRQRE FFCFGMDILL KLDLPATRRF FDAFFDLEPR
    escu    ELSTAVWKDL WPIERRRQRE FFCFGMDILL KLDLPATRRF FDAFFDLEPR
    arab    QLSAEVWRDL WPIERRRQRE FFCFGMDILL KLDLDATRRF FDAFFDLQPH
    narc    DLSADVWKDL WPIERRRQRE FFCFGMDILL KLDLEGTRRF FDAFFDLEPR
    syne    ELATQAWRGL WPTEKIRKHY IYQFGLEKLM RFSEAQLNHH FQTFFGLPKE
agrocrty    AIDRARRDRF LRLLN..... .....RMLFR GCAPDRRYTL LQRFYRMPHG
   flavo    AIDRADRDRF LRLLN..... .....RMLFR GCPPDRRYRL LQRFYRLPQP
eho10(1)    AERHWRRQGF FRLLN..... .....RMLFL AGREENRWRV MQRFYGLPEP
eho10(2)    AERHWRRQGF FRLLN..... .....RMLFL AGREENRWRV MQRFYGLPEP
   eho13    AHERWQQQRF FRMLN..... .....RMLFL AGPADSRWRV MQRFYGLPED
    ured    ARERWQQQGF FRMLN..... .....RMLFL AGPADSRWRV MQRFYGLPED
  longus    ARRHWSKTGY YRLLA..... .....RFLFF AAKPEKRVKV FQRFYGLREG
sgris(1)    A..RLMDAVL LRALDSGRVD GADFFHRLFR HIPGERLLSF MDGRSQLHED
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          501                                                  550
  arabep  MWQGFLGSTL TSGDLVLFAL YMFVISPNNL RKGLINHLIS DPTGATMIKT
  escuep  MWQGFLGSSL SSADLMLFAF YMFIIAPNDM RKGLIRHLLS DPTGATLIRT
     cap  YWHGFLSSRL FLPELIVFGL SLFSHASNTS RLEIMTK..G TLPLVHMINN
    escu  YWHGFLSSRL FLPELIVFGL SLFSHASNTS RFEIMTK..G TVPLVNMINN
    arab  YWHGFLSSRL FLPELLVFGL SLFSHASNTS RLEIMTK..G TVPLAKMINN
    narc  YWHGFLSSRL FLPELVPFGL SLFSHASNTC KLEIMAK..G TLPLVNMINN
    syne  QWYGFLTNTL SLPELIQAML RLFAQAPNDV RWGLMEQ..Q GREL.QLFWQ
agrocrty  LIERFYAGRL SVAD.....Q LRIVTGKPPI PLGTAIRC.L PERPLLKENA
   flavo  LIERFYAGRL TLAD.....R LRIVTGRPPI PLSQAVRC.L PERPLLQERA
eho10(1)  TVERFYAGRL SLFD.....K ARILTGKPPV PLGEAWRAAL NHFPDRRDKG
eho10(2)  TVERFYAGRL SLFD.....K ARILTGKPPV PLGEAWRAAL NHFPDRRDKG
   eho13  LISRFYAGKL TLTD.....R LRILSGKPPV PVLAALQAIM TTHR......
    ured  LIARFYAGKL TLTD.....R LRILSGKPPV PVLAALQAIM TTHR......
  longus  LIERFYAARS NTFD.....K VRVLWGEPPV AIHSAILAMF KSGPALKSEK
sgris(1)  LLIGLRTPMV PMLRTVFELP FRTRRARPAA PFPPHRPPPK GAPHDPVAR.

          551               570
  arabep  YLKV..... ..........
  escuep  YLTF..... ..........
     cap  LLQDKE.... ..........
    escu  LLQDKE.... ..........
    arab  LVQDRD.... ..........
    narc  LVQDRD.... ..........
    syne  AIAAR..... ..........
agrocrty  .......... ..........
   flavo  .......... ..........
eho10(1)  .......... ..........
eho10(2)  .......... ..........
   eho13  .......... ..........
    ured  .......... ..........
  longus  SDRGVAQAAL DEELQTEKRP
sgris(1)  .......... ..........
```

Aligned deduced amino acid sequences from the lycopene cyclase genes of plants, cyanobacteria and bacteria. The dinucleotide binding motif is shown (Cunningham *et al.*, 1994; Van Beeuman *et al.*, 1991). The key to symbols is as follows:

p=polar or charged D,E,K,R,H,S,T,Q,N	x=any AA
s=small and hydrophobic A,I,L,V,M,C	a=acidic D or E

Abbreviations are as follows: arabep *Arababidopsis thaliana* epsilon cyclase (Cunningham *et al.*, 1996), escuep *Lycopersicon esculentum* epsilon cyclase (Hirschberg 1997), cap *Capsicum annuum* (Hugueney *et al.*, 1995), escu *Lycopersicon esculentum* (Cunningham *et al.*, 1996), arab *Arababidopsis thaliana* (Scolnik and Bartley, 1995), narc *Narcissus pseudonarcissus* (Al-Babili *et al.*, 1996), syne *Synechococcus sp.* (Cunningham *et al.*, 1994), agrocrty *Agrobacterium aurantiacum* (Misawa *et al.*, 1995b), flavo *Flavobacterium* ATCC21588 (Pasamontes *et al.*, 1997), eho10 (1) *Erwinia herbicola* Eho10 (Hundle *et al.*, 1994), eho10 (2) *Erwinia herbicola* Eho10 (Hundle *et al.*, 1994), eho13 *Erwinia herbicola* Eho13 (To *et al.*, 1994), ured *Erwinia uredovora* (Misawa *et al.*, 1990), longus *Erythrobacter longus* (Matsumara *et al.*, 1997), sgris(1) *Streptomyces griseus* (Schumann *et al.*, 1996).