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BIOSYNTHESIS OF FLEXIRUBIN: INCORPORATION OF PRECURSORS BY THE BACTERIUM *FLEXIBACTER ELEGANS*

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Abstract—Biosynthesis of flexirubin (**1**) by *Flexibacter elegans* (Cytophagaceae) is closely linked to growth. Out of a number of experimental conditions tested, only lowering of the pH of the medium to slightly acid conditions led to a reduced specific pigment content. The latter returned very slowly to normal when the pH was increased again. Under no conditions was a stimulation of pigment synthesis observed. The following ^{14}C-labelled compounds were incorporated into flexirubin with sufficiently high efficiency to accept them as precursors: acetate, malonate, tyrosine and methionine.

INTRODUCTION

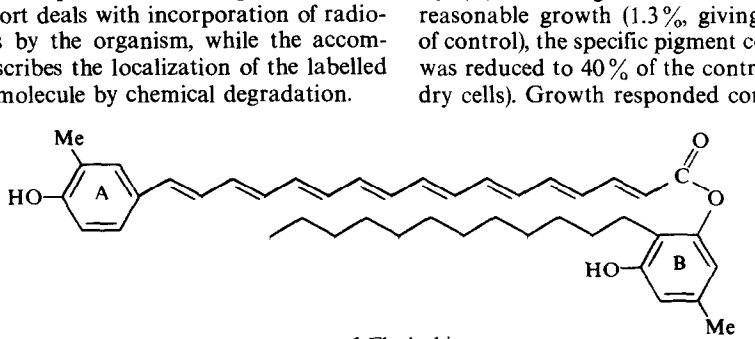
Gram-negative gliding bacteria of the *Cytophaga–Flexibacter* group produce a novel type of yellow pigment. The first member, flexirubin, has been isolated from *Flexibacter elegans* strain *Fx el* [1], and its structure has been elucidated [2] and confirmed by total synthesis [3, 4] (**1**). These novel pigments seem to have a rather limited distribution among bacteria, and may thus serve as valuable chemosystematic markers. So far they have been identified in strains of *Cytophaga*, *Flexibacter*, and *Sporocytophaga* from soil and freshwater, and in several so-called flavobacteria, the taxonomic position of which is, however, ill-defined at the moment. They are usually lacking in marine cytophagas. Interestingly, each of these taxonomic groups seems to be characterized by a special structural modification of their flexirubin-type pigments. For example, the pigments from *Cytophaga* lack the methyl group in ring A, and have a large variety of different hydrocarbon substituents in ring B, although never a methyl group as in flexirubin [5]. Furthermore, all bacteria producing flexirubin-type pigments also contain chlorinated derivatives with the chlorine in the *meta*-position of ring A [5, 6]. The flexirubins are located in the outer membrane of the cell wall [7] and are synthesized only by growing cells [1]. Their biological function is still unknown.

In view of its unusual structure, it was of interest to study the biosynthesis of flexirubin. As a first approach, we tried to identify the precursors of the pigment molecule. This first report deals with incorporation of radioactive compounds by the organism, while the accompanying paper describes the localization of the labelled atoms within the molecule by chemical degradation.

RESULTS AND DISCUSSION

Experiments were performed with *Flexibacter elegans* strain *Fx el*, because this organism produces a relatively simple pigment pattern consisting of *ca* 95% flexirubin (**1**), 3% chloroflexirubin, and a small amount of a pigment mixture which travels as a uniform band on silica gel thin layers and can be separated quantitatively from the other two pigments. All other organisms investigated so far contain much more complex and often inseparable mixtures of different pigments [4, 5]. We restricted our study to the main pigment, flexirubin.

All efforts to block flexirubin biosynthesis either by varying the culture conditions (different media, temperatures and oxygen levels, illumination), or by adding inhibitors known to interfere with carotenoid biosynthesis (diphenylamine, nicotine, antimycin A, CPTA or 2-(4-chlorophenylthio)-triethylamine, herbicide San 6706 or 4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone) [8] were essentially unsuccessful. While some of the inhibitors actually reduced the specific pigment content of the bacteria (diphenylamine, CPTA), they did so only at concentrations which were already severely growth limiting. A simulation of the synthesis of polyenic antibiotics has been observed upon addition of fatty acids or of natural oils [9–11]. Addition of 2–4% (v/v) of olive oil to a peptone medium (cas l.m.) had no effect on growth and pigment synthesis by *Fx el*. Oleic acid inhibited growth already at low concentrations (0.16 %, v/v); at the highest concentration which still allowed reasonable growth (1.3%, giving 30% of growth yield of control), the specific pigment content of the bacterium was reduced to 40% of the control (i.e. to 0.3 μg per mg dry cells). Growth responded considerably less to addi-



1 Flexirubin

Table 1. Effect of pH on specific flexirubin content of *Flexibacter elegans*

pH of medium	Age of culture (hr)	OD of culture (at 623 nm, 1 cm light path)	Specific pigment content (μg flexirubin/mg dry cells)
6.5	9	1.70	0.29
6.8	8	2.39	0.58
7.2	8	1.80	0.66
7.6	8	1.94	0.65
7.9	8	1.94	0.72
8.3	9	1.10	0.62

Table 2. Response of specific pigment content of *Flexibacter elegans* to a pH shift

pH of medium	Age of culture (hr)	OD of culture (at 623 nm, 1 cm light path)	Specific pigment content (μg flexirubin/mg dry cells)
6.5	23	2.15	0.29
This culture was diluted with fresh medium and the pH shifted at the same time:			
7.4	0	0.80	0.29
7.5	2	1.26	0.24
7.6	5	1.60	0.47
7.5	9	2.15	0.64
7.4	24	2.95	0.71

tions of saturated fatty acids (myristic, palmitic, stearic acid), but again no stimulation of pigment synthesis was observed. Neither could we obtain increased pigment synthesis by addition of sodium acetate (0.05–0.1 %), a likely precursor of flexirubin. At concentrations higher than 0.1 % acetate inhibited growth steeply.

The only feasible method found so far to control flexirubin biosynthesis is via the pH of the medium. As observed earlier [1], addition of a variety of sugars to the growth medium leads to a lower specific pigment content of the bacterium. The effect was supposed to be due to production of acid from sugar, and this could now be proved unequivocally by growing the bacterium in a laboratory fermenter at a constant and regulated pH in sugar-free peptone medium (Table 1). Without pH regulation the medium would in this case become alkaline due to production of ammonia by the organism. If the pH of a culture grown under slightly acid conditions was shifted to a higher pH, the specific pigment content of the bacterium increased again, but only very gradually over several hours and in the course of cell growth (Table 2). In conclusion then, flexirubin biosynthesis is strictly linked to growth: specific pigment content can be manipulated experimentally only within narrow limits, and, once reduced, does not return to higher levels quickly when cultivation conditions are changed. Consequently all labelling experiments have to be done with cultures growing under conditions as optimal as possible, which means in complex, peptone containing media.

The results of the labelling experiments are shown in Table 3. Acetate is taken up by the bacterium rather efficiently and readily incorporated into flexirubin. The

Table 3. Uptake of labelled compounds by *Flexibacter elegans* and their incorporation into flexirubin

Labelled compound (specific activity in Ci/mol)	Radioactivity added to culture (μCi/ml)	Incubation time (hr)	Total radioactivity found in the cells (% of added radioactivity)	Incorporation into flexirubin (a) Absolute amounts (DPM/μg flexirubin)	(b) Efficiency (% of radioactivity taken up by cells)
D-Glucose[U-^{14}C] (260)	0.1	3	25	570	0.52
		8	29	330	0.32
Sodium acetate [1-^{14}C] (59.5)	0.3	1	22	1890	0.49
		2	18	1390	0.51
		5	17	1920	0.96
Sodium acetate [U-^{14}C] (59)	0.25	8	19	1290	0.62
Sodium malonate [2-^{14}C] (17)	0.5	8	2.0	250	0.73
L-Phenylalanine [U-^{14}C] (486)	0.5	8	4.4	14	0.03
Cinnamic acid [3-^{14}C] (238)	0.3	3	1.6	7	0.2
L-Tyrosine [U-^{14}C] (486)	0.1	3	3.0	80	0.43
		5	5.2	100	0.41
		8	7.3	160	0.60
	0.5	8	7.9	930	0.63
DL-Tyrosine [2-^{14}C] (46)	0.33	9	5.6	320	0.82
L-Tyrosine [1-^{14}C] (59)	0.25	8	12	530	0.72
L-Methionine [methyl-^{14}C] (53)	0.17	8	5.0	250	0.68

Table 4. Incorporation of DL-mevalonic acid [2-^{14}C]* into various lipid fractions of *Cytophaga johnsonae*.

Lipid fraction	DPM/fraction
Quinones	13650
Cytophaga-flexirubins†	0
Zeaxanthin†	10600

* Specific activity: 22.8 Ci/mol; added: 0.11 μCi/ml, 4.3% of which was taken up by the cells.

† The molar ratio between the cytophaga-flexirubins and zeaxanthin was *ca* 7:3, amount of zeaxanthin was 8.5 μg.

proportion of label from acetate in flexirubin relative to other labelled cell constituents seems to increase with incubation time. This probably means that part of the label enters flexirubin via other compounds synthesized from acetate before. While the uptake of malonate is low, the efficiency of its incorporation into flexirubin is as high as with acetate. Both labelling experiments indicate that at least part of the flexirubin molecule comes from fatty acid or/and polyketide biosynthesis.

Glucose is taken up and metabolized very efficiently by *Fx el*, but in comparison with acetate much less of its labelled carbon appears in flexirubin. This suggests that glucose enters flexirubin only via other metabolites, one certainly being acetate [12]. Phenylalanine is taken up with reasonable efficiency, regarding the presence of peptone in the medium; less so is cinnamic acid. Neither compound enters flexirubin.

Tyrosine, on the other hand, seems to be incorporated specifically into flexirubin, probably supplying one or both of the aromatic rings. As the efficiency of labelling remains the same whether C1-, C2-, or U- labelled tyrosine is offered, the compound seems to enter with all 3 chain carbon atoms. High labelling efficiency was observed also with methyl labelled methionine, so that probably one or both of the two methyl groups are introduced by transmethylation. Mevalonic acid is not incorporated into flexirubin by *Fx el*, as has been reported before [1]. The experiment was repeated with *Cytophaga johnsonae* strain *Cy j1*, because this organism synthesizes carotenoids (mainly zeaxanthin [5]) in addition to flexirubin-type pigments. While the carotenoids and menaquinones became heavily labelled, the flexirubin-type pigments remained totally free of radioactivity (Table 4).

EXPERIMENTAL

Organisms and culture condition. Flexibacter elegans strain *Fx el* [1] and *Cytophaga johnsonae* strain *Cy j1* [5] were used. Media and culture conditions were as described before [1, 5]. Experiments to determine the pH dependence of flexirubin biosynthesis were performed in a laboratory fermenter (Biostat) with 1 l. vol.; pH was regulated with HOAc and NH_4OH, respectively. Inhibitors for pigment synthesis were in the concentration range 0.5–5000 μM. The influence of temperature on flexirubin biosynthesis was tested between 20 and 35°.

Quantitative determination of pigment was done photometrically using $E^{1\%}_{1\,cm} = 1565$ (451 nm, Me_2CO) [1].

Labelling experiments were performed in shake cultures with 50 or 100 ml Fx A l.m. [1, 5]. Radioactive compounds were purchased from Amersham-Buchler (Braunschweig) with the exception of methionine and cinnamic acid, both from Isotopendienst West (C.I.S.). Mevalonic acid was applied as its DBED-salt. Pigments were extracted with Me_2CO, and separated and purified to constant radioactivity by TLC (Si gel; first run with: toluene–petrol (60–80°)–Me_2CO, 25:25:16; second run of eluted pigments on fresh plate with: toluene–EtOAc, 9:1). Radioactivity was determined in a scintillation counter with quench correction by use of an external standard.

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