The regulation of fatty acid biosynthesis in some estuarine strains of *Flexibacter*

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The mechanism of unsaturated fatty acid biosynthesis in two estuarine strains of *Flexibacter* was investigated. Addition of cyclic AMP (cAMP) inhibited the incorporation of radiolabelled acetate into fatty acids of late-exponential phase cultures of strain Inp2. Cerulenin selectively inhibited the incorporation of radioactive acetate into the major fatty acid, 16:1. When *Flexibacter* strain Inp3 was grown in a medium of high osmotic strength, polyunsaturated fatty acids were present, but they were absent from cultures grown in media with an osmotic strength close to that of sea water. Addition of cAMP to cultures growing at high osmotic strength suppressed the formation of polyunsaturated fatty acids; the uncoupler 2,4-dinitrophenol had a similar effect. The findings are discussed in the context of the suggestion made by Intriago and Floodgate (*Journal of General Microbiology* 137, 1503–1509, 1991) that *Flexibacter* strains possess both the anaerobic and aerobic pathways for unsaturated fatty acid biosynthesis, the activity of the latter being modulated by the intracellular concentration of cAMP.

Introduction

Only two biochemical mechanisms are recognized for the introduction of a double bond into a fatty acid. In the anaerobic pathway, which can be considered part of the fatty acid synthetase system, a double bond is formed when the chain generally has 10 or 12 carbon atoms. The chain is then lengthened to produce eventually either C16:1w7 or C16:1w9 respectively (Goldfine & Bloch, 1961; Erwin & Bloch, 1964; Fulco, 1983). This pathway is found in all anaerobes, facultative anaerobes and some aerobic bacteria (Harwood & Russell, 1984; Schweizer, 1989). In contrast, the aerobic or oxygen-dependent pathway introduces double bonds by desaturation of existing chains, usually 16 or 18 carbon atoms in length. This latter pathway is found in some aerobic bacteria, especially Gram-positive ones, as well as non-parasitic eukaryotes (Fulco, 1970; Schweizer, 1989). The position of the first double bond in plants and microbes is usually at the 9,10 position (Bloomfield & Bloch, 1960; Russell, 1978). Scheuerbrandt & Bloch (1962) suggested that the

anaerobic and the aerobic pathways were mutually exclusive. However, Wada *et al.* (1989) have recently demonstrated the presence of both pathways in a psychrotrophic *Pseudomonas*.

The presence of polyunsaturated fatty acids (PUFAs) in marine bacteria has been demonstrated (Delong & Yayanos, 1986; Wirsén *et al.*, 1987; Yazawa *et al.*, 1988; Intriago & Floodgate, 1991), but the mechanism controlling their biosynthesis is unknown. A potential regulator such as cyclic AMP (cAMP) is known to affect cell metabolism in different ways, including regulation of gene expression (Pauli *et al.*, 1974), control of cellular morphogenesis (Larsen & Sypherd, 1974) and phosphorylation of proteins (Saha *et al.*, 1988). In contrast, there are few reports in the literature about the effect of cAMP on fatty acid composition. Dallas *et al.* (1976) showed that the fatty acid composition of *Escherichia coli* was regulated by cAMP and Alaniz *et al.* (1976) found that incubation of cultured mouse cells with cAMP decreases the desaturation of linolenic acid.

Cerulenin was used in the present study because it is known to reduce fatty acid synthesis in *E. coli* by inhibiting β -ketoacyl-ACP synthase (D'Agnolo *et al.*, 1973). Butkke & Ingram (1978) suggested that β -ketoacyl-ACP synthase I, which elongates *cis*-decenoyl-ACP to C16:1w7 in *E. coli*, is more sensitive to cerulenin than is β -ketoacyl-ACP synthase II, which elongates C16:1 to C18:1. Thus, inhibition of unsaturated fatty

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Abbreviations: DNP, 2,4-dinitrophenol; UFA, unsaturated fatty acid; PUFA, polyunsaturated fatty acid; p.p.t., parts per thousand (salinity).

acid (UFA) synthesis by cerulenin can be considered as an inhibition of the anaerobic pathway.

The rationale of this study was to examine the effect of cAMP, cerulenin and high osmotic strength on the incorporation of radioactive acetate into exponentially growing cultures of *Flexibacter* strain Inp2, a PUFA-containing strain, and to compare their effects on the fatty acid composition of *Flexibacter* strain Inp3, which is deficient in PUFAs.

Methods

Culture conditions. The *Flexibacter* strains Inp2 and Inp3 used in this study were isolated after continuous cultivation of the wild-type *Flexibacter* strain Inp on high salinity medium (Intriago & Floodgate, 1991). Besides the differences in fatty acid composition, no nutritional, physiological or morphological differences between the strains were found. The culture conditions were as described by Intriago & Floodgate (1991). The carbon source was 12% (w/v) sucrose, 0.5% (w/v) glucose or 0.5% (w/v) soluble starch. Salinity was increased by adding the artificial sea-water salts formulated by Reichenbach & Dworkin (1981). The doubling time of all strains in each of the media was 12 h. When the cultures reached exponential phase (about 40 h), sodium [^{14}C]acetate [Amersham; specific radioactivity 50–60 mCi mol $^{-1}$ (1.85–2.22 GBq mol $^{-1}$)] was added to the medium to give a final concentration of 1.0 $\mu\text{Ci ml}^{-1}$ (37 kBq ml $^{-1}$) and incubated for 4 h before harvesting the bacteria.

When either cAMP (1 mM, Aldrich) or cerulenin (5 $\mu\text{g ml}^{-1}$, Sigma) were included in the incubation mixtures for Inp2, they were added at the same time as the radioisotope.

When cAMP (1 mM) or the uncoupler 2,4-dinitrophenol (DNP; 1 μM ; BDH) were added to the culture medium of Inp3 they were sterilized by filtration and added to the growth medium at the beginning of the experiment.

Lipid extraction. Bacteria were harvested as described in Intriago & Floodgate (1991). Lipids were extracted by the method of Bligh & Dyer (1959), saponified with 2 ml 5% (w/v) KOH in 50% (v/v) methanol in water for 1 h at 100 °C, cooled and acidified with 6 M-HCl. The fatty acids were extracted twice with 1 ml chloroform and dried under nitrogen.

Gas-liquid chromatographic analysis. Fatty acids of Inp3 were methylated and analysed as described in Intriago & Floodgate (1991).

Radio-HPLC analysis. Fatty acids of Inp2 were derivatized using a technique adapted from that of Lam & Gruska (1985). Derivatization was started by adding 20 μl 5% (w/v) KOH in 50% (v/v) methanol in water to the dried lipid sample, and then evaporating the methanol and water using a stream of nitrogen gas. The fatty acid potassium salts so formed were dissolved in 1 ml acetonitrile, plus 150 μl *p*-bromophenacyl bromide (Sigma) (50 mg ml $^{-1}$) and 150 μl 18 crown 6 (dicyclohexano-18-crown-6) (Aldrich) (5 mg ml $^{-1}$) dissolved in acetonitrile. The reaction mixture was heated for 25 min at 85 °C. Excess reagent after derivatization was eliminated by adding 2 ml water and 2 ml hexane to the reaction vial and mixing vigorously. The hexane layer was removed and placed in a second vial and evaporated using nitrogen gas. The fatty acid methyl esters were redissolved in 100 μl acetonitrile, and 10 μl was injected into a Varian 2050 HPLC, fitted with a C18 ODS2 3 μm 150 cm $\times$ 4.6 mm column (Spherisorb, Alltech). An isocratic programme with acetonitrile/water (85:15, v/v) as mobile phase was used, with a flow rate of 1.5 ml min $^{-1}$ equivalent to 120 bar, and was monitored at a wavelength of 254 nm. The radioactive detector was a

Berthold HPLC radioactivity monitor (LB506C-1), connected to a Compaq-Desk PRO 386/20 computer. A typical run time was 30 min. Identification of peaks was based on comparison with pure standards, and compared against gas-liquid chromatographic analyses.

The mobile phase used, acetonitrile/water (85:15, v/v), did not completely resolve C16:0 from C18:1, and they were reported together, but this did not affect the aims of this study.

It is relevant to point out that whereas fatty acid analysis by gas-liquid chromatography agrees more closely with weight percentages, the values of fatty acid phenacyl derivatives quantified by HPLC are closer to mole percentages (Wood & Lee, 1983).

Results

Fatty acid biosynthesis in *Flexibacter* strain Inp2

The fatty acid composition of *Flexibacter* strain Inp2 was broadly similar whether it was grown in 12% sucrose or 0.5% glucose (cf. Tables 1 and 2). Both C18:2 and C18:3

Table 1. Percentage fatty acid composition and distribution of radioactivity among fatty acids in *Flexibacter* strain Inp2 after growth on 12% sucrose

Values represent two separate experiments, after correcting for quenching and background.

Fatty acid	Composition by mass (%)	Radioactivity (%)
14:0	3.5–1.7	3.9–5.9
14:1	4.8–1.2	4.0–4.5
i15:0	4.6–5.1	0.0–0.0
a15:0	5.3–5.0	0.0–0.0
15:0	0.0–0.0	0.0–0.0
16:0/18:1	8.1–7.8	25.2–32.2
16:1	61.5–57.2	53.5–54.2
18:2	8.7–7.3	8.0–3.1
18:3	3.0–3.8	3.2–0.0
Unknown	0.6–0.4	0.0–0.0
		Total c.p.m. 12280–33210

Table 2. Percentage fatty acid composition and distribution of radioactivity among fatty acids in *Flexibacter* strain Inp2 after growth on 0.5% glucose

Values represent two separate experiments, after correcting for quenching and background.

Fatty acid	Composition by mass (%)	Radioactivity (%)
14:0	3.1–2.8	4.9–2.3
14:1	7.0–11.1	0.0–0.0
i15:0	4.4–7.2	0.0–0.0
a15:0	8.1–0.0	0.0–0.0
15:0	0.0–0.0	0.0–0.0
16:0/18:1	8.1–13.0	34.5–56.4
16:1	56.8–55.5	54.8–41.2
18:2	7.3–6.9	0.0–0.0
18:3	3.4–0.0	0.0–0.0
Unknown	1.1–3.2	0.0–0.0
		Total c.p.m. 9940–14060

Table 3. Effect of cAMP on fatty acid composition and distribution of radioactivity among fatty acids in *Flexibacter* strain Inp2 after growth on 12% sucrose

Values represent two separate experiments, after correcting for quenching and background. cAMP (1 mM) was added together with the radioactive precursor 4 h before harvesting the bacteria.

Fatty acid	Composition by mass (%)	Radioactivity (%)
14:0	2.4-3.6	36.5-20.9
14:1	4.3-6.4	0.0-0.0
i15:0	2.3-4.6	0.0-0.0
a15:0	4.5-6.4	0.0-0.0
15:0	0.0-0.0	0.0-0.0
16:0/18:1	35.7-9.4	48.1-36.0
16:1	36.4-57.5	14.2-37.3
18:2	12.4-7.2	0.0-0.0
18:3	0.0-2.8	0.0-0.0
Unknown	1.0-2.4	0.0-0.0
		Total c.p.m. 12400-15300

Table 4. Effect of cerulenin on fatty acid composition and distribution of radioactivity among fatty acids in *Flexibacter* strain Inp2 after growth on 12% sucrose

Values represent two separate experiments, after correcting for quenching and background. Cerulenin (5 µg ml⁻¹) was added together with the radioactive precursor 4 h before harvesting the bacteria.

Fatty acid	Composition by mass (%)	Radioactivity (%)
14:0	2.7-3.3	0.0-0.0
14:1	10.9-7.3	0.0-0.0
i15:0	5.1-4.7	0.0-0.0
a15:0	9.4-7.0	0.0-0.0
15:0	0.0-0.0	0.0-0.0
16:0/18:1	8.1-11.9	100.0-100.0
16:1	54.4-54.6	0.0-0.0
18:2	4.0-4.6	0.0-0.0
18:3	2.9-3.1	0.0-0.0
Unknown	2.7-1.8	0.0-0.0
		Total c.p.m. 1960-640

were present in bacteria grown in either medium. However, none of the radiolabelled acetate appeared in these acids in glucose-grown cells after incubation with the precursor for 4 h, whereas a significant amount of radioactivity was incorporated into these acids in sucrose-grown cultures (Tables 1 and 2).

Addition of cAMP to the sucrose medium did not affect bacterial growth, but prevented the biosynthesis of PUFAs. The relative percentages of radiolabelled material incorporated from acetate into myristic and palmitic/oleic acids were increased compared with the control whilst the incorporation into palmitoleic/oleic acids was decreased (cf. Tables 1 and 3).

Table 5. Effect of cerulenin on fatty acid composition and distribution of radioactivity among fatty acids in *Flexibacter* strain Inp2 after growth on 0.5% glucose

Values represent two separate experiments, after correcting for quenching and background. Cerulenin (5 µg ml⁻¹) was added together with the radioactive precursor 4 h before harvesting the bacteria.

Fatty acid	Composition by mass (%)	Radioactivity (%)
14:0	3.5-2.1	26.5-11.6
14:1	8.8-3.7	1.7-1.0
i15:0	5.8-2.1	8.9-2.8
a15:0	0.0-4.0	0.0-0.0
15:0	0.0-0.3	0.0-1.8
16:0/18:1	31.7-32.6	43.9-49.0
16:1	34.4-41.5	16.0-21.8
18:2	13.1-10.6	2.0-7.9
18:3	0.0-1.3	0.0-2.6
Unknown	2.6-1.7	0.0-0.0
		Total c.p.m. 8450-6140

Addition of cerulenin to the sucrose medium resulted in a marked decrease of fatty acid synthesis; radio-labelled acetate was incorporated only into the palmitic/oleic acids fraction (Table 4). Cerulenin had a different effect on cultures grown in glucose medium (cf. Tables 4 and 5). In contrast to bacteria grown in 12% sucrose, incorporation of radioactive acetate into C16:1 and total fatty acids was only partly inhibited, and the precursor was readily incorporated into both PUFAs (Table 5).

Effect of osmotic strength on *Flexibacter* strain Inp3

A comparison of the fatty acid composition of *Flexibacter* strain Inp3 cultured in glucose medium (Table 6) with that of strain Inp, described in Intrigato & Floodgate (1991), under the same conditions highlights the dissimilarities between these two strains: whereas PUFAs were practically absent in Inp3, they represented up to 20% of the total fatty acids in Inp. However, the data in Table 6 illustrates that synthesis of PUFAs was restored once Inp3 was grown in a high osmotic strength medium such as 12% sucrose or starch-Inp medium at 60 p.p.t. salinity.

Table 7 shows the fatty acid composition of Inp3 cultured in five different experiments, with starch-Inp containing betaine as basic medium, and four individually added salts, at the concentrations given for the artificial sea-water mixture described by Reichenbach & Dworkin (1981). In another experiment 30 g KCl l⁻¹ (approximately 0.4 M, final concentration) was added to the medium to compare the effects of equal weight concentrations of NaCl and KCl. Addition of NaCl alone led to PUFA levels similar to those obtained with

Table 6. *Fatty acid composition of Flexibacter strain Inp3 after growth on different media*Values in parentheses represent means $\pm$ 1 SD from two separate experiments. ND, Not detected.

Fatty acid	Fatty acid composition (percentage of total fatty acids)						
	Glucose (5 g l ⁻¹)	Starch (5 g l ⁻¹)	Sucrose (12%)	Sucrose (12%) + 1 mM-cAMP	Starch (60 p.p.t.)	Starch (60 p.p.t.) + 1 mM-cAMP	Starch (60 p.p.t.) + 1 μ M-DNP
14:0	ND	2.1(0.2)	1.3(0.6)	1.8(0.3)	2.2(0.6)	1.9(0.2)	2.3(0.4)
i15:1	4.3(0.6)	7.9(1.0)	1.6(0.3)	4.4(0.1)	2.0(0.3)	6.3(0.1)	5.3(1.1)
a15:0	0.2(0.1)	0.8(0.2)	0.2(0.1)	0.4(0.3)	0.5(0.2)	0.3(0.4)	0.9(0.3)
15:0	0.4(0.2)	0.4(0.6)	0.4(0.2)	0.8(0.5)	0.8(0.3)	0.5(0.7)	0.7(0.1)
16:0	25.0(1.2)	23.1(0.8)	28.4(0.0)	23.3(4.3)	28.5(0.4)	21.3(4.6)	17.8(1.1)
16:1 ω 9	ND	ND	0.5(0.2)	0.2(0.3)	2.4(0.4)	ND	ND
16:1 ω 7	1.0(0.3)	2.5(0.0)	1.2(0.2)	1.6(0.6)	2.1(0.1)	3.0(1.3)	2.8(0.6)
16:1 ω 5	60.8(0.2)	57.9(0.1)	13.3(1.5)	43.5(2.4)	22.1(4.7)	45.6(11.4)	59.3(0.5)
18:0	1.3(0.9)	0.6(0.3)	4.1(1.0)	2.4(0.4)	4.7(0.3)	4.9(1.1)	3.6(3.4)
18:1 ω 9	0.8(0.3)	0.9(0.0)	10.0(1.1)	5.2(2.1)	6.8(0.7)	6.8(4.7)	1.5(0.1)
18:1 ω 7	0.2(0.1)	0.1(0.1)	2.2(0.3)	1.2(0.7)	5.8(1.1)	2.2(3.1)	0.3(0.1)
18:2 ω 6	0.2(0.0)	0.2(0.1)	9.8(0.8)	2.3(0.9)	6.1(0.9)	2.9(0.9)	0.9(0.5)
18:3 ω 3	ND	ND	1.0(0.2)	0.6(0.4)	2.0(0.2)	0.7(0.0)	0.3(0.2)
20:1 ω 9	ND	ND	2.1(0.8)	ND	ND	ND	ND
Unknown	ND	ND	10.2(2.5)	ND	ND	ND	ND

Table 7. *Fatty acid composition of Flexibacter strain Inp3 after growth on starch-Inp medium plus salts*

Flexibacter strain Inp3 was grown on starch-Inp medium containing 10 mM-betaine together with individually added salts at concentrations corresponding to those in the artificial sea-water mixture described in Methods. ND, Not detected.

Fatty acid	Fatty acid composition (percentage of total fatty acids)				
	NaCl (30 g l ⁻¹)	KCl (0.7 g l ⁻¹)	MgSO ₄ / MgCl ₂ (11.2 g l ⁻¹)	CaCl ₂ (1.0 g l ⁻¹)	KCl (30 g l ⁻¹)
14:0	1.2	0.6	1.1	1.7	1.5
i15:0	0.9	6.5	5.0	6.9	4.1
a15:0	ND	0.6	ND	ND	0.1
15:0	ND	ND	ND	ND	0.2
16:0	39.0	21.7	21.3	19.9	14.9
16:1 ω 9	ND	ND	ND	ND	0.7
16:1 ω 7	ND	2.3	1.4	1.5	0.4
16:1 ω 5	18.0	62.6	59.4	55.7	61.4
18:0	25.2	1.3	2.8	2.4	1.5
18:1 ω 9	5.8	0.5	1.9	2.7	5.2
18:1 ω 7	ND	ND	ND	0.3	0.8
18:2 ω 6	6.1	0.4	2.2	1.4	1.0
18:3 ω 3	1.8	ND	ND	0.6	0.4

the full artificial sea-water complex. Both MgSO₄/MgCl₂ and CaCl₂ slightly increased the PUFA content, whereas no change was found when KCl was added at either concentration.

Effect of cAMP and DNP on Flexibacter strain Inp3

Addition of cAMP to the culture medium lowered the proportion of PUFAs in *Flexibacter* strain Inp3 growing at a high salt concentration in 12% sucrose medium

(Table 6). Addition of cAMP reduced the proportion of all acids with an 18-carbon chain length in Inp3. The effects of the uncoupler DNP (1 μ M) and cAMP on the fatty acid composition of Inp3 were similar (Table 6). Although DNP did not prevent the growth of Inp3 it reduced the growth rate approximately twofold.

Discussion

Intriago & Floodgate (1991) regarded the presence and inverse relationship in *Flexibacter* strain Inp between the two major fatty acids C16:1 ω 5 and C18:1 ω 9 as evidence for the presence of both the aerobic and anaerobic pathways for synthesis of unsaturated fatty acids in this bacterium. If the anaerobic pathway were present the antibiotic cerulenin should inhibit synthesis by this pathway. Fatty acid metabolism in *Flexibacter* strain Inp2 was affected by cerulenin in two ways: firstly, cerulenin inhibited fatty acid synthetase, and with it the synthesis of C16:1; secondly, it enhanced PUFA synthesis. These two effects could be the result of the presence of both the aerobic and anaerobic pathways for UFA synthesis. It also shows that as the flow of substrate from one pathway rises, the flow of substrate from the other is lowered. Wada *et al.* (1989) demonstrated the presence of both pathways for fatty acid synthesis in *Pseudomonas* E-3. The selectivity of cerulenin to inhibit UFA synthesis by the anaerobic pathway, is well documented (Buttke & Ingram, 1978; Wada *et al.*, 1989). The partial inhibition of C16:1 synthesis by cerulenin in glucose medium may be due to either a more resistant β -keto-acyl synthase I present in bacteria grown in

glucose, or the palmitoleate detected was an isomer other than C16:1w5, and was synthesized by the aerobic pathway.

The results of Intriago & Floodgate (1991), together with these from the present study, show that high osmotic strength stimulates PUFA production in *Flexibacter* Inp and related strains, and that this is prevented by addition of cAMP to the medium. Piovanti & Lazdunski (1975) demonstrated that media with high osmotic strength, such as 12% sucrose, caused a fourfold decrease in the intracellular cAMP level in *E. coli*, and Brownlie *et al.* (1985) found that a high NaCl concentration in the medium inhibited adenylate cyclase in *Bordetella pertussis*. Thus it seems possible that high osmotic strength media lower the intracellular levels of cAMP in *Flexibacter* Inp strains, resulting in the observed increase in PUFA content. However, it is not clear whether the effect of cAMP on Inp2 and Inp3 was involved in facilitating the transcription of genes encoding for proteins, as has been reported for other organisms (Buettner *et al.*, 1973; Botsford, 1981; Roy *et al.*, 1988), or in the activation of a cAMP-dependent protein kinase. Although protein kinases have been thought to be absent in prokaryotes (Dadssi & Cozzzone, 1985), some exceptions have been found (Muller *et al.*, 1985; Saha *et al.*, 1988). More recently, Kinney *et al.* (1990) demonstrated regulation of phospholipid biosynthesis by a cAMP kinase in *S. cerevisiae*.

The present study has demonstrated that PUFA synthesis is restored in the normally PUFA-deficient *Flexibacter* strain Inp3 by increasing the osmotic strength in the medium either with ionic solutes such as NaCl or non-ionic ones such as sucrose. Nevertheless, it is clear from the data that NaCl and sucrose are more effective in increasing the proportion of PUFAs than was KCl. However, whilst sea-water medium containing sucrose gives an extra 0.225 g-mol osmotic units to that of the sea water (0.473 g-mol), 30 g l⁻¹ of both NaCl and KCl gives 0.523 g-mol and 0.404 g-mol osmotic units respectively (Weast, 1987). Hence it can be concluded that osmotic strength is not the only factor controlling PUFA biosynthesis.

Addition of the uncoupler DNP has been shown to raise the cAMP level in the yeast *Saccharomyces cerevisiae* (Thevelein *et al.*, 1987). Fatty acid analysis of protonophore-resistant bacteria has revealed variation between species. Thus, whilst unsaturated fatty acids decreased in *Bacillus subtilis* (Krulwich *et al.*, 1987), they increased in *E. coli* (Herring *et al.*, 1985). Furthermore, Kogure & Tokuda (1986) showed that the growth of marine bacteria can depend on either the proton- or sodium-motive force. On this basis, it may be suggested that the inhibition by DNP of PUFA synthesis in *Flexibacter* Inp3, as seen in the present study, was caused

by an increase in cellular cAMP, which in turn was regulated by changes in membrane electrochemical potential.

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