Fatty acids synthesized by oral treponemes in chemically defined media

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Abstract

OMIZ-W68, a chemically defined medium that contains no long-chain fatty acids and yet supports *in vitro* proliferation of a wide range of fastidious oral anaerobes, is described. The type strains of *Treponema denticola*, *Treponema lecithinolyticum*, *Treponema maltophilum*, *Treponema pectinovorum*, *Treponema socranskii*, and an as yet unpublished canine *Treponema* species could be propagated indefinitely in this medium with sugar supplements for the saccharolytic species. Analysis of the cellular fatty acids (CFA) of these treponemes by gas chromatography demonstrated the synthesis of C14, C15, C16, and C17 fatty acids (linear-, iso-, and anteiso-forms) in various proportions, but neither hydroxy- nor unsaturated fatty acids. However, between 0% and 40% of the eluted material could not be identified. The proportions of CFAs differed not only between species but also between the eight strains of *Treponema denticola* investigated. Replacing OMIZ-W68 by a derivative minimal essential medium (OMIZ-M/TDCDK) developed for *Treponema denticola* had little effect on the CFA profiles. In contrast, the CFA profiles of treponemes grown in OMIZ-W68 showed at best minor similarity to the strains from the Moore library of the Virginia Polytechnic Institute, which had been grown in media containing serum, peptones, and yeast extract.

Introduction

Treponemes are anaerobic spirochaetes associated with humans and animals. The type species *Treponema pallidum* is an obligate pathogen and has resisted attempts to axenic cultivation for over a century. In the human oral cavity, *c.* 60 species-level treponemal phylotypes have been recognized and appear to be associated with periodontal diseases. However, less than a quarter of these have so far been grown in culture (Wyss *et al.*, 2004). Until they become amenable to *in vitro* cultivation and genetic manipulation, research into the physiology and virulence of this plethora of host-associated organisms remains severely hampered (Norris *et al.*, 2003).

The natural habitat of these organisms is not only extremely complex in terms of numbers of individual chemical species (Aas *et al.*, 2005) but also ill understood as regards the individual and interactive roles of the components. Therefore, failures to grow fastidious bacteria in artificial media may have multiple causes, including simple lack of an essential nutrient, the presence of inhibitors (or overdose of essentials), and lack of balancing or neutralizing compounds (and any combination thereof). Through development of chemically defined media, factorial analysis becomes feasible and allows assessment of an organism's capacity for biosynthesis of cellular components like fatty acids.

Cellular fatty acids (CFA) constitute an important part of the treponemal cell mass, predominantly as phospholipids in the two membranes, which surround the cell body and enclose the periplasmic space with peptidoglycan and the terminally anchored endoflagellae. Fatty acid composition of phospholipids is regarded as a taxonomically relevant character, although it is often subject to environmental regulation. It is a major determinant of membrane flexibility and resistance, as well as activity of membrane-embedded proteins. In addition to the structural and physiological functions, unusual fatty acids have been detected in compounds affecting the immune system of the host (Yotis *et al.*, 1993; Sela *et al.*, 1997; Schultz *et al.*, 1998; Schröder *et al.*, 2000, 2001; Hashimoto *et al.*, 2003).

Fatty acids are costly to synthesize but are readily available to oral treponemes from saliva and crevicular fluid. Therefore, results from early studies that indicated that oral treponemes had lost the ability to synthesize these essential compounds were readily accepted and perpetuated (Olsen *et al.*, 2000; Norris *et al.*, 2003).

Here, the content and consequently synthesis of major CFAs is reported for a range of oral treponemes, grown in

newly developed chemically defined media containing no fatty acids, except the essential volatile iso-butyric and 2-methylbutyric acids.

Materials and methods

Bacterial strains

The origin, characteristics, and cultivation of the following treponemal strains have been described previously (Correia *et al*., 2003; Wyss *et al*., 2004): *Treponema denticola* (ATCC 35405^T, ATCC 33520, ATCC 33521, ATCC 35404, OMZ 830, OMZ 834, CD-1, and CDK), *Treponema lecithinolyticum* (OMZ 684^T), *Treponema maltophilum* (OMZ 679^T), *Treponema pectinovorum* (ATCC 33768^T), and *Treponema socranskii* ssp. *socranskii* (ATCC 35536^T).

OMZ 796 is a novel saccharolytic treponeme with one endoflagellum per pole, isolated from canine dental plaque (C. Wyss, unpublished). It was grown and maintained as described for the other isolates, as were strains from other taxa mentioned in the results section.

Media

Table 1 lists the composition of the two novel media developed in this study. OMIZ-W68 is a general-purpose basal medium for fastidious anaerobes derived from medium OMIZ-W1 (Wyss, 1992). OMIZ-M/TDCDK is derived from OMIZ-W68 by elimination of all compounds nonessential for both the type strain ATCC 35405^T and strain CDK of *Treponema denticola*. Preparation of the media was as described for OMIZ-W1, with appropriate changes of concentrations in the stock solutions for vitamins and trace salts.

CFA analysis

For CFA analysis, cells were grown in either OMIZ-W68 or OMIZ- M/TDCDK at 37 °C in an atmosphere of 10% H_2/5% CO_2/85% N_2, collected by centrifugation, and transferred in physiological saline into Eppendorf tubes. Except for *Treponema denticola*, medium OMIZ-W68 was supplemented with 2 g L^{-1} each of D-arabinose, D-galacturonic acid, D-glucose, D-maltose, D-mannose, D-ribose, D-xylose, and L-fucose.

Pellets of 50–80 μL were lyophilized and sent to DSMZ, Braunschweig, Germany (http://www.dsmz.de). Saponification, methylation, extraction, and gas chromatographic analysis (GC) were kindly performed by Dr R.M. Kroppenstedt using the Sherlock® Microbial Identification System (MIDI, Newark, DE), hereafter called the 'MIDI system'. To assess the presence of hydroxy fatty acids, extracts were analyzed by GC before and after passage through silica columns.

Table 1. Composition of two novel chemically defined media: OMIZ-W68 and OMIZ-M/TDCDK

	Medium	
Compounds	OMIZ-W68	OMIZ-M/TDCDK
$NaHCO_3$	1000	1000
Vitamin C (ascorbic acid)	1000	1000
Glutathione, reduced	1536	1536
$FeSO_4 \cdot 7H_2O$	13.901	13.901
L-alanine	45	45
L-arginine	1044	1044
L-asparagine	150	150
L-aspartic acid	133	133
L-citrulline	175	
L-cysteine-HCl	352	352
L-glutamic acid	294	
L-glutamine	680	680
L-glycine	75	
L-histidine	620	620
L-isoleucine	131	131
L-leucine	131	131
L-lysine-HCl	182	182
L-methionine	149	149
L-ornithine-HCl	168	
L-phenylalanine	165	165
L-proline	115	115
L-serine	525	
L-threonine	119	119
L-tryptophan	102	102
L-tyrosine	90	90
L-valine	117	117
ACES (good buffer)	1822	1822
N-acetylglucosamine	500	
D, L-carnitine	200	
Citric acid, Na_3	200	200
Formic acid, Na	300	
Fumaric acid, Na_2	500	500
D, L-lactic acid 30% solution (mL L^{-1})	1.4	
Putrescine 2HCl	5	
Pyruvic acid, Na	550	550
Spermidine-3HCl	5	
iso-butyric acid (μL L^{-1})	10	10
2-methylbutyric acid (μL $^{-1}$)	10	10
Adenine	1.35	1.35
2-deoxythymidine	2.4	
Hypoxanthine	1.4	
Inosine	2.7	2.7
Uracil	1.1	1.1
Uridine	2.4	
$CaCl_2 \cdot 2H_2O$	147	147
$CuSO_4$	0.000798	0.000798
KCl	2267	2267
$MgCl_2 \cdot 6H_2O$	1016.5	1016.5
$MnSO_4 \cdot H_2O$	1.6902	1.6902
2-Na_2SeO_3	0.0173	0.0173
$NaH_2PO_4 \cdot H_2O$	140	140
$NaVO_3$	0.00061	0.00061
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0124	0.0124
NH_4Cl	267	267

Table 1. Continued.

Compounds	Medium OMIZ-W68	OMIZ-M/TDCDK
$NiSO_4 \cdot 6H_2O$	0.000131	0.000131
$SnCl_2 \cdot 2H_2O$	0.000118	0.000118
$ZnSO_4 \cdot 7H_2O$	0.287	0.287
D(+) − biotin	0.05	0.05
Choline chloride	50	
Coenzyme A, Na	1	1
1,4-dihydroxy-2-naphthoic acid		0.408
Flavin adenine dinucleotide	1	1
Folic acid	0.25	
Folinic acid, Ca	1	1
Myo-inositol	50	
β-nicotinamide adenine dinucleotide	1	
Nicotinic acid	1	1
Pantetheine	0.66	
D(+)-pantothenate, calcium	5	
Pyridoxal phosphate	5	5
Pyridoxal-HCl	5	
Pyridoxine-HCl	0.05	
Riboflavine	0.03	
Thiamine 2HCl	5	
Thiamine pyrophosphate	25	25
Vitamin B12	0.05	
Phenol red, Na	10	10
Final pH	6.9	6.9

Unless otherwise indicated, compound concentrations are indicated in $mg\,L^{-1}$.

Results and discussion

Need for improved defined culture media

The media used to isolate fastidious organisms like treponemes were largely aimed to reproduce their natural environment. For the few organisms for which this approach was successful, the complexity of the medium with poorly controllable components like sera, peptones, and yeast extract sets limits to the stringency of results from nutritional studies. These pioneering studies provided an essential starting base for the development of improved media. The present study is part of the efforts to develop a defined *in vitro* model of the oral microbiota involved in the development of periodontal diseases. Ecological theory predicts differences in growth characteristic between the members of complex consortia to be important for long-term community stability. To understand metabolic interactions in these consortia, it is important to study concurrently as wide a spectrum of fastidious oral bacteria as possible. In view of a special interest in oral treponemes, additionally, a nonoral spirochaete, *Borrelia burgdorferi*, the agent of lyme disease, was at times included in the cultural studies (Wyss & Ermert, 1996).

This approach not only led to the recognition of specific requirements and sensitivities of already cultured species but also to novel media permitting growth of previously uncultured organisms. The previously developed chemically defined OMIZ-W1 medium supports the long-term *in vitro* proliferation of a number of laboratory-adapted oral treponemes (Wyss, 1992). However, isolation of treponemes from human dental plaque was only successful when OMIZ-W1 was enriched with complex additives like serum, yeast extract, and peptones (Wyss *et al.*, 2004). Ensuing nutritional studies of such newly available clinical *Treponema* isolates, as well as a range of other fastidious oral anaerobes led to sequential changes in the basic formulation. These include addition of compounds to satisfy newly identified requirements and corrections in compound concentration to avoid inhibition and/or improve satisfaction of requirements, which optimally led to the replacement of undefined components. As an example, pantetheine, an intermediate in coenzyme A synthesis, was found to be necessary because *Treponema lecithinolyticum* proved to be unable to use coenzyme A, which is, however, essential for *Treponema denticola* and *Treponema* 'vincentii'. Some components of the medium had to be added at much higher concentrations than in OMIZ-W1 as they were found to be limiting the growth of some organisms. This specifically applies for ferrous sulfate, which was increased to 5×10^{-5} M for the sake of treponemes, and to manganese sulfate, which was increased to 1×10^{-5} M for the sake of iron-independent streptococci and *B. burgdorferi*. The efforts to modify OMIZ-type media to support *B. burgdorferi* led to an enhanced recognition of the importance of osmolarity and pH on top of specific chemical requirements (Wyss & Ermert, 1996). Sometimes, the concentration range is narrow between sufficient for growth of an auxotrophic species and inhibitory to another organism. This was seen for riboflavin, which, at $0.03\,mg\,L^{-1}$, was still limiting for *Fusobacterium nucleatum* ssp. *fusiforme* (NCTC 11326) but at $0.1\,mg\,L^{-1}$ inhibited proliferation of some spirochetes.

Description and characteristics of media OMIZ-W68 and M/TDCDK

All these observations and extensive additional testing (not shown) led to the formulation of medium OMIZ-W68 listed in Table 1. To enhance experimental flexibility, save costs, and avoid inhibitory effects, many compounds essential only for some specific organisms were not included in the basic formula. This includes the carbohydrates required only by saccharolytic organisms, or *N*-acetylmuramic acid, which is uniquely an essential nutrient for *Tannerella forsythia* (Wyss, 1989). Table 1 also includes the much-reduced formulation of medium OMIZ-M/TDCDK, which is a minimal essential medium for *Treponema denticola*. This formulation was

obtained with ATCC 34405^T and CDK as test organisms by the sequential elimination of nonessential compounds from OMIZ-W68. During this process, the requirement of *Treponema denticola* for selenite was identified (Rother *et al.*, 2001). Some compounds are included, which are essential only for one of the strains. In particular, uracil is nonessential for the type strain and aspartic acid is not required by CDK. Different strains of *Treponema denticola* proved to differ in growth requirements: of the eight strains analyzed for fatty acid content, only CD1, CDK, ATCC 35405^T, and ATCC 33520 could be grown in OMIZ-M/TDCDK. A factorial analysis to determine which components of OMIZ-W68 need to be added to OMIZ-M/TDCDK to achieve growth of the other strains has not yet been performed. However, during investigations on the effects of defined peptides on the growth of different strains of *Treponema denticola*, it was found that millimolar concentrations of some dipeptides like threonyl-valine and valyl-aspartate initiated the growth of strain ATCC 33521 in OMIZ-M/TDCDK. While this indicates considerable metabolic versatility of *Treponema denticola*, other strains of *Treponema denticola* appear to be more fastidious: some clinical isolates would not even grow in OMIZ-W68 unless supplied with some of the complex ingredients included in medium OMIZ-Pat (Wyss *et al.*, 1996). Such additions usually also enhanced the growth of the treponemes described in this report. The identification of these metabolic differences remains a daunting challenge, but is of obvious importance to further expand the range of cultivable bacteria and to improve performance *in vitro*.

Interestingly, choline was not required by the two *Treponema denticola* strains and thus grown contained no detectable phosphatidylcholine. Consistent with the findings of Kent *et al.* (2004), who abrogated phosphatidylcholine synthesis by gene deletion, these cells were not visibly affected in morphology or growth *in vitro*, although normally *c.* 30% of treponemal phospholipid is phosphatidylcholine (Livermore & Johnson, 1974).

For the treponemal isolates used in this study, the growth rate as measured by intervals between consecutive passages (using dilutions of 10^{-7} v/v) in liquid media ranged between 3 days for *Treponema denticola* and 7 days for *Treponema lecithinolyticum*, while the growth yield as estimated by a hemocytometer ranged from 5×10^7 to 2×10^8 cells mL^{-1}. As indicated above, enrichment of OMIZ-W68 with further components may enhance the performance of many strains, or even be essential, as for some clinical treponemal isolates or for *B. burgdorferi*. Through such supplements, the chemically defined basal medium becomes equally complex and undefined as conventional 'Treponema' media such as TYGVS of Mangan *et al.* (1982). However, usually only a few components may be required on top of the rich but defined OMIZ-W68, which therefore makes identification by fractionation of undefined components like yeast extract analytically feasible (Wyss & Ermert, 1996).

Long-chain CFA content of treponemes propagated in OMIZ-W68 or M/TDCDK

In view of the absence of preformed long-chain fatty acids in the two novel media reported in Table 1, it was of interest to determine the CFA content of cells grown in these media. The range of oral treponemes studied and their CFA content as determined by the commercial MIDI system are listed in Table 2. The eight strains of *Treponema denticola* were similar in having major proportions of C14 : 0, C16 : 0, and anteiso C15 : 0 fatty acids, but they differed widely in their content of isoC14 : 0 and of nonidentified eluates (ECL 13.646, ECL 14.114). Furthermore, *Treponema denticola* OMZ 830 (ATCC 700768) was the only oral *Treponema* strain containing major amounts of C15 : 0. This strain is also exceptional in displaying a monoclonal antibody-defined epitope otherwise characteristic of a *Treponema pallidum* flagellar protein (Wyss *et al.*, 2004).

The only treponeme for which the present GC analysis identified all eluted components of the saponified and methylated cell extracts (both from OMIZ-W68 and from OMIZ-M/TDCDK cultures) was the nonmotile *Treponema denticola* variant strain CDK. The two analyses of CD-1, the motile parent of CDK, and the 12 analyses of the other (motile) strains yielded between 6% and 42% of eluted material that could not be identified by the MIDI system. It is interesting to speculate that there may be a functional connection: the rotation of the endoflagellae within the periplasmic space will impose a constant dynamic stress on the membranes and it could well be that the 'missing, unidentified' compounds function to stabilize the membranes and are not synthesized in the absence of motility-related stress.

In addition to *Treponema denticola*, five other species of oral treponemes, represented by their type strains, have been analyzed (Table 2). Definitely, all these species are capable of fatty acid synthesis. In how far the interspecies differences could be used for taxonomic purposes remains to be determined. It may be noted that the phylogenetically closely related species *Treponema maltophilum* and *Treponema lecithinolyticum*, show major differences in the proportion of C14 : 0 and C16 : 0 fatty acids. For systematic purposes, a dedicated library of CFA values would have to be collected, as the fatty acid profiles determined in the present study with cells grown in chemically defined media had at best a marginal match to the corresponding data collected in the Moore library of the Virginia Polytechnic Institute. Notably, the latter data set is used as reference by the MIDI system. It does not contain any entries of the novel

Table 2. Long-chain fatty acids of oral treponemes grown in chemically defined media*

		ECL†														
		CFA‡	12.546	12.926	13 : 0	13.646	14 : 0 iso	14 : 0	14.114	14.575	15 : 0 anteiso	15 : 0	16 : 0	16 : 0 iso	17 : 0 anteiso	% not identified
Strain		Medium														
T. denticola																
	35405^T	W68	1.5	0.6	0	0	**12.3**	**12.4**	**4.8**	0	**37.0**	1.9	**12.7**	**14.3**	2.5	**6.9**
	35405^T	M/TDCDK	2.2	0.8	0.2	**4.4**	**7.5**	**13.2**	**7.1**	0	**34.8**	1.9	**11.7**	**11.8**	2.2	**14.5**
	33520	W68	1.6	0.6	0.3	0	**10.0**	**6.5**	**10.0**	0	**35.5**	2.0	**14.9**	**11.0**	2.9	**12.1**
	33521	W68	1.4	1.4	1.3	**4.9**	3.1	**19.0**	**6.0**	0	**36.1**	**9.3**	**10.9**	**4.3**	1.2	**13.6**
	35404	W68	**5.3**	**5.8**	0	**24.8**	0	**10.3**	**4.8**	1.2	**28.8**	0.9	**12.2**	3.4	1.5	**41.9**
	830	W68	0.7	0.9	**4.7**	3.5	0	**14.4**	**4.4**	0	**30.4**	**30.5**	**6.6**	1.8	0.7	**9.6**
	834	W68	1.6	1.9	0.4	**6.4**	0	**15.3**	**7.0**	0.6	**26.6**	**5.3**	**22.5**	**7.4**	2.1	**17.5**
	CD-1	W68	**4.3**	**4.6**	2.0	**14.0**	0	**24.1**	**6.2**	0	**27.6**	**5.4**	**6.3**	3.4	0.9	**29.1**
	CD-1	M/TDCDK	0	2.8	0.8	**15.7**	0	**16.9**	**4.9**	0	**34.8**	**5.4**	**8.9**	**4.2**	1.0	**23.5**
	CDK	W68	0	0	0.2	0	**9.2**	**8.4**	0	0	**31.3**	2.7	**20.0**	**23.1**	**4.3**	0
	CDK	M/TDCDK	0	0	0.1	0	**13.0**	**12.4**	0	0	**36.4**	1.8	**16.0**	**17.0**	2.7	0
T. lecithinolyticum																
	684^T	W68+sugar	2.2	1.8	0	**5.2**	0	**16.7**	**10.8**	3.9	**19.2**	0.0	**30.3**	**5.2**	2.0	23.9
T. maltophilum																
	679^T	W68+sugar	1.2	1.1	0.1	2.2	**4.8**	**45.0**	**7.5**	**14.9**	**14.3**	0.2	**4.2**	1.2	0	**27.1**
T. pectinovorum																
	864^T	W68+sugar	3.2	0	0	**5.8**	0	2.8	**15.1**	**4.8**	**21.9**	0	**9.8**	**21.1**	2.6	**28.8**
T. socranskii ssp. *socranskii*																
	35536^T	W68+sugar	1.3	0	0	0	**15.0**	0.8	**7.3**	**22.9**	**21.8**	0.4	**5.4**	**13.5**	**11.2**	**31.6**
T. sp.																
	796	W68+sugar	2.6	0	0	0	**14.8**	3.7	**19.7**	**18.3**	**17.4**	0.2	2.6	**17.5**	2.1	**40.6**

*Given as % of total eluates; values higher than 4% are in bold type.
†ECL, elution chain length equivalent of eluates not identified by the MIDI system.
‡CFA, cellular fatty acid, indicating carbon chain length:unsaturation and branching position.

oral treponemes isolated in media related to OMIZ-W68 (Wyss *et al.*, 2004).

Further investigations are required to identify the eluates for which the MIDI system assigned no structure and that together represented up to 40% of the treponemal lipids of motile strains (e.g. ATCC 35404 in Table 2). Other compounds reportedly present in oral treponemes, e.g. C18 : 1, which has been reported to represent up to 20% in *Treponema denticola* (Livermore & Johnson, 1974), or iso- and anteiso-3-hydroxy fatty acids found in the FM-strain of *Treponema denticola* as part of an LPS-like structure (Dahle *et al.*, 1996), were not detected. This suggests differences in the composition of the cells used in this and previous studies that most likely are due to differences in growth conditions. These compounds are apparently not essential (at least above the detection limit of the MIDI system) for treponemal life *in vitro*. It remains to be determined whether the biosynthetic spectrum for lipids is reduced in the chemically defined media or whether treponemes rely for some lipids on exogenous compounds only available in the complex media. The differences observed in the fatty acid profiles of strains of *Treponema denticola* grown in OMIZ-W68 vs. OMIZ-M/TDCK (Table 2) clearly suggest some environmental control of cellular lipids and potential for further analysis.

The present data confirm and extend the earlier conclusion that at least some oral treponemes have the ability to synthesize their own long-chain fatty acids (Wyss, 1992). With respect to *Treponema denticola*, this is consistent with the recent genome data (Seshadri *et al.*, 2004). The contrasting view that oral treponemes had lost the ability to synthesize these essential compounds is based on early nutritional studies in complex media (Johnson & Eggebraten, 1971; VanHorn & Smibert, 1982). However, demonstration of a requirement does not exclude the ability for *de novo* biosynthesis in a different context. It is possible that the intricate balance between inhibitory and stimulatory components in such complex media led to inhibition of synthesis and consequent dependency on exogenous fatty acid. The presence of a fatty acid transporter (FadL) and a fatty acid acyl-CoA synthetase (FadD) in the *Treponema denticola* genome (Seshadri *et al.*, 2004) supports the possibility of switching between costly biosynthesis and salvaging of exogenous fatty acids. This is also consistent with the ability for metabolic labeling with exogenous tritiated oleic acid (Sela *et al.*, 1997). In some media, however, free fatty acids may prove inhibitory, as seen for *Treponema lecithinolyticum*, which is inhibited by the fatty acids liberated by its own phospholipases (Wyss *et al.*, 1999). Such toxicities are well known and buffered *in vivo* by binding to proteins like serum albumen.

The present study underlines the need for extensive physiological data to complement the rapidly growing information from genomic studies. Regulatory or toxic aspects of media components or novel enzymatic reactions like the obligatory salvage of MurNac by *Tannerella forsythia*, even if predictable from genomic data, need confirmation through studies on living cells.

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