Phytochemistry, 1974, Vol. 13, pp. 905 to 910. Pergamon Press, Printed in England.

SPECIFICITY EFFECTS IN THE BIOSYNTHESIS OF FATTY ACIDS IN *BACILLUS ACIDOCALDARIUS*

MARIO DE ROSA and AGATA GAMBACORTA

Laboratorio per la Chimica e Fisica di Molecole di Interesse Biologico del C.N.R., Arco Felice, Napoli, Italia

and

JOHN D. BU'LOCK

Microbial Chemistry Laboratory, Department of Chemistry, University of Manchester, Manchester M13 9PL, England

(*Received* 16 *September* 1973. *Accepted* 22 *October* 1973)

Key Word Index—*Bacillus acidocaldarius*; Bacteria; fatty acids; ω-cyclohexyl fatty acids; new fatty acid types; biosynthesis.

Abstract—Addition to *Bacillus acidocaldarius* of acids which can act as primers for fatty acid synthesis promote the synthesis of corresponding fatty acids competitively. The effective acids are *n*-C_5 to -C_7 (not C_4 or C_8), *iso*- and *anteiso*-C_5 and -C_6 (not C_4), and a range of cyclic acids from cyclobutylacetic and cyclopentanecarboxylic to cycloheptylacetic. New non-natural ω-cyclobutyl-, ω-cyclopentyl-, and ω-cycloheptyl-fatty acids are obtainable. The range of acceptable primers and the range of fatty acids produced therefrom indicate, respectively, the substrate specificities of the transacylase which introduces acyl species into fatty acids synthesis and the one which removes them. The specificity of the primer transacylase may be similar to that in some rumen anaerobes.

INTRODUCTION

Bacillus acidocaldarius is an unusual thermophilic acidophilic organism.[1,2] The fatty acid mixture from its lipids[3] comprises a major proportion of unique acids, 11-cyclohexylundecanoic and 13-cyclohexyltridecanoic, together with acids more typical of *Bacillus* species[4] viz *iso*-C_{15} to -C_{17}, *anteiso*-C_{15} and -C_{17}, and *n*-C_{16}. We have shown that the ω-cyclohexyl acids are formed by homologation of cyclohexanecarboxylic acid, synthesized in *B. acidocaldarius* by deoxygenation of shikimic acid.[6] The rather complex effects of temperature, pH, and aeration on the fatty acid composition are described elsewhere.[5]

In bacteria generally, the proportions of the biogenetically different types of fatty acid reflect competition between the different available acyl-CoA species for a transacylase which attaches the "primer" acyl residues to acyl carrier protein. This has been demonstrated by studies of fatty acid synthesis in various cell-free systems, but comparable

[1] DARLAND, G. and BROCK, T. D. (1971) *J. Gen. Microb.* **67**, 9.
[2] DE ROSA, M., GAMBACORTA, A. and BU'LOCK, J. D. (1971) *Giornale di Microbiologia* **19**.
[3] DE ROSS, M., GAMBACORTA, A., MINALE, L. and BU'LOCK, J. D. (1971) *Chem. Commun.*, 1334.
[4] KANEDA, T. (1967) *J. Bact.* **93**, 894.
[5] DE ROSA, M., GAMBACORTA, A. and BU'LOCK, J. D. (1973) *J. Bact.* in press.
[6] DE ROSA, M., GAMBACORTA, A., MINALE, L. and BU'LOCK, J. D. (1972) *Biochem. J.* **128**, 751.

evidence can generally be obtained by studying the effects on fatty acid synthesis of adding an excess of the primer acids to the bacterial cultures. We have used this approach to show that there is only a single transacylase of this type in *B. acidocaldarius*, and to establish its specificity for different types of primer including cyclic acids similar to cyclohexanecarboxylic.

RESULTS

The results of GLC analyses of methyl esters from *B. acidocaldarius* fatty acids following incubations with different fatty acid precursors are presented in Tables 1–3. Where possible, precursors were tested at 0·5 mM, but some measurements were made for 0·1 mM additions particularly for the more toxic acids which at 0·5 mM drastically reduced cell multiplication. All the tabulated results are actual yields of fatty acids, i.e. in mg per g of cells, rather than percentage composition of the mixtures, so that changes in the amounts as well as the relative proportions of the different acids can be followed; biogenetically homologous acids are grouped together but were of course measured separately.

The following new acids, from prolonged incubations (see below) with cyclobutylacetic acid, cyclopentylacetic acid, and cycloheptanecarboxylic acid respectively, were isolated as their methyl esters by preparative GLC and characterized spectroscopically (NMR, MS) and by GLC (see experimental): 12-cyclobutyldodecanoic, 12-cyclopentyldodecanoic, 11-cycloheptylundecanoic. Other acids were identified by the GLC retention times of their methyl esters as read off on straight-line plots of their equivalent chain length (see Experimental).

TABLE 1. EFFECTS OF PRIMERS FOR THE SYNTHESIS OF NATURAL FATTY ACIDS ON FATTY ACID PRODUCTION BY *Bacillus acidocaldarius**

Precursor added (mM)	Acids recovered (mg/g lyophilized cells)				
	n-C_{14}, C_{16}	*iso*-C_{16}	*iso*-C_{15}, C_{17}	*anteiso*-C_{15}, C_{17}	ω-cyclohexyl-C_{17}, C_{19}
None (control)	1·0	2·0	3·3	7·3	18·9
Butyric (0·5)	1·2	1·7	3·4	7·0	20·1
Hexanoic (0·5)	**19·2**	*0*	*2·0*	*1·8*	*16·8*
Hexanoic (0·1)	**6·7**	*0·9*	*2·8*	*3·9*	*18·0*
Octanoic (0·1)	1·5	1·8	4·2	7·9	19·1
Isobutyric (0·5)	0·9	1·8	3·6	7·1	19·0
Isocaproic (0·5)	*0*	**21·0**	*0*	*0*	*17·3*
Isovaleric (0·5)	*0·2*	*0*	**30·4**	*1·1*	18·2
2-Methylbutyric (0·5)	*0·3*	*0·2*	*1·9*	**20·1**	*17·4*
Cyclohexanecarboxylic (0·5)	*0*	*0*	*0*	*0*	**43·1**
Cyclohexanecarboxylic (0·1)	*0·1*	*0*	*0·2*	*0·2*	**44·2**
2-Cyclohexylpropionic (0·1)	*0*	*1·1*	*2·7*	*5·2*	**24·1**

* Grown at 60°, pH 3·5, and harvested at end of log phase; precursors added at inoculation. Acids significantly more than controls in **bold**; significantly less in *italics*.

Precursors of typical fatty acids

The typical fatty acid composition of *B. acidocaldarius* is seen in control data: 57% of ω-cyclohexyl-C_{17} and -C_{19} acids, 22% of *anteiso*-C_{15} and -C_{17}, 10% of *iso*-C_{15} and -C_{17}, 6% of *iso*-C_{16}, and 3% of *n*-C_{14} and -C_{16}. Suitable precursors selectively increase

the production of each class of fatty acids as shown in Table 1, and the effects are specific for certain chain-lengths in the precursor. Thus hexanoic, but not butyric or octanoic, increases the yield of *n*-C_{14} and -C_{16} acids; isovaleric (C_5), isocaproic (C_6), and α-methylbutyric (C_5) increase the yields of *iso*-C_{15} and -C_{17}, *iso*-C_{16}, and *anteiso*-C_{15} and C_{17} respectively, but isobutyric acid (C_4) has no effect. Cyclohexanecarboxylic acid has a much larger effect than 3-cyclohexyl-propionic acid as a precursor for the ω-cyclohexyl-C_{17} and -C_{19} acids.

Precursors of un-natural fatty acids

Within similar specificity limits, other acids to *B. acidocaldarius* cause substantial synthesis of fatty acids not normally found, as shown in Table 2. Both valeric (C_5) and heptanoic acids, but not propionic or nonanoic, are converted into the odd-numbered *n*-C_{15} and -C_{17} acids which are normally negligible; similarly cyclohexylacetic acid (and to a much lesser degree, 4-cyclohexylbutyric acid) is converted into the non-natural even-numbered ω-cyclohexyl-C_{18} and -C_{20} acids. Other types of cyclic acid can be used, as Table 2 shows, giving rise to ω-cyclobutyl-, ω-cyclopentyl-, and ω-cycloheptyl-acids of the indicated chain lengths, and there are similar specificity-requirements for these precursors, since cyclopropanecarboxylic, cyclopropylacetic, and cyclobutanecarboxylic acids are all ineffective.

TABLE 2. EFFECT OF PRIMERS FOR THE SYNTHESIS OF NON-NATURAL FATTY ACIDS ON FATTY ACID PRODUCTION BY *Bacillus acidocaldarius**

Precursor added (mM)	Acids recovered (mg/g lyophilized cells)					
	n-C_{14}, C_{16}	*iso*-C_{16}	*iso*-C_{15}, C_{17}	*anteiso*-C_{15}, C_{17}	ω-cyclohexyl-C_{17}, C_{19}	Other
Propionic (0·5)	1·2	1·3	2·9	7·1	19·2	—
Valeric (0·5)	0·5	1·1	1·9	3·6	17·0	***n*-C_{15}, C_{17} 14·9**
Valeric (0·1)	0·7	1·9	3·5	7·1	19·1	***n*-C_{15}, C_{17} 1·8**
Heptanoic (0·5)	0·4	1·2	1·6	3·8	16·3	***n*-C_{15}, C_{17} 20·9**
Heptanoic (0·1)	0·9	2·1	3·2	7·4	18·4	***n*C_{15}, C_{17} 1·3**
Nonanoic (0·1)	1·1	2·0	3·6	7·7	18·4	—
Cyclopropanecarboxylic (0·5)	1·1	2·1	3·0	7·6	17·4	—
Cyclopropylacetic (0·5)	1·4	1·9	3·1	7·1	19·5	—
Cyclobutanecarboxylic (0·5)	1·1	2·1	2·9	7·5	18·2	—
Cyclobutylacetic (0·5)	0·2	0·4	0·4	0·5	14·2	**ω-cyclobutyl-C_{16}, C_{18} 24·1**
Cyclopentanecarboxylic (0·5)	0·1	0·7	1·6	1·9	16·1	**ω-cyclopentyl-C_{16}, C_{18} 20·1**
Cyclopentylacetic (0·5)	0·4	0·9	1·4	2·0	17·1	**ω-cyclopentyl-C_{17}, C_{19} 16·2**
Cyclohexylacetic (0·5)	0	0	0	0	17·2	**ω-cyclohexyl-C_{18}, C_{20} 18·4**
Cyclohexylacetic (0·1)	0	0	1·1	2·3	19·4	**ω-cyclohexyl-C_{18}, C_{20} 15·2**
4-Cyclohexylbutyric (0·1)	0	1·5	3·1	5·2	18·2	**ω-cyclohexyl-C_{18}, C_{20} 5·1**
Cycloheptanecarboxylic (0·5)	0·1	0·1	0·4	0·5	10·1	**ω-cycloheptyl-C_{18}, C_{20} 25·2**
Cycloheptanecarboxylic (0·1)	0·5	1·0	2·6	3·1	17·0	**ω-cycloheptyl-C_{18}, C_{20} 7·1**
Cycloheptylacetic (0·1)	0·7	1·2	2·6	3·4	17·5	**ω-cycloheptyl-C_{19}, C_{21} 8·2**

* Details as Table 1.

Precursor competition effects

The data of Tables 1 and 2 suggest that added acids which fulfil the specificity requirements compete with natural precursors in a synthetic system common to all the fatty acid types; each increase in the amount of a particular product is accompanied by decreases in the yields of all other types. There is however a net increase in fatty acid yield in each case, i.e. the increase in one type of product is attained partly by competition with other types and partly by an increase in total fatty acid synthesis.

If fermentations with added precursor are carried on for a further 24–30 hr after the end of the logarithmic phase of growth the yield of acids corresponding to the added precursor is even further increased and that of the remaining acids lowered; typical results are summarized in Table 3. This is ascribed to further competition by unused precursor during a phase of fatty acid turnover. By such prolonged incubations, up to 70% of, e.g. cycloheptanecarboxylic acid added at 0·1 mM can be converted into the corresponding 11-cycloheptylundecanoic acid.

Table 3. Effect of added primers on fatty acid synthesis in prolonged incubations with *Bacillus acidocaldarius**

Added precursor (0·5 mM)	Yield at end of log phase, mg/g New acids	Others (total)	Yield 24–30 hr later, mg/g New acids	Others†
Cyclobutylacetic	ω-cyclobutyl-C_{16}, C_{18} 24·1	15·7	38·5	7·4
Cyclopentylacetic	ω-cyclopentyl-C_{17}, C_{19} 16·2	21·8	33·2	4·8
Cycloheptanecarboxylic	ω-cycloheptyl-C_{18}, C_{20} 25·2	11·2	29·2	3·8

* Grown at 60°, pH 3·5 and harvested at the end of log phase (data from Table 2) or 24–30 hr later.
† All ω-cyclohexyl-C_{17} and -C_{19}.

Chain-length of products

The chain-length of the fatty acids produced in these incubations is specified independently of the requirements for precursors, apart from the obvious consequence that precursors with an odd number of C atoms give rise to odd-numbered fatty acids. Thus both valeric and heptanoic acids give rise to the same mixture of *n*-C_{15} and -C_{17} acids; other data in Tables 1 and 2 show the same pattern.

Effect of aminoacids

The effect of added α-aminoacids is the same as that of the acids to which they would give rise, in the form of CoA esters, by deamination and decarboxylation, i.e. leucine and isoleucine increase the formation of the *iso*- and *anteiso*-C_{15} and -C_{17} acids, whereas additions of valine have no effect on the yield of the *iso*-C_{16} acids. This helps to confirm the supposition that the apparent primer specificities relate to the actual acyl-CoA transacylase and not, e.g. to the specificity of a system for converting the added acids into their CoA derivatives, which would be bypassed by supplying the aminoacids.

DISCUSSION

Although in some mammalian systems for fatty acid synthesis the type of fatty acid produced is largely determined by the type of acyl-CoA primer available,[7] the type of fatty acid produced in bacterial systems appears to be mainly determined by the specificity of the primer transacylase which first attaches an acyl group to the acyl carrier protein. For example, in *B. subtilis* the primer transacylase will accept the CoA derivatives of isobutyric, isovaleric, and 2-methylbutyric acids as substrates, but not acetyl-CoA, whereas the subsequent stages of fatty acid synthesis will proceed satisfactorily if provided with the *S*-acetyl derivative of the acyl carrier protein; on the other hand in *Escherichia*

[7] Horning, M. G., Martin, D. B., Karmen, A. and Vagelos, P. R. (1961) *J. Biol. Chem.* **236**, 669.

coli the initial transacylase discriminates against branched-chain primers and in favour of acetyl while the subsequent steps are similarly discriminatory but to a less marked extent.[8] In *B. subtilis* the relative effectiveness of different acyl-CoA as primers closely parallels the composition of the fatty acid mixture which the system produces, and conversely we feel justified in the present case in using the fatty acid data as an index of the specificity of primer transacylation. The apparent competition between all the types of precursor used suggests that only one transacylase is involved in our system.

From the data it is apparent that the *B. acidocaldarius* system will accept straightchain acids with 5, 6, or 7 carbon atoms, branched chain acids with 5 or 6 carbon atoms, and cyclic acids with 6–9 carbon atoms (i.e. from cyclobutylacetic and cyclopentanecarboxylic to cycloheptylacetic acids). Apparently the cyclic acids of larger carbon number can be accommodated in the substrate-binding region of the enzyme because they are relatively more compact than the acyclic acids. The utilization of such cyclic acids has not been investigated in other bacteria but it is clearly important in *B. acidocaldarius* in which cyclohexane–carboxylic acid is formed endogenously from shikimate.[6]

The specificity for branched-chain acids is not identical with that of the *B. subtilis* system.[8] Both utilise the C_5 acids isovaleric and 2-methylbutyric, but whereas in *B. subtilis* the *iso*-C_{16} product arises from isobutyric acid, the present system only utilizes its C_6 homologue isocaproic. Isobutyryl-CoA would be available by oxidative decarboxylation of the valine precursor, α-ketoisovalerate,[9] but no endogenous source of isocaproic acid in *B. acidocaldarius* can be suggested. The comparison with *B. subtilis* shows a similar pattern when the utilization of straightchain acids is considered. Additions of propionic, butyric, and valeric acids cause competitive increments in the corresponding *n*-C_{14} to -C_{17} acids in *B. subtilis*[10] whereas in *B. acidocaldarius* the effective range of precursor acids is from C_5 to C_7.

It is interesting that the apparent preference for primer acids of intermediate chain-length shown by *B. acidocaldarius* is closely paralleled in *Selenomonas ruminantium*, a Gram-negative rumen anaerobe in which *n*-C_5 to -C_8 acids are the preferred primers for synthesis of the normal fatty acids;[11] the only other source of the ω-cyclohexyl acids of *B. acidocaldarius* is from an unidentified component of rumen flora.[12] Another rumen anaerobe, *Bacterioides succinogenes*, which like several such species requires primer fatty acids for growth, likewise utilizes *n*-C_5 to -C_8 primers for the synthesis of *n*-C_{13} to -C_{16} fatty acids but will accept only isobutyric and 2-methylbutyric acids for synthesis of branched-chain acids.[13]

The chain-length of the "finished" fatty acids in the *B. acidocaldarius* system is, as already noted, determined independently of the size of the primer species. However, as with the primer transacylase, the specificity of this terminal step is such that longer chains are accommodated when the molecule has a cyclic ω-terminal. The preferred chain-lengths are: for *n*-acids, C_{15} and C_{16}; for *iso*- and *anteiso*-acids, C_{16} and C_{17}, for ω-cyclobutyl, C_{16}; for ω-cyclopentyl, C_{17} and C_{18}; for ω-cyclohexyl, C_{17} to C_{19};

[8] BUTTERWORTH, P. H. W. and BLOCH, K. (1970) *European J. Biochem.* **12,** 496.
[9] KANEDA, T. (1965) *Canad. J. Microbiol.* **12,** 501.
[10] KANEDA, T. (1963) *J. Biol. Chem.* **238,** 1229.
[11] KANEGASAKI, S. and NUMA, S. (1970) *Biochim. Biophys. Acta* **202,** 436.
[12] HANSEN, R. P. (1967) *Chem. Ind.* 1640.
[13] WEGNER, G. H. and FOSTER, E. M. (1963) *J. Bact.*, **85,** 53.

for ω-cycloheptyl, C_{18} and C_{19}. Dimensionally, all these molecules are of approximately the same length, a feature which doubtless underlies the specificity of the enzyme which removes them from the system of fatty acid synthesis.

EXPERIMENTAL

Precursor acids. The precursor acids were purchased commercially except for cyclopropyl-, cyclobutyl, and cycloheptyl-acetic acids which were prepared by Arndt-Eistert homologation from cyclopropane-, cyclobutane-, and cycloheptane-carboxylic acids respectively; after purification the products gave satisfactory MS and NMR spectra.

Identification of acids. Cultures of *Bacillus acidocaldarius* were grown at 60° in sparged flasks containing 100 ml of medium[2,6] (0·1% glucose, 0·1% yeast extract, ammonium and other salts, H_2SO_4 to pH 3·5) plus 0·5 or 0·1 mM precursor acid. At the end of log phase growth the cells were centrifuged, washed with H_2O, lyophilized and weighed (30–40 mg). After addition of a reference standard of methyl stearate the cells were saponified (10% KOH in 1 : 1 aq. MeOH, 5–6 hr reflux), and the recovered acids (30–44 μg/mg cells) methylated and analysed by GLC on a Carlo Erba Fractovap GV using a 2 m × 5 mm silanized column packed with 10% diethyleneglycol succinate on 100–120 mesh Chromosorb P at 200°.

Larger-scale recovery of acids. The organism was grown on 25 l. of the same medium in a 30 l. fermentor with 12·5 l/min air sparging and with 0·5 mM cyclobutylacetic, cyclopentylacetic, or cycloheptanecarboxylic acid. Some 30 hr from the end of log phase the cells were centrifuged down, washed, lyophilized, and saponified; the recovered acids were methylated and after preliminary purification on a silica gel column the major components were recovered, 97–98% pure, by preparative-scale GLC (2 m × 10 mm column with 25% DEGS on Chromosorb P 30–60 at 180° with N_2 at 130 ml/min). The esters obtained all showed no C–Me signal in the NMR and ran as saturated esters on silica gel–$AgNO_3$. Methyl 12-cyclobutyldodecanoate, M^+/e 268, showed cyclobutane proton signals at $\delta = 2{\cdot}13$; methyl 12-cyclopentyl-dodecanoate. M^+/e 282, showed cyclopentane proton signals at $\delta = 1{\cdot}53$; methyl 11-cycloheptylundecanoate showed cycloheptane proton signals at $\delta = 1{\cdot}61$.

Equivalent chain lengths. From the GLC data for known esters for the above cyclic esters and for homologous esters from the small-scale incubations, plots of log (retention time) vs chain-length were linear, and a table of equivalent chain lengths was drawn up. For methyl esters of acids with y carbon atoms in the chain the equivalent chain lengths are: *n*-series, $y + 0{\cdot}00$; *iso*-series, $y - 0{\cdot}43$; *anteiso*, $y - 0{\cdot}37$; ω-cyclobutyl series, $y + 0{\cdot}22$; ω-cyclopentyl series, $y + 0{\cdot}65$; ω-cyclohexyl series, $y + 1{\cdot}90$; ω-cycloheptyl series, $y + 2{\cdot}60$.

Acknowledgements—We are grateful to E. Esposito and S. Sodano for technical assistance and to A. Milone for GLC operation.