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ISOLATION AND PARTIAL CHARACTERIZATION OF CAROTENOID MUTANTS OF *CORYNEBACTERIUM POINSETTIAE* ATCC 9682

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ABSTRACT

Carotenoid pigments were extracted from wild-type *Corynebacterium poinsettiae* ATCC 9682 and from mutants obtained by exposure of a streptomycin resistant strain to ultra violet (UV) irradiation or N-methyl-N′-nitro-N-nitrosoguanidine. Three major pigments isolated by high performance liquid chromatography (HPLC) were characterized by their absorption maxima, partition ratios in light petroleum ether/95 per cent methanol and nuclear magnetic resonance (NMR). High performance liquid chromatography was used to compare pigments of the wild-type with those of the mutants. Mutants were found which may serve as potential candidates for the study and in depth understanding of carotenoid biosynthetic pathway(s) in this bacterium and related species at the molecular level.

KEYWORDS: *Corynebacterium poinsettiae*, Carotenoid mutants, isolation, characterization, biosynthesis.

INTRODUCTION

Studies on the biosynthesis of carotenoids, mainly in plant systems, indicate that there is a general pathway for the formation of the primary C_{40} unit, phytoene, from low molecular weight precursors (Liaaen-Jensen and Andrewes, 1972; and Goodwin, 1980). Most of the over 600 different carotenoids that are known presently consist of 40 carbon atoms (Goodwin, 1980). The enzymatic reactions leading to their formation are well understood in plants (Sandmann, 2001) and bacteria (Armstrong, 1997). The subsequent desaturation of phytoene to lycopene which is the branch point from the typical C_{40} pathway to the elongated carotenoids appear to be of a general nature (Krubasik *et al*. 2001). Very little is known about the details of the subsequent reactions including the insertion of oxygen functions and appear to be specific for different groups of organisms. The biosynthesis of carotenoids in non-photosynthetic bacterial involves the formation of carotenoids with 45 and 50 carbon atoms as well as the C_{40} compounds found in other systems (Goodwin, 1980). The first C_{50} carotenoid decaprenoxanthin, was discovered in *Flavobacterium dehydrogenans* (Weeks *et al*, 1969) and also was later found in a few other bacteria (Goodwin, 1980; and Miki *et al*; 1994). They all carry an additional isopentenyl residue at the C-2 and C-2′ positions and can be acyclic, monocyclic or bicyclic with either two alpha rings or two beta rings (Goodwin, 1980). The biosynthetic pathways of the C_{45} and C_{50} carotenoids follow similar but not identical routes in all organisms thus far examined. Several carotenogenic gene clusters have been Isolated (Krügel *et al*; 1999; Krubasik and Sandmann, 2000; and Viveiros *et al*. 2000). However, all the genes found to date encode only enzymes that synthesize C_{40} carotenoids. A number of pigment mutants have been obtained by transposon mutagenesis from *F. dehydrogenans* and *Corynebacterium glutamicum* (Vertes *et al*; 1994). The transposon mutants of *C. glutamicum* have been used to clone the carotenogenic genes of the C_{50} biosynthetic pathway in order to understand more about carotenoid metabolism (Krubasik *et al*; 2001).

Corynebacterium poinsettiae, a phytopathogen causing wilts in the leaves of poinsettia plant, was the first non-photosynthetic bacterium in which carotenoid was shown to protect cells from photodynamic killing (Kunisawa and Stanier, 1958). It is now well known that carotenoid pigment not only protect cells against photodynamic killing but also serve as antioxidants and membrane stabilizers (Mathews and Sistrom, 1959; Mathews, 1963; Dieringer *et al*. 1977; Inlag and Linn 1988; Krubasik *et al*, 2001). A pathway for the biosynthesis of a bicyclic C_{50}-diol from lycopene in *C. poinsettiae* based on the structures of the six major pigments, has been proposed by Norgard *et al*. (1970) but very little is known about the details of the reactions. We are not aware of any studies to identify the carotenogenic genes or clone pigment mutants of this bacterium. Therefore, the purpose of this investigation is to report on the isolation and partial characterization of pigment mutants of *C. poinsettiae* ATCC 9682 which differ from the wild type strain in carotenoid composition. It is hoped that mutants so obtained may assist in further investigations of the biosynthetic pathway in this bacterium at the molecular level.

MATERIALS AND METHODS

Organism: *Corynebacterium poinsettiae* ATCC 9682 used in this study was obtained from the American Type Culture Collection.

Media: Cultures were maintained on tryptic soy agar (TSA) (Difco Laboratories, Detroit, Michigan) slants and were subcultured every 3 weeks. Tryptic soy broth (TSB) (Difco) was used for growing cells in most experiments. The thiamine deficient medium of Starr and Saperstein (1953), in which cells produce two major pigments C.p. 450 and C.p. 496 was used to confirm the identity of these pigments in our strain.

Chemicals: Mutants, termed chemical mutants (CM) were induced with N′-methyl-N□-nitrosoguanidine (NTG)(Sigma). Reagent-grade methanol was used for extraction of pigments. Sodium chloride (NaCl reagent grade), was used occasionally

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to facilitate transfer of the pigments to the organic phase after saponification of extracted pigments with 5% aqueous KOH. Light petroleum ether (b.p. 36-57°C) was used for partitioning pigment saponified in methanol.

Culture .Conditions: Seed flasks of TSB were inoculated from stock slants and incubated under fluorescent light (0.2 mJs^{-1} cm^{-2} from four 20W lights 80cm above the flasks) on a rotary shaker at 30°C for 15h. After purity checks, the optical density of the cultures were read at 580nm with a spectrophotometer (Bausch and Lomb digital double-beam spectronic 210 UV) and adjusted to 0.3 with sterile distilled water when necessary. A 1% (V/V) inoculum was used to inoculate the test flasks. The flasks were incubated as described above and harvested by centrifugation at 7710g for 10 min. The cells were washed three times with distilled water before extraction.

Isolation of a Streptomycin Resistant (Str^R) Strain: To diminish the chances of isolating pigmented contaminants when pigment mutants were sought, and to establish a genetically distinct strain, a Str^R mutant of the wild type was isolated. The wild-type was sensitive to 25µg ml^{-1} streptomycin sulphate. A Str^R mutant was obtained by streaking a 48h culture which, in the presence of 50µg streptomycin ml^{-1}, did not grow in 24h but did grow in 48h.

Isolation of Pigment Mutants: Pigment mutants of the Str^R *C. poinsettiae* were obtained by treating of 15-24h cultures with ultraviolet light irradiation and by treatment with NTG. Using a UV-light (Model 420-U1 George W. Gates & Co.), intensity 186 x 10 3µJcm^{-2} positioned 30cm above 20ml constantly mixed culture in a 15 x 60mm glass petri dish, the exposure time for 99% kill was 6 min. To isolate pigment mutants, cells were exposed for 6 min., serially diluted and plated out under red light on TSA containing 50µg streptomycin ml^{-1} and incubated in the dark at 30°C for 24h to prevent photoreactivation. After 24h, the plates were counted and then exposed to continuous fluorescent light for 3-5 days and pigment mutants determined by visual inspection. The spread plate technique was used for counting. For NTG mutagenesis, 20 mg NTG was dissolved in 5ml sterile TSB and twofold serial dilutions from 2^{-1} to 2^{-10} were made in 5ml TSB at pH 6.5. One drop of an overnight culture of the Str^R strain of *C. poinsettiae* was pipetted aseptically into each tube. The tubes were incubated at 20°C for 24h. Ten-fold serial dilutions of the 2^{-4}, 2^{-5} and 2^{-6} dilutions which showed some growth after 24h of incubation were made in sterile water, and 0.1ml volumes of the 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated out in quintuplicate using the spread plate technique. The plates were incubated in the light at 30°C for 48h. Mutants detected after the UV or NTG treatment were picked aseptically and purified by repeated streaking on TSA containing 50µg streptomycin ml^{-1} until only one colony type was observed.

Extraction of Pigments: Pigment from cells from 1 litre culture was extracted repeatedly with methanol (5ml portions) until the cells became colourless. The extracted pigment in the pooled methanol extracts was saponified by adding a volume of 10% aqueous KOH equal to the volume of pigment extract in methanol to make a final concentration of 5% KOH. The mixture was left to stand for 1h. The pigment was then extracted from the mixture with light petroleum ether (b.p. 36-57°C). Occasionally, it was necessary to add a small amount of NaCl to ensure that all the pigments were transferred to the organic phase. The light petroleum ether extract was washed five times with 5-10 times its volume of water and dried over anhydrous sodium sulphate. The light petroleum ether was then evaporated to dryness under reduced pressure and the pigments were dissolved in a volume of hexane to give a final pigment concentration of approximately 300µg ml^{-1} (assuming an absorption coefficient A = 2500).

High Performance Liquid Chromatograph (HPLC): Pigment mixtures in hexane were resolved on a micro porasil column with a Waters ALC 200 series liquid chromatograph with a model H50 variable wavelength detector. An isocratic elution solvent mixture of hexane/acetone/methylene chloride (11.35 : 1.73 : 1.00 by vol.) was used (Kester & Thompson, 1984). Operating conditions were: chart paper speed, 2.54cm min^{-1}; flow rate, 2ml min^{-1}; Sensitivity 0.04 absorbance units full scale; detection, maximum absorbance of sample in the visible range. Injection volume for analysis of crude pigment extract was 10-15µl of a sample containing approximately 0.3µg $µl^{-1}$. Two hundred to 300µl were injected when peaks were to be collected for further analysis. All solvents for HPLC were HPLC grade. Fractions collected for NMR analysis and visible absorption spectrum determinations were evaporated to dryness in a stream of nitrogen and redissolved in HPLC grade acetone.

Partition Coefficients: Partition coefficients were determined by measuring the amount of pigment in each phase when partitioned between equal volumes of 95% aqueous methanol saturated with light petroleum ether and light petroleum ether saturated with 95% aqueous methanol.

NMR Sample Preparation: The solvent was removed in the dark under a stream of nitrogen. The dry residue was dissolved in 0.7ml of $CDCl_3$ (99.96 D, MSD isotopes) and transferred to a 55mm NMR tube).

NMR Parameters: NMR spectra were recorded in 5 mm NMR tubes in $CDCl_3$ ppm with a Varian VXR-300 NMR spectrometer at 25°C. Typical ^1H parameters were: 300 MHz spectrometer frequency; 2400-4000 Hz spectral width; 30,016 data points, zero-filled to 32K; 30-90° pulse width; no relaxation delay; and 1200-3000 acquisitions. Total experiment time was from 1.2 – 3.2h. All chemical shifts are relative to TMS based on the residual proton of $CDCl_3$ at 7.24 ppm. Typical ^{13}C parameters were: 75 MHz spectrometer frequency; 17,500 Hz spectral width; 30,016 data points, zero-filled to 32K; 65° pulse width; no relaxation delay; and 50,000 – 65,000 acquisitions. Total experiment time was from 12-15h. All chemical shifts are relative to TMS based on $CDCl_3$ at 77.00 ppm.

RESULTS

Twenty five microgram of streptomycin ml^{-1} was found to be the minimal lethal dose. Streptomycin resistant mutants were isolated by streaking TSA plates containing 50µg streptomycin ml^{-1} from those tubes in which growth appeared in 48h but not 24h.

The growth characteristics of the isolated Str^R strain indicated that the exponential growth phase at which cell were harvested occurred during the period of 9 to 15h after incubation. From the determination of the number of cells ml^{-1} of culture of the Str^R strain, it was determined that exposure of the str^R *C. poinsettiae* culture to UV-light irradiation for 360s resulted in a 99% kill.

Fifteen pigments were detected from *C. poinsettiae* (wt) by HPLC (Fig. 1). Three of the major pigment peaks were tentatively identified as C.p. 496, C.p. 473 and C.p. 450 based on absorption maxima and partition ratios (Table 1). Also

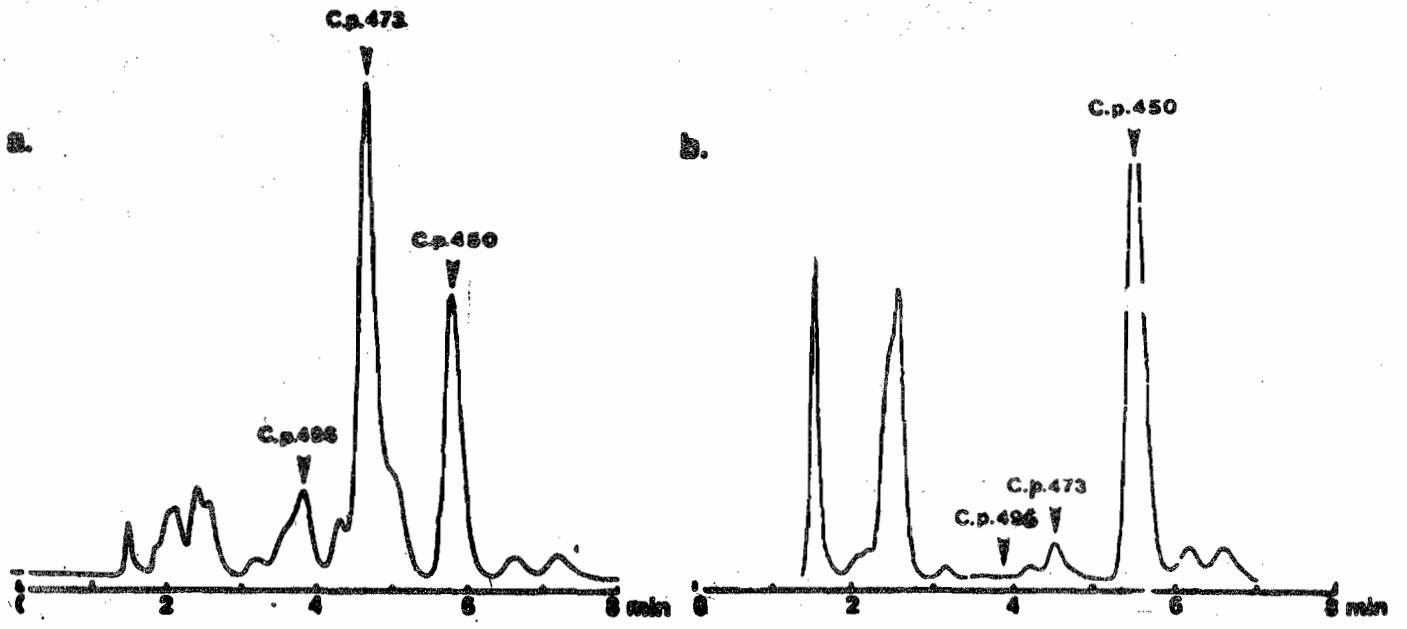

Fig. 1 ^{1}H-NMR and ^{13}C-NMR spectra of C.p. 496, C.p. 473, C.p. 450.

Table 1.

Identification of Major pigments of C. poinsettiae by Absorption maxima and partition ratios. Pigments were separated by HPLC and collected as described in METHODS. Light petroleum Saturated with methanol/water 95:5, v/v) and methanol/water (95:5, v/v) saturated with light petroleum was used for partitioning.

Pigment	Partition Ratio		Absorption Maxima (NM) in Acetone	
Assignment	[a]Observed	[b]Reported	[a]Observed	[b]Reported
C.p. 450	30 : 70	27 : 73	320, (426), 453, 480	(427), 464 481
C.p. 473	31 : 69	32 : 68	(366), 453, 476, 507	(365) 451, 478, 509
C.p. 496	34 : 66	40 : 60	326, 389, 470, 496, 529	(390), 471. 498, 533

a; Observations from this study.

b; Data reported by Norgard et al (1970).

included in Table 1 are parallel data reported by Norgard *et al.* (1970). Figures 2a and 2b represent the ^{1}H-NMR and ^{13}C-NMR spectra of C.p. 496 in 100% $CDCl_3$ respectively. Figure 2c represents the ^{1}H-NMR spectrum of C.p. 473 in 100% $CDCl_3$ and Figures 2d and 2e represent ^{1}H-NMR and ^{13}C-NMR spectra of C.p. 450 respectively. Table 2 represents the observed ^{13}C chemical shifts for pigment C.p. 450 and that reported by Britton et al. (1985). Table 3 indicates parallel data observed and that reported by Britton *et al.* (1985) of ^{13}C chemical shifts for pigment C.p. 496.

Mutants obtained by UV irradiation and NTG treatment were determined by visual observation of colour differences of colonies on agar plates after incubation for 48h under continuous light. Four phenotypic groups were isolated: colourless or white, yellow, red and orange.

DISCUSSION

Norgard *et al.* (1970), in their investigation of carotenoids produced by *C. poinsettiae* (wt) have assigned structures to the 6 major pigments and proposed a

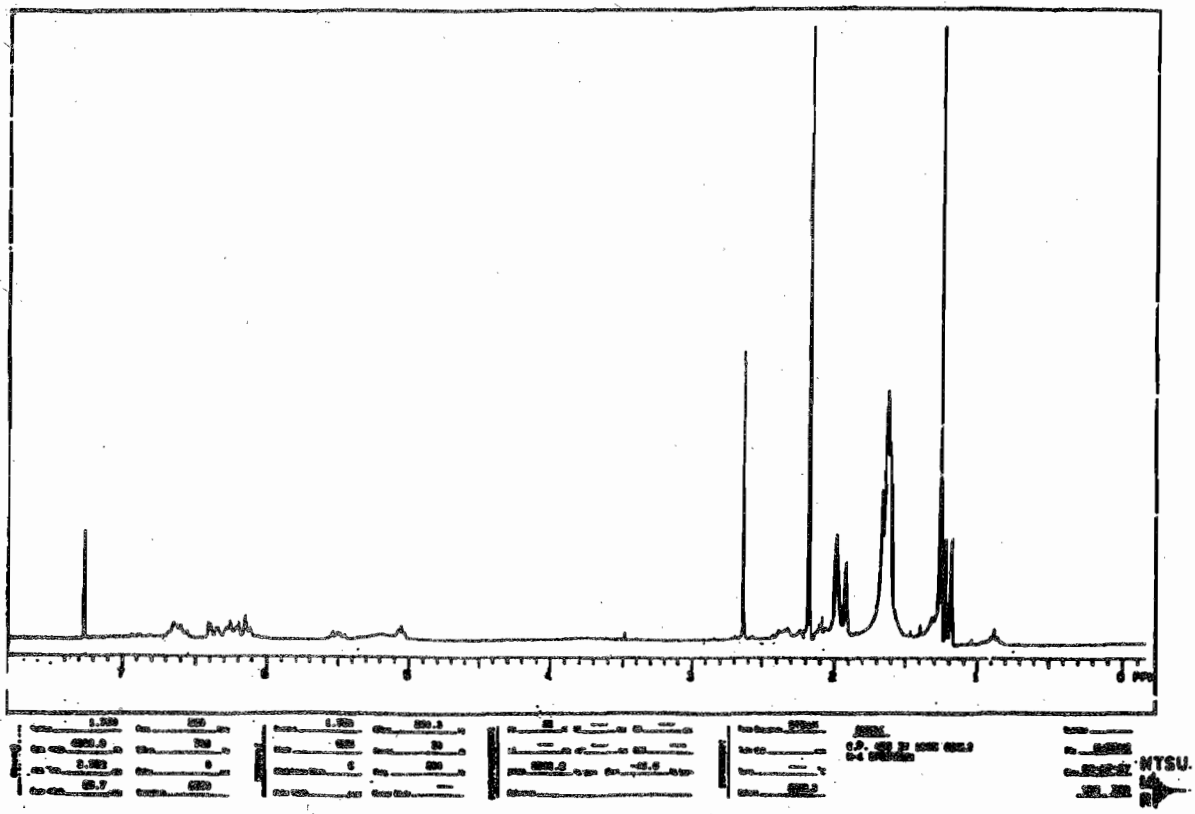

Fig. 2a ^{1}H-NMR spectrum of C.p. 496 in 100% $CDCl_3$.

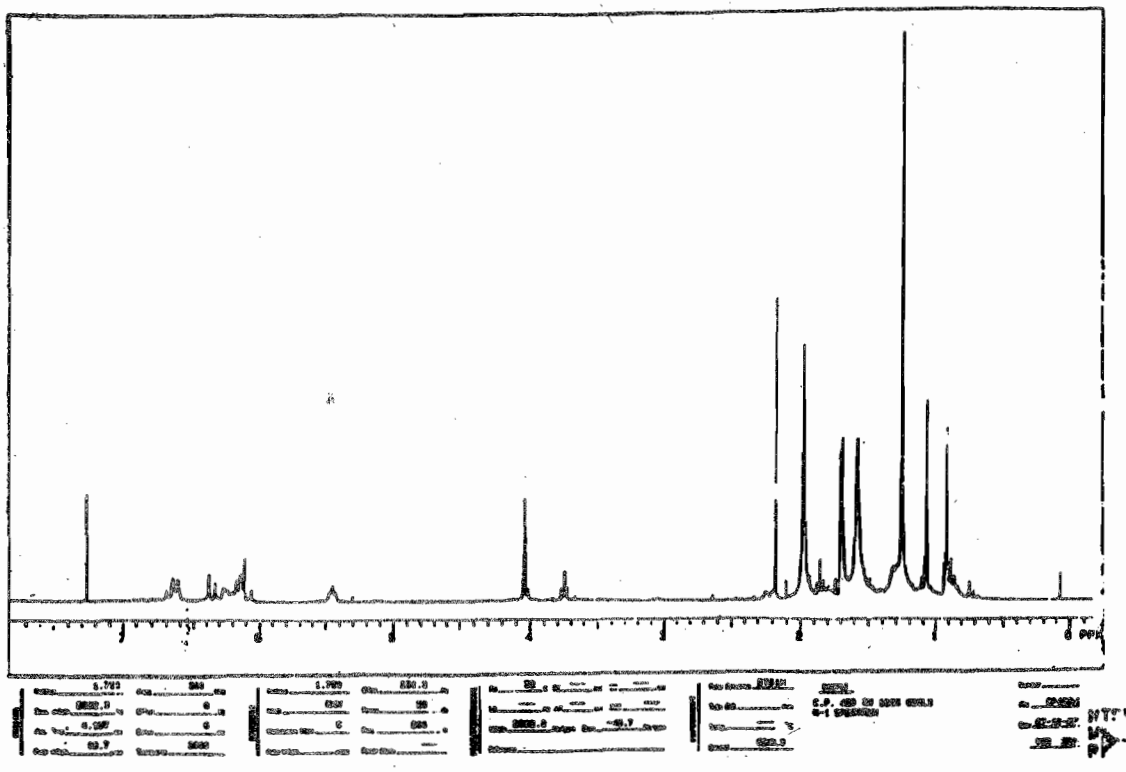

Fig. 2b ^{13}C-NMR spectrum of C.p. 496 in 100% $CDCl_3$

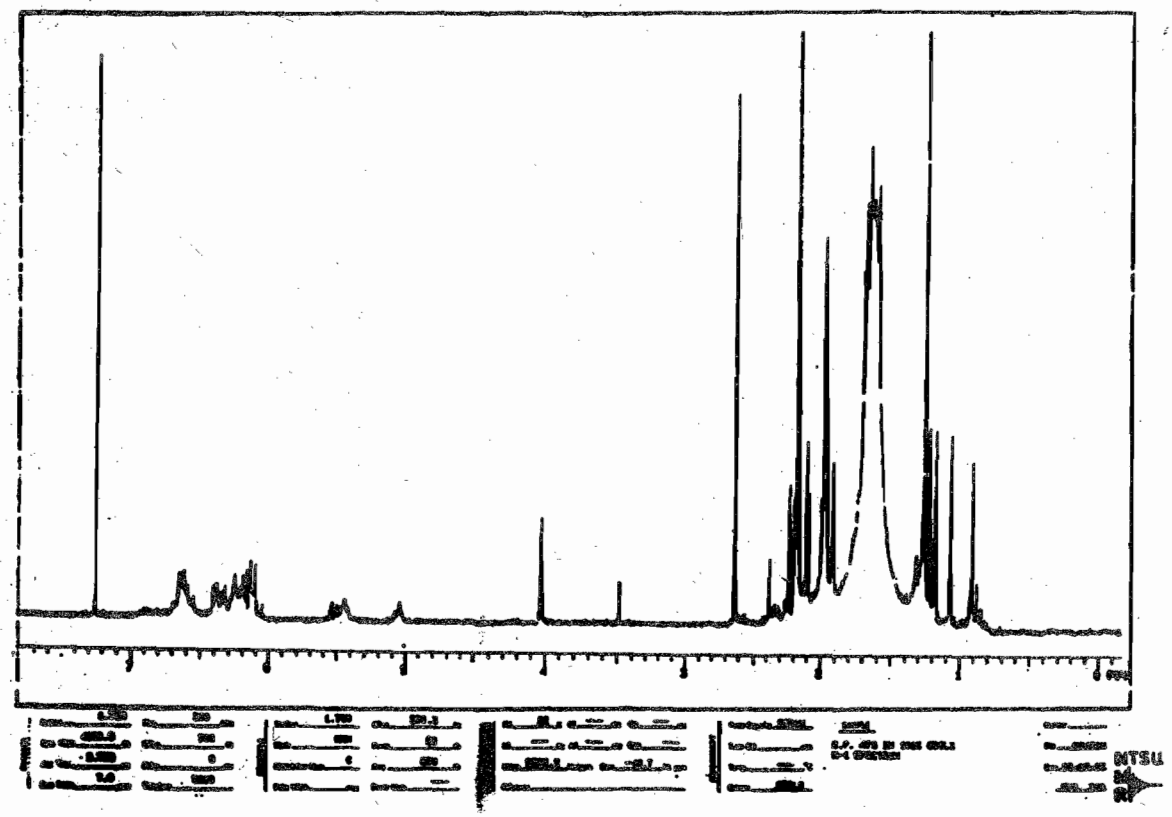

Fig 2c ^{1}H-NMR spectrum of C.p. 473 in 100% $CDCl_3$

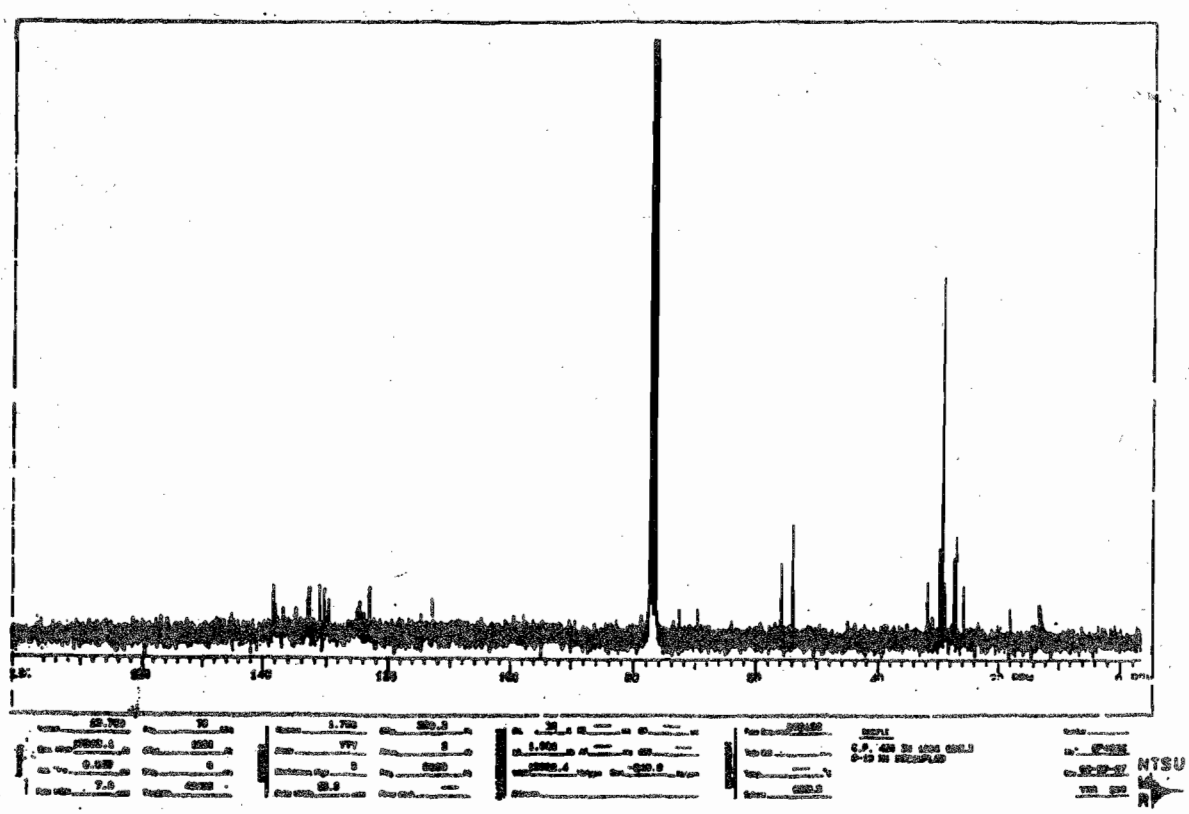

Fig 2d ^{1}H-NMR spectrum of C.p. 450 in 100% $CDCl_3$

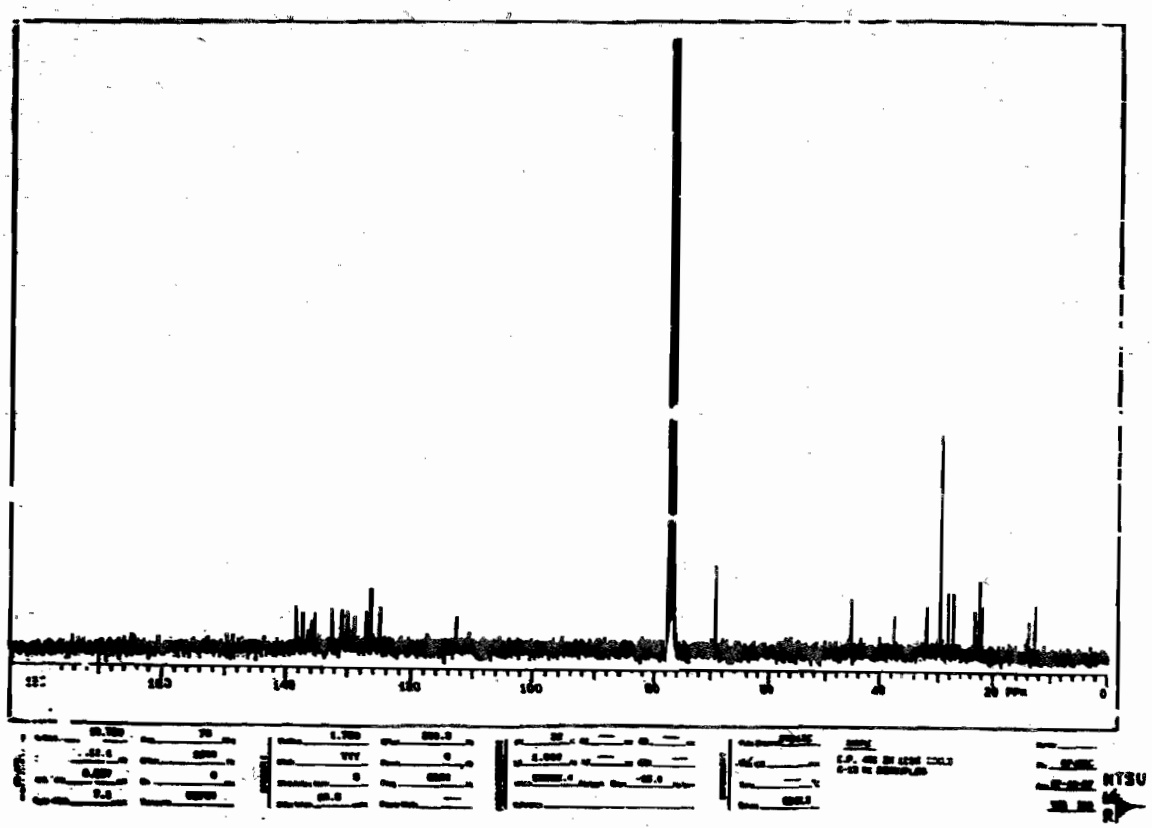

Fig 2e ^{13}C-NMR spectrum of C.p. 450 in 100% $CDCl_3$

Table 2: Observed ^{13}C Chemical Shifts for pigment C.p. 450 and That reported by Britton et al. (1985)

Carbon[a]	Observed Chemical shift[b]	Literature Value[c]
1	37.66 ppm	37.72 ppm
2	45.34	45.45
3	23.32	23.40
4	32.13	32.15
5	128.91	128.90
6	138.38	138.32
7	127.12	127.16
8	138.45	138.47
9	135.90	135.90
10	130.86	130.90
11	125.00	125.04
12	137.33	137.35
13	136.47	136.48
14	130.01	130.05
15	132.45	132.45
1"	28.32	28.36
2"	126.40	126.41
3"	135.32	135.35
4"	69.16	69.17
1-CH_3 a	22.47	22.53
1-CH_3 b	27.40	27.47
5-CH_3	21.92	21.89
9-CH_3	12.82	12.82
13-CH_3	12.82	12.82
3" -CH_3	13.88	13.87
Acetone	29.71	

[a] Numbering scheme as used by Britton (1985).

[b] Chemical shifts relative to TMS based on $CDCl_3$ at 77.00 ppm.

[c] From Britton (1985).

Table 3: **Observed ^{13}C chemical shifts for Pigment C.p. 496 and that reported by Britton et al. (1985)**

Carbon[a]	Observed Chemical Shift[b]	Literature Value[c]
1	72.76 ppm	72.72 ppm
2	55.70	55.68
3	129.48	129.44
4	138.04	138.04
5	138.08	138.44
6	131.19	131.17
7	124.52	124.46
8	137.86	137.85
9	134.81	135.30
10	138.19	138.20
11	125.09	125.01
12	138.19	138.20
13	136.68	136.67
14	130.30	130.29
15	132.80	132.80
1"	27.44	27.72
2"	122.97	122.94
3"	132.80	132.80
4" a	25.77	25.78
4" a	17.96	17.96
1-CH_3	27.01	26.95
5-CH_3	13.06	13.05
9-CH_3	12.90	12.89
13-CH_3	12.80	12.82
d	136.71	
"	133.07	
"	132.83	
"	129.64	
"	129.59	
"	124.82	
"	112.77	
"	69.55	
"	53.77	
"	31.74	
"	29.70	
Acetone	29.26	
d	28.83	

[a] Numbering scheme as used by Britton (1985)
[b] Chemical shifts relative to TMS based on $CDCl_3$ at 77.00 ppm.
[c] From Britton (1985)
d Unidentified peaks

biosynthetic pathway from lycopene, a C_{40} aliphatic to C.p. 450, a C_{50}-diol, bicyclic. In our study, 15 pigments were detected by HPLC (Fig. 1). Three of the major pigment peaks were tentatively identified as C.p. 496, C.p. 473 and C.p. 450 based on absorption maxima and partition ratio (Table 1). Presumably C.p. 482 and C.p. 470, because of their lower polarity are found in the earlier eluting pigments. In collecting the pigments from the HPLC column, C.p. 496 was collected after the shoulder was eluted. For C.p. 473, collection was stopped before the trailing shoulder was eluted. Slowing the rate to 1.3 ml min^{-1} permitted better, though not complete resolution of pigment C.p. 496 and C.p. 473 from contaminating pigments indicated by the shoulders in Figure 1a. Conclusive identification of C.p. 496, C.p. 473 and C.p. 450 was obtained by NMR spectroscopy. The ^{13}C NMR spectrum for the peak labelled C.p. 496, the only pink pigment produced in this organism (Norgard *et al.* 1970), contained 36 resolved resonances. Of those, 24 of the resonances fit well with the resonances reported earlier by Britton *et al.* (1985) for the acyclic half of C.p. 473. The ^{1}H spectrum is consistent with this assignment. There is a broad triplet at 5.05 ppm for the 2" vinylic proton, a doublet of doublets at 5.49 ppm for the vinylic protons at position 3, as well as singlets for each of the required methyl groups. As with the ^{13}C spectrum, there are additional ^{1}H resonances, but these remain unassigned. There are no additional resolved vinylic protons in the 4.5 – 5.5 range, although there is a very broad resonance centered at 5.2ppm. The 300 MHz ^{1}H spectrum was also consistent with the ^{1}H spectra reported earlier for C.p. 473 (Andrewes and Liaaen-Jensen, 1984 and Britton *et al.* 1985). There were two broad triplets of equal intensity at 5.44 and 5.05 ppm for the 2" and 2"' respectively, a singlet at 4.03 ppm for 4" methylene protons, as well as resonances of appropriate relative magnitude for all the methyl groups.

Pigment C.p. 450 was identified based on the 75 MHz ^{13}C NMR spectrum. The 25-line spectrum denoted a symmetrical C_{50} carotenoid. All resonances had the same chemical shift within 0.1 ppm, as those reported earlier for C.p. 450 by Britton *et al.* (1985). The only additional resonance was a large peak at 29.7 ppm, which was apparently due to residual acetone. The ^{1}H resonance in the 300 MHz spectrum were not all resolved, but were consistent with those reported by Britton *et al* (1985) at 400 MHz. In particular, there is a broad triplet at 5.44 ppm for the 2" protons, a singlet at 4.03

ppm for the 4" methylene protons, and singlets at the appropriate chemical shifts for each of the methyl groups. Although not apparent in the ^{13}C spectrum, there are also some additional smaller resonances apparently due to small amounts of an unidentified pigment.

HPLC analysis of 59 pigment mutants Kmbiacluster analysis using the "Fastclus" procedure of the Statistical Analysis System (SAS) (Sarle, 1982) revealed seven cluster groups. One cluster, group 2, exemplified by pigment mutant 16 (pm 16) deserves special mention because it appears to be a possible candidate for the study of carotenoid biosynthesis in this bacterium at the molecular level. The pigment of this cluster group when examined by HPLC, showed C.p. 496 to be absent, C.p. 473 at a greatly reduced level and C.p. 450 at an increased level (Fig. 1b). Assuming the pathway of Norgard *et al.* (1970) to be correct, this would mean that end products are present in the absence of presumed precursors. The possible suggestions from this finding are:

i) that the precursors are present at levels too low to detect by HPLC;
ii) the enzymatic conversion of the precursor is very fast compared to the reaction forming the precursor, so that no precursor accumulates, or
iii) a possible alternative pathway exists.

No methods have been developed to study enzyme kinetics in this system. However, more sensitive methods for pigment detection are available and these were used with pigment mutant 16 (pm 16). HPLC can detect less than 1µg in 10µl carotenoid pigment (Kester and Thompson, 1984) because the pigments have very high molar extinction coefficient when measured at their visible absorption maxima. The absence of C.p. 496 when this method of detection was used is a good evidence that C.p. 496 is not produced in this mutant group. Wild type *C. poinsettiae* is enriched in pigments C.p. 496 and C.p. 450 when grown in the low thiamine medium of Starr and Saperstein (1953). When pm 16 was grown in this medium, C.p. 496 was still undetectable by HPLC.

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