SAPERSTEIN, S., STARR, M. P. & FILFUS, J. A. (1954). *J. gen. Microbiol.* 10, 85-92.

Alterations in Carotenoid Synthesis accompanying Mutation in *Corynebacterium michiganense*

BY S. SAPERSTEIN, M. P. STARR AND JOYCE A. FILFUS

Department of Bacteriology, University of California, Davis, California, U.S.A.

SUMMARY: Colour mutants of the tomato pathogen, *Corynebacterium michiganense*, provided an opportunity to determine the nature of this micro-organism's pigments, and the effect of mutation and nutrition thereon. The major pigments of *C. michiganense* are carotenoids. The parent and mutant strains show distinct qualitative differences in pigment synthesis. The naturally occurring yellow parent type produces cryptoxanthin and lycopene. A pink mutant forms lycopene and spirilloxanthin. A red back-mutant produces only lycopene. An orange mutant synthesizes cryptoxanthin, β -carotene and canthaxanthin. Carotenoids were not detected in the colourless mutants.

Carotenoid synthesis in the yellow strain of *C. michiganense* is affected by thiamine in a manner similar to that previously described for *C. poinsettiae*: cryptoxanthin and lycopene are synthesized only at relatively high thiamine concentrations. The formation of lycopene, relative to spirilloxanthin, in the pink mutant is favoured by high thiamine concentrations. Similarly, the red strain forms appreciably more lycopene at higher thiamine concentrations than at lower. The formation of carotenoids in the orange strain appears not to be influenced by external thiamine concentration, possibly because this culture synthesizes an excess of the vitamin. No other nutritional factor which was examined caused detectable alterations in colour of the cells.

Colour variants of *Corynebacterium michiganense* (E. F. Smith) Jensen were described by the earliest students of this phytopathogenic coryneform organism. Bryan (1930, 1931) isolated 'spontaneously' occurring white and pink strains of this normally yellow organism, and similar colour variants were obtained by Ark (1946, 1951) from broth cultures to which had been added acenaphthene or uranium salts.

Nutrition is known to affect pigmentation of this species. The naturally occurring yellow type of *C. michiganense* was reported by Braun (1949) to produce either a cream or a yellow growth, depending upon whether the concentration of thiamine in the growth medium was low (0.1 $\mu\text{g./100 ml.}$) or high (1-100 $\mu\text{g./100 ml.}$), respectively. Braun noted that a white mutant of this organism produced no pigment, regardless of the thiamine concentration. In the related species, *C. poinsettiae* Starr & Pirone, thiamine was observed to affect pigmentation (Starr & Pirone, 1942; Starr, 1949); later a more intensive examination of this effect was made by the present writers (Starr & Saperstein, 1953). At low thiamine concentration (0.1 $\mu\text{g./100 ml.}$) the cells of *C. poinsettiae* are pink; whereas, at higher concentration (100 $\mu\text{g./100 ml.}$) the cells are orange-yellow; these colour changes have been related to qualitative and quantitative alterations in carotenoids.

There has been no study, before the one reported here, on the nature of the

individual pigments of *C. michiganense*; in fact, Braun (1949) does not mention whether they are carotenoids. Consequently, it is not known whether the pigments in the mutants are related to those of the parent strain. The present investigation was undertaken with the following objectives: (a) to characterize the major individual pigments of the naturally occurring yellow type and of several colour variants of *C. michiganense*; (b) to determine whether the colour shift accompanying the change in thiamine concentration is related to the situation in *C. poinsettiae*; (c) to uncover any other connexion between the pigments and the nutrition of individual strains; (d) to provide data bearing upon the mechanism of microbial carotenoid synthesis.

MATERIALS AND METHODS

Cultures. The cultures used throughout this study were authentic strains of *C. michiganense* obtained from Prof. P. A. Ark, University of California, Berkeley. Culture 4702 is a yellow strain isolated from tomato. Cultures 4944, 4938 and 4939—pink, orange, and colourless, respectively—are colour mutants isolated by Prof. Ark following treatment of a parent culture (4702) with uranium sulphate. Culture 4999 is a colourless mutant isolated by the same investigator following treatment of the same parent strain with 5-amino-acenaphthene. Culture 4999RR is a red variant, probably resulting from spontaneous reverse mutation, isolated by the present writers from the colourless culture 4999. All cultures were pathogenic for tomato at the start and conclusion of this investigation; in this connexion, we substantiated the findings of Fawcett & Bryan (1934) who found that pink variants usually are less virulent than either the yellow or the colourless strain.

Medium. For mass cultivation, a medium having the following composition per 100 ml. was used: peptone 1 g.; glucose 1 g.; pH adjusted to 6.8. For studies on the effect of thiamine on carotenoid production, a medium was used which had the following composition per 100 ml.: acid-hydrolysed casein (vitamin-free casamino acids, Difco) 0.5 g.; KH_2PO_4 , 0.5 g.; sodium citrate, 0.01 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g.; 'trace metals solution' (Starr, 1946) 1 ml.; biotin 0.1 μg .; nicotinic acid, 10 μg .; thiamine, 0.2–200 μg .; glucose (added separately after autoclaving) 1 g.; pH 6.8. The procedure for mass cultivation and harvesting of the cells was identical with that described in an earlier report (Starr & Saperstein, 1953).

Nutritional studies. The nutritive requirements of the cultures were determined by conventional means; the technical details were similar to those in previous studies of phytopathogenic micro-organisms (Starr, 1946, 1949). Cultures were incubated at 28° on a shaking machine to increase the cell yield. Estimations of turbidity were made with the Klett-Summerson photoelectric colorimeter using the 660 $m\mu$. filter.

Solvents. The solvents employed were as follows: methanol, light petroleum (35–60°), acetone, benzene (C. P., Baker), chloroform (Reagent, Merck), hexane (Standard mixed hexanes, Standard Oil Company of California) redistilled to yield the 62–65° fraction. Where necessary, solvents were purified by conventional methods.

Extraction and separation of the carotenoids. The cell mass was dried with 7 to 9 vol. of acetone, powdered in a ball mill, and then extracted with a mixture of methanol and light petroleum. The extracted fractions were combined, and the acetone and methanol removed by washing with water in a separating funnel. Saponification was carried out by adding an equal volume of alkaline methanol (18 % (w/v) KOH in 90 % (v/v) methanol) to the light petroleum solution and agitating the mixture under nitrogen by means of a magnetic stirrer for at least 20 hr. at room temperature. After thorough washing, the light petroleum solution containing the mixture of carotenoids was dried over anhydrous sodium sulphate.

RESULTS

Chromatographic separation of the polyenes

The dried light petroleum solutions of the mixture of carotenoids were chromatographed on columns of magnesia+filter aid mixed 2 : 1 by weight (Westvaco Chemical Company, 2641 adsorptive powdered magnesia; Hyflo Super Cel of Johns Manville Company). The chromatograms were developed with 5–8 % (v/v) acetone in light petroleum. Records of typical chromatograms are shown in Table 1. The columns were cut to separate the individual bands and the pigments were eluted with a mixture of chloroform+methanol or

Table 1. *Records of typical chromatograms showing pigments present in Corynebacterium michiganense mutants*

Bands on magnesia + filter aid column, listed from top downwards.					
Organism 4702 (parent; yellow)			Organism 4944 (pink)		
Band			Band		
Width (mm.)	Colour		Width (mm.)	Colour	
10	Yellow-orange	Cryptoxanthin	3	Red	All-trans-spirilloxanthin
20	Diffuse band, pink	All-trans-lycopene	1	Clear	
	Faint yellow eluant	cis-Lycopene	10	Pink	cis-Spirilloxanthin
			2	Clear	
			3	Orange	All-trans-lycopene
			3	Yellow	cis-Lycopene
Organism 4938 (orange)			Organism 4999 RR (red)		
Band			Band		
Width (mm.)	Colour		Width (mm.)	Colour	
2	Orange	Cryptoxanthin	4	Red-orange	All-trans-lycopene
8	Purple	Canthaxanthin	1	Clear	
1	Clear		3	Yellow-orange	cis-Lycopene
2	Dark red	cis-Canthaxanthin			
30	Clear				
2	Orange	β -Carotene			

Organisms 4939, 4999 (colourless)

No pigment

benzene + methanol (1 : 1). The pigment from each band was chromatographed separately to insure chromatographic purity. For this purpose the eluates were evaporated nearly to dryness under reduced pressure, and then redissolved in light petroleum. The light petroleum solutions were percolated through columns of $\text{Ca}(\text{OH})_2$ + filter aid (2 : 1 by weight) and the chromatograms developed with 2–8% (v/v) acetone in light petroleum.

Identification of the pigments

The individual pigments (listed in Table 1 and depicted in Fig. 1) were characterized by their absorption spectra (Table 2), by their position on chromatographic columns relative to known carotenoids, and by their behaviour when partitioned between aqueous methanol and light petroleum. More positive identification was obtained by demonstrating chromatographic homogeneity with known pigments.

From the original yellow culture, 4702, two pigments were detected and isolated: cryptoxanthin and lycopene. These pigments were present only when the concentration (c. 200 $\mu\text{g.}/100\text{ ml.}$) of thiamine in the medium was well above the concentration necessary for minimal growth. When the concentration of thiamine was diminished to a level which permits only minimal growth (c. 0.2 $\mu\text{g.}/100\text{ ml.}$), the cells were cream to white in colour and only traces of carotenoid pigments were detected. These results are in accord with Braun's (1949) finding that a shift in colour of the cell mass was mediated by the thiamine concentration.

The pink mutant 4944 produced spirilloxanthin and lycopene, whereas the

Fig. 1. Structural formulae of the principal carotenoids of *Corynebacterium michiganense* (Karrer & Jucker, 1950; Bonner, 1950). The structure of canthaxanthin is not known with certainty (Saperstein & Starr, 1954). 'R' signifies the common $\text{C}_{20}\text{H}_{24}$ central portion of the molecule.

red back-mutant 4999RR produced only lycopene. With organism 4944, there was no apparent change in colour at high or low thiamine concentrations. The scanty sediment which developed in the control culture (no added thiamine, but probably containing traces of it) was pink in colour. Since a considerable change in pigment production might have occurred without a concomitant change in the colour of the cell mass, the relative concentrations of spirilloxanthin and lycopene were estimated in the cells of 4944 grown at two concentrations of thiamine (0.1 and 100 $\mu\text{g.}/100\text{ ml.}$). A distinct difference

Table 2. *Spectral characteristics of the carotenoid pigments observed in Corynebacterium michiganense*

Pigment	Absorption maxima (black), minima, and inflexions (brackets)*	
	In benzene ($m\mu$.)	In hexane ($m\mu$.)
Cryptoxanthin	493, 481, 464, [439]	477, 467, 451, [426], 358.
Lycopene		502.5, 488, 471, 454, 445, 377
Spirilloxanthin	546, 530, 510, 490, 480, 410, 397	529, 514, 495, 476, 467, 400, 388, 378
β -Carotene	492, 482, 464, [440], 360	478, 468, 451, [426], 358
Canthaxanthin	480	468

* Obtained with the Beckman photoelectric spectrophotometer Model DU.

was observed: the content of spirilloxanthin relative to that of lycopene was significantly different at the two concentrations of thiamine. The ratios of the optical densities at 530 $m\mu$. (spirilloxanthin) and 442 $m\mu$. (lycopene) in the light petroleum (60–70°) from unchromatographed extracts were as follows:

Low-thiamine grown cells: $\text{O.D.}_{530}/\text{O.D.}_{442} = 0.72$.

High-thiamine grown cells: $\text{O.D.}_{530}/\text{O.D.}_{442} = 0.38$.

Since it has already been shown that the lycopene concentration in the naturally occurring yellow parent strain undergoes an absolute decrease when the cells are grown at the lower thiamine concentration, it is likely that the alteration in relative concentration of lycopene in the pink mutant 4944 is brought about in the same way.

Organism 4999RR, which forms only lycopene, showed no visible change in colour when the thiamine concentration was diminished; however, when the pigment content of the cells was estimated by the method of Stahly, Sesler & Brode (1942), we detected an increase in pigment concentration of approximately 36% at high thiamine concentrations. This strain does not require thiamine as an obligatory nutrient, but is stimulated by its addition to the medium (see below).

The orange mutant 4938 produced three carotenoid pigments: cryptoxanthin, β -carotene and canthaxanthin. Only one of these, cryptoxanthin, was found in the original yellow strain. Canthaxanthin, previously isolated from the fungus *Cantharellus cinnabarinus* by Haxo (1950), has not before been reported from bacteria. The characteristics of this ketonic pigment are the subject of a separate detailed report (Saperstein & Starr, 1954). With the

orange mutant there was no change in pigment production when the quantity of thiamine added to the medium was varied; it should be mentioned that strain 4938 synthesizes thiamine (see below).

The colourless mutants 4939 and 4999 showed no pigment production during this study at any concentration of thiamine in the growth medium (Table 1).

No fluorescent zones were found when any of the chromatographic columns were examined, at various stages of development, with ultraviolet radiation at a distance of approximately 3 cm. (Hanovia utility ultraviolet lamp, blue filter). The percolates showed no characteristic absorption curves to suggest the presence of colourless polyene pigments.

Nutritional requirements

To determine whether there was a change in nutritional patterns of the mutants as compared with the naturally occurring yellow strain, and to see whether such changes might reflect any alteration in carotenogenesis, all the organisms were screened for vitamin and amino acid requirements. Other cultures of the naturally occurring yellow type and a white variant had been shown previously to require biotin, thiamine and nicotinic acid (or nicotinamide) for minimal growth in a casein hydrolysate medium (Starr, 1949).

The colourless strains 4939 and 4999, and the yellow strain 4702, showed the same vitamin requirements (biotin, thiamine, and nicotinic acid) as the white and yellow cultures studied by Starr (1949). The orange mutant, 4938, did not require any accessory growth factors for minimal growth, and did in fact excrete biotin, thiamine and nicotinic acid (or nicotinamide) into the growth medium, as shown by microbiological assay. Strain 4944 required only biotin and thiamine for minimal growth, and strain 4999RR required only riboflavin obligatorily for minimal growth, but growth was increased considerably upon the addition of nicotinic acid, biotin and thiamine.

In the presence of the required vitamins, all the cultures used in the present study were able to utilize monosodium glutamate (1 g./l.) as the sole source of nitrogen; indeed, glutamate was found to be essential for growth in chemically defined media. Ammonium chloride alone, or in a combination with mixtures of amino-acids lacking glutamate, did not support growth of any of the *C. michiganense* cultures. Methionine can replace glutamate in strain 4702.

Lowering the concentration of the required growth factors other than thiamine did not result in any change in colour of the cell mass, although the growth was markedly diminished. Addition to the medium of L-leucine, an amino-acid shown by Goodwin & Lijinsky (1951) to increase carotenoid production in *Phycomyces blakesleeana*, did not affect pigmentation in *Corynebacterium michiganense* strains, nor in *C. poinsettiae*, when pigment concentration was estimated by the method of Stahly *et al.* (1942).

DISCUSSION

It is quite clear from the foregoing that *C. michiganense* contains carotenoid pigments, and that the differences in colour between the naturally occurring yellow culture and the mutants reflect qualitative changes in carotenoid

production. The qualitative manifestation of pigment production in *C. michiganense* could not, however, be correlated with any change in amino acid or vitamin requirements, other than the thiamine effect first demonstrated for this species by Braun (1949).

One interesting aspect of our results is the demonstration that the shift in pigmentation from cream to yellow in the parent strain 4702 which is mediated by thiamine, was due to the formation of cryptoxanthin under conditions of high thiamine concentration in the medium. In this respect, the organism behaved like *C. poinsettiae* (Starr & Saperstein, 1953) where the formation of cryptoxanthin was shown to be influenced by the concentration of thiamine in the culture medium. Furthermore, *C. poinsettiae* showed a slight decrease in lycopanthin (3-hydroxylycopene) production when grown in a low thiamine medium. Formation of the related carotenoid lycopene by the naturally occurring yellow strain of *C. michiganense*, as well as by the colour mutants 4944 and 4999RR, was diminished by low thiamine concentrations sufficient only to support minimal growth. These parallels suggest the operation of similar mechanisms for the production of these pigments by the two species.

Of interest, too, is the demonstration that the orange mutant of *C. michiganense* produces β -carotene, cryptoxanthin and canthaxanthin, while the pink mutant produces only lycopene and spirilloxanthin, and the red back-mutant, lycopene alone. This may indicate an independent genetic control for the production of carotenoids with cyclohexenyl rings, and carotenoids with non-cyclic structures, respectively. The absence of colourless polyenes from the coryneform organisms we have studied does not support the mechanism for carotenogenesis proposed by Porter & Lincoln (1950), by Turian (1950) and by Bonner *et al.* (1946), which assumes that the more saturated polyenes are precursors of the less saturated carotenoids, which result from the step-wise removal of hydrogen atoms from the former. The differences which exist between the individual carotenoids of the parent and mutant strains suggest that alterations in polyene structure probably occurs prior to the formation of the completed C_{40} chain. This hypothesis has been further developed elsewhere (Saperstein, 1953).

We wish to thank Prof. P. A. Ark, of the University of California at Berkeley, for the cultures of *Corynebacterium michiganense*, and Miss Barbara Brack, of our laboratory, for performing the microbiological assays.

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(Received 29 June 1953)