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Synthesis of the major unsaturated fatty acids of an estuarine Flexibacterium

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Abstract

Fatty acid composition of the *Flexibacter* strains Inp2 and Inp3 were found to be influenced by the presence of cAMP. Whilst cAMP inhibited the synthesis of linoleic and linolenic acid in Inp2, cerulenin inhibited the synthesis of C16:1. This suggests that Inp strains possess both the aerobic and anaerobic pathway for synthesis of unsaturated fatty acids (UFAs).

Introduction

Synthesis of UFAs in bacteria may occur by either an aerobic or anaerobic pathway. Unlike the former pathway which involves the presence of molecular oxygen and introduction of a double bond by desaturation, the anaerobic pathway does not require oxygen and a double bond is generally formed when the chain has ten or twelve carbon atoms (GOLFINE and BLOCH 1961, ERWIN and BLOCH 1964, FULCO 1983). SCHEUERBRANDT and BLOCH (1962) suggested that both pathways were mutually exclusive. However, more recently WADA et al. (1989) have demonstrated the presence of both pathways in bacteria. Although the presence of polyunsaturated fatty acids (PUFAs) in bacteria has been shown (DELONG and YAYANOS 1986, YAZAWA et al. 1988, INTRIAGO 1990), the mechanisms regulating their synthesis are not known.

The rationale of this study was to examine the incorporation of a radioactive precursor into Inp2 grown in high seawater osmotic strength and natural seawater osmotic strength medium, and to examine the effect of cAMP and cerulenin on this incorporation. Additionally the effect of high osmotic medium with and without cAMP on the fatty acid composition of Inp3 was also examined.

The organisms used in the present study *Flexibacter* Inp2 and Inp3 have been described by INTRIAGO (1990).

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Material and methods

Culture techniques

Cultures were maintained at 25 °C on ZoBell 2216-E solid medium (OPPENHEIMER and ZOBELL 1952). With the exception of the carbon source the medium consisted of 2 g of ammonium sulphate; 0.2 g dipotassium hydrogen phoshate; 0.5 g of Tris; 1ml of trace metal solution (REICHENBACH and DWORKIN 1981), in 1 liter of seawater of salinity 30 ppt. Cycloheximide (0.1 g/L) was used to inhibit eukaryotic growth. The pH was adjusted to 7.6. The experiments were carried out in 50 ml of medium in 250 ml flasks, using 5 ml of inoculum. Cultures were bubbled with 100:20 ml/minute of an air:nitrogen mixture and incubated at 24 °C in a water bath. When it was required, salinity was increased by adding the mixture of marine salts described in REICHENBACH and DWORKIN (1981).

Cells were harvested during late log phase by centrifugation at 10000 g for 15 minutes at 10 °C, washed once in NaCl (0.5 M), resuspended in 10 ml of phosphate buffer (50 mM ph 7.5) (WHITE et al. 1979) and stored at -20 °C.

Fatty acid composition of Inp3

Either starch or glucose (0.5 %), or 12 % sucrose were used as carbon source. When cAMP was used, it was filter sterilized and added at the beginning of the experiments.

Lipids of Inp3 were extracted by adding enough chloroform:methanol (1:2) to the unfrozen cell pellet to obtain a single phase chloroform:methanol:phosphate buffer mixture (1:2:0.8). This extract was left at 4 °C overnight, then water and chloroform were added to form two separate phases (BLIGH and DYER 1959, WHITE et al. 1979).

Fatty acids were extracted using a technique based on MOSS et al. (1969). The lipid extract was dried under a stream of nitrogen and then saponified with either 5 % (w/v) NaOH or KOH in 50 % (v/v) methanol, at 100 °C for 45 minutes. These methanolysates were cooled, and adjusted to pH 2 with HCl (6 M). 1 ml of chloroform was then added and shaken vigorously. The upper phase was discarded and the lipid fraction dried under a stream of nitrogen. Fatty acids were esterified with 2 ml of 10 % (v/v) BCl_3 in methanol at 80 °C for 10 minutes. After cooling, the fatty acids methyl esters (FAMEs) were extracted by adding 1 ml of a saturated solution of NaCl and 2 ml of hexane:chloroform (4:1). The extracts were evaporated under a stream of nitrogen, redissolved in chloroform and spotted onto a TLC plate (Merck precoated silica gel 60) to separate FAMEs from other reaction products. The plates were developed with hexane:diethyl ether (90:10) for 1 hour 30 minutes in a continuous elution tank. FAMEs were scraped off the plates and dissolved in chloroform:methanol (2:1), evaporated to dryness and redissolved in 200 µl of hexane.

FAMEs analyses were carried out in a Carlo Erba gas chromatograph (model Vega 6180) using on-column injection, fitted with a 30 m x 0.25 mm ID DB-225 fused silica column (J and W Scientific), or an Econo-cap, Carbowax 30 m x 0.25 mm ID capillary column (Alltech, Ass. Inc) using hydrogen as a carrier gas (2.0 ml/min). The oven was temperature programmed from 50 °C to 150 °C at a ramp rate of 49.9 °C per minute and then 3 °C per minute to 230 °C, and held for 10 minutes. The FID temperature was 250 °C. Uniform response was assumed for all components. Quantification was based on comparison of peak areas with

an internal injection standard (C23:0). FAMEs were identified by comparision of retention times with known standards.

Radioactive experiments

Culture conditions were as described above. Either sucrose 12 % (w/v) or glucose 0.5 % (w/v) was used as carbon source. The doubling time of Inp2 in both media was 12 hours. When the cells reached log phase (about 40 hours), either [1-[14-C]] acetate, specific activity 50-60 mCi/mmol or [1-[14-C]] palmitic acid, specific activity 50-60 mCi/mmol (Radiochemical Centre, Amersham, England), was added to the medium to give a final concentration of either 1.0 µCi/ml of [14-C] acetate or 0.1 µCi/ml of [14-C] palmitic acid. The bacteria were incubated for four hours before harvesting. When either cAMP 1mM (Aldrich. chem. Co), or cerulenin 5.0 µg/ml (Sigma, Co) were included in the experiment, they were added together with the radioisotopes.

Fatty acids were saponified with 2 ml of 5 % (w/v) KOH in 50 % methanol:water (w/v) for one hour at 100 ºC, cooled and acidified with HCl (6M). The lipids were extracted with 1 ml of chloroform and dried under nitrogen. Fatty acids were derivatisated by a technique adapted from LAM and GRUSKA (1985). Derivatisation was started by adding 20 µl of the KOH solution used above to the dried lipids, the methanol and water evaporated under nitrogen and the fatty acid potassium salts formed dissolved in 1 ml of acetonitrile. One hundred and fifty µl of both p-bromophenacyl bromide (Sigma, Co) (50 mg/ml) and dicyclohexano- 18-crown- 6 (Aldrich. chem. Co) (5 mg/ml) were added and heated for 25 minutes at 85 ºC. Both reagents were dissolved in acetonitrile.

Excess of reagent after derivatisation was eliminated by adding 2 ml of both water and hexane to the reaction vial which was mixed vigorously. The hexane layer was placed in another vial and evaporated under nitrogen and redissolved in 100 µl of acetonitrile. Ten µl were injected into a Varian 2050 HPLC, fitted with a C18 ODS2 3µ 150 mm x 4.6 mm column (Spherisorb, Alltech, Co). An isocratic program with acetonitrile:water (85:15) as mobile phase was used, with a flow rate of 1.5 ml/min equivalent to 120 bars and monitored at a wavelength of 254 nm. The radioactive detector was a Berthold HPLC radioactivity monitor (LB506C-1) using solid scintillation, and connected to a compaq desk PRO 386/20 computer. A typical run time was 30 minutes. Identification of peaks was based on comparison with pure standards, and compared against gas chromatograph analyses.

The mobile phase used, acetonitrile:water (85:15), did not completely resolve C16:0 from C18:1, therefore these two were reported together. This did not affect the aims of this study.

The stock solution of [14-C] palmitic acid was stored in ethanol:water (1:1) at -20 ºC. All reagents used were analytical grade (Sigma and BDH). Solvents were from Rathburn.

Results and discussion

Both linoleic and linolenic acid were present when Inp2 was grown in either 12 % sucrose or 0.5 % glucose medium (Tables 1, 2). However, unlike cells grown in 12 % sucrose, there was no incorporation of radioactivity within 4 hours into PUFAs with either radiolabelled precursor in cells grown in glucose medium. This suggests that synthesis of PUFAs occurred faster in 12 % sucrose than in

Table 1. Fatty acid profiles (expressed as a percentage of the total fatty acids) and distribution of radioactivity among fatty acids in Inp2 after growth on 12 % sucrose. Numbers represent the average of two different experiments, after correcting for quenching and background, +/- one standard deviation. Fatty acids are presented in elution order.

		Sucrose 12 %					
				+ 1mM cAMP		+5µg/ml cerulenin	
		[14-C]					
Fatty acids %	UV 254nm	acetate	palmitate	acetate	palmitate	acetate	palmitate
i15:0	5.1(0.7)	0	0	0	0	0	0.2(0.3)
18:3	3.4(0.6)	1.6(2.3)	0	0	0	0	0.9(0.6)
14:0	2.6(1.2)	4.9(1.4)	2.4(0.9)	28.7(11.3)	0	0	1.8(0.5)
16:1	59.4(3.0)	53.9(0.5)	7.5(1.3)	25.8(16.3)	3.0(1.1)	0	0
18:2	8.0(1.0)	5.6(3.5)	0	0	0	0	3.3(0.6)
16:0/ 18:1	8.0(0.2)	28.7(5.0)	88.0(0.9)	42.1(8.6)	93.7(0.6)	100(0)	92.0(0.0)
Total radioactivity incorporated into fatty acids							
cpm		22750 (14800)	17790 (6110)	13850 (2050)	127470 (24890)	1300 (930)	112980 (28180)

Table 2. Fatty acid profiles (expressed as a percentage of the total fatty acids) and distribution of radioactivity among fatty acids in Inp2 after growth on 0.5 % glucose. Numbers represent the average of two different experiments after correcting for quenching and background, +/- one standard deviation. Fatty acids are presented in elution order.

		Glucose 0.5 %			
				+5µg/ml cerulenin	
		[14-C]			
Fatty acids %	UV 254nm	acetate	palmitate	acetate	palmitate
i15:0	5.8(2.0)	0	0	5.9(4.3)	0
18:3	1.7(2.4)	0	0	6.6(0.9)	1.4(2.0)
14:0	3.0(0.2)	3.6(1.8)	0	19.1(10.5)	4.2(3.5)
16:1	56.2(1.0)	48.1(9.7)	0	18.9(4.1)	0
18:2	7.1(0.3)	0	0	5.0(4.2)	0
16:0/ 18:1	10.6(3.3)	45.5(15.4)	97.4(0.1)	46.5(3.6)	86.5(5.7)
Total radioactivity incorporated into fatty acids					
cpm		12000 (2910)	35910 (1800)	7300 (1630)	47060 (10880)

0.5 % glucose. However, it is unknown whether this is due to a carbon or high osmotic strength medium effect. A comparison of the fatty acid composition of Inp3 cultured in glucose medium with the wild strain Inp (Table 3) highlights the dissimilarities between these strains. However, the proportion of PUFAs in Inp3 was partly restored once Inp3 was cultured in high osmotic strength media (Table 4).

Whilst addition of cAMP to the sucrose medium inhibited the synthesis of PUFAs (Table 1), cAMP reduced the proportion of all acids with an 18 carbon length chain in Inp3 (Table 4). This suggests that cAMP may regulate PUFA synthesis

Table 3. Fatty acid profiles (expressed as a percentage of the total fatty acids) in the wild strain Inp and Inp3 after growth on different conditions. Value in parenthesis represents +/- one standard deviation from two different experiments. Symbols. - means not detected.

Fatty acids %	Wild strain Glucose 0.5 %	Inp3 Glucose 0.5 %	Inp3 Starch 0.5 %
14:0	0.55(0.02)	-	2.12(0.22)
i 15:0	3.34(0.37)	4.31(0.56)	7.93(0.95)
16:0	18.02(2.26)	25.04(1.16)	23.09(0.78)
16:1w5	30.11(2.50)	60.75(0.24)	57.90(0.07)
18:0	3.23(1.11)	1.28(0.88)	0.57(0.26)
18:1w9c	16.08(1.16)	0.81(0.27)	0,93(0.01)
18:1w7	2.60(0.40)	0.16(0.09)	0.05(0.06)
18:2w6	17.77(1.26)	0.16(0.01)	0.19(0.09)
18:3w3	2.25(0.89)	-	-

Table 4. Fatty acid profiles (expressed as a percentage of the total fatty acids) of Inp3 after growth on different conditions. Value in parenthesis represents +/- one standard deviation from two different experiments. Symbols. * position of double bond not determined. - means not detected.

Fatty acids %	Sucrose 12 %	Sucrose 12 % + 1mM cAMP	Starch 60ppt	Starch 60ppt + 1mM cAMP
14:0	1.28(0.63)	1.80(0.30)	2.18(0.64)	1.93(0.21)
i 15:0	1.58(0.30)	4.40(0.09)	2.03(0.33)	6.29(0.13)
16:0	28.39(0.04)	22.32(4.34)	28.50(0.42)	21.30(4.60)
16:1w5	13.25(1.45)	43.49(2.37)	22.05(4.73)	45.56(11.36)
18:0	4.05(0.99)	2.44(0.37)	4.65(0.29)	4.85(1.10)
18:1w9c	10.03(1.11)	5.24(2.06)	6.83(0.67)	6.80(4.66)
18:1w7	2.20(0.27)	1.21(0.71)	5.80(1.13)	2.17(3.06)
18:2w6	9.82(0.76)	2.34(0.89)	6.06(0.93)	2.88(0.88)
18:3w3	0.98(0.16)	0.61(0.44)	1.95(0.20)	0.73(0.00)
16-oxo-18:2w6	10.22(2.48)	-	-	-

by decreasing both synthesis and elongation of C16:0. PIOVANT and LAZDUNSKI (1975) demonstrated that 12 % sucrose medium caused a decrease in the intracellular cAMP level in *Escherichia coli,* and BROWNLIE et al. (1985) have shown that high NaCl concentrations inhibited the adenylate cyclase of *Bordetella pertussis.* Thus, it is possible that high osmotic strength medium lowers the intracellular levels of cAMP in Inp strains and hence increases the PUFA content.

In general, cerulenin affected Inp2 in two ways; it inhibited the fatty acid synthetase, and with it the synthesis of C16:1. Secondly, it enhanced both PUFA synthesis, and the total radioactivity incorporated from [14-C] palmitic acid. Cerulenin was used in the present study because it is known to inhibit the B-ketoacyl-ACP synthetase in *Escherichia coli* (D'AGNOLO et al. 1973). BUTTKE et al. (1978) have shown that B-ketoacyl-ACP synthetase I, which elongates cis-decenoyl-ACP to C16:1w7 in *E. coli,* was more sensitive than the B-ketoacyl-ACP synthetase II, which elongates C16:1 to C18:1. Thus, inhibition of UFA synthesis by cerulenin can be considered as an inhibition to the anaerobic pathway.

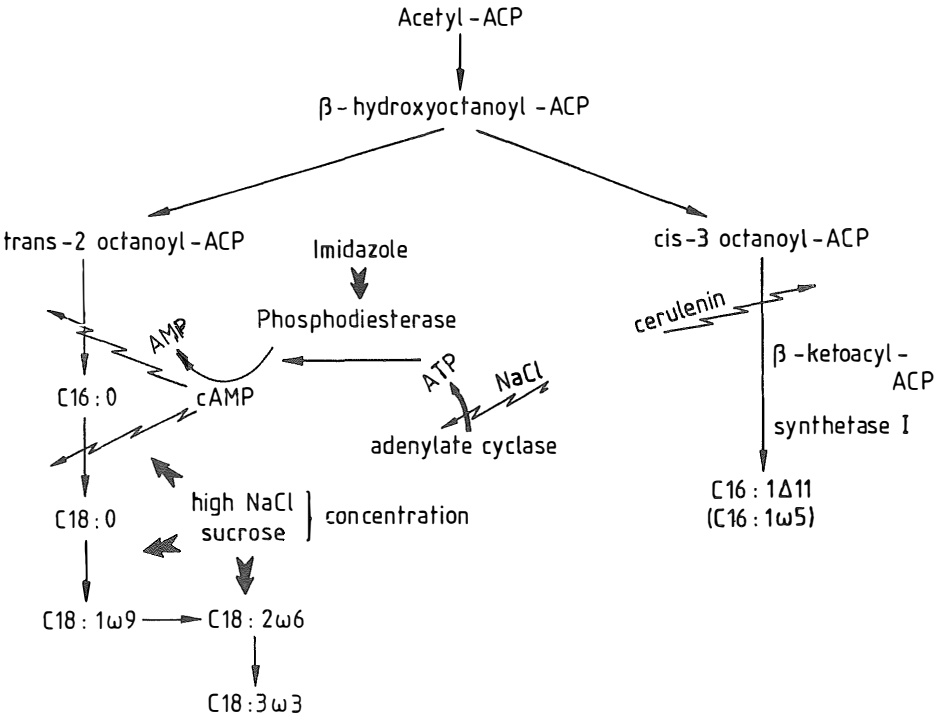

Fig. 1. Hypothetical fatty acid synthesis pathways adapted from *E. coli,* in Inp strains. ß-hydroxydecanoyl-ACP was replaced by ß-hydroxyoctanoyl-ACP because cerulenin inhibited C16:1w5 rather than C16:1w7.

The inhibition of the incorporation of radioactive precursor into C16:1 by cerulenin, which is known to inhibit the synthesis of UFAs by the anaerobic pathway strongly suggests the presence of the anaerobic pathway in Inp2. Additionally, presence of PUFAs involve desaturation and hence presence of the aerobic pathway in Inp2 and Inp3. Although it is not known whether the effect of cAMP on Inp2 was in the activation of protein synthesis as reported in other organisms (BOTSFORD et al. 1981) or in an cAMP dependent protein kinase, it is clear that the intracellular concentration of cAMP plays a major role in the synthesis of the major fatty acids in Inp and related strains. Results of this study have been incorporated into a flow diagram (Fig. 1) of the fatty acid synthesis of *E. coli* taken from HARWOOD and RUSSELL (1984) to show the possible routes of UFA synthesis in Inp strains.

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