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Biochemical and molecular genetic studies of uncoupler-resistant mutants of *Bacillus subtilis*

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BIOCHEMICAL AND MOLECULAR GENETIC STUDIES OF UNCOUPLER-RESISTANT MUTANTS OF *Bacillus subtilis*

by

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Abstract

BIOCHEMICAL AND MOLECULAR GENETIC STUDIES

OF UNCOUPLER-RESISTANT MUTANTS OF *Bacillus subtilis*

by

Eugene Alexander Dunkley, Jr.

Adviser: Professor Terry Ann Krulwich

The chemiosmotic hypothesis holds that oxidative phosphorylation is driven solely by an electrochemical gradient of protons, the $\Delta\bar{\mu}_{H+}$, which can be dissipated by protonophores. Protonophore-resistant mutants of *B. subtilis* and *B. megaterium* are of bioenergetic significance because they grew in the presence of concentrations of CCCP that were inhibitory to growth of the wild-type. Furthermore, as shown in earlier work from this laboratory, the mutants synthesized more ATP than the wild-type at sub-maximal levels of the $\Delta\bar{\mu}_{H+}$ in experiments utilizing starved whole cells or membrane vesicles re-energized by electron donors, but not by artificial gradients.

Extensive characterization of the protonophore-resistant mutants and revertants by others in the laboratory indicated that protonophore resistance correlated with a decreased ratio of unsaturated fatty acids to saturated fatty acids in the membrane phospholipids of the mutants; restoration of the level of unsaturated fatty acids using exogenous palmitoleic acid abolished the resistance. In the current study, it was first established that exogenous $C_{16:1}$ abolished protonophore resistance in duramycin-resistant strains of protonophore-resistant mutants. These strains have markedly deficient levels of

phosphatidylethanolamine and cardiolipin, which are the major sites of incorporation of unsaturated fatty acids in *B. subtilis*. Assays of the fatty acyl-CoA desaturase of wild-type *B. subtilis* indicated that the activity resembled the fatty acyl-CoA desaturase of eukaryotic organisms in that it required O_2 and an electron donor, was cyanide-sensitive and carbon monoxide-insensitive. This O_2-,NADH-dependent formation of unsaturated fatty acids was deficient in the protonophore-resistant mutants. Temperature-sensitive protonophore-resistant revertants exhibited fatty acid desaturation that was active at temperatures which were non-permissive for protonophore-resistant growth, and inactive at temperatures which were permissive for protonophore-resistant growth.

Five libraries of insertional mutations introduced into *B. subtilis* by Tn917-mediated transposition were screened for CCCP resistance to determine whether the site(s) of insertion unrelated to the desaturase could lead to protonophore resistance, and to identify the genes encoding or positively regulating components of the fatty acid desaturase. Twenty five protonophore-resistant strains were isolated. Nineteen of these isolates were phenotypically reversible by the addition of palmitoleic acid to the growth medium, while the remainder were not reversible. Six of the "$C_{16:1}$-reversible" and three of the "non-reversible" strains were assayed for fatty acyl-CoA desaturase activity. All of the strains assayed were deficient in desaturase activity in comparison to the wild-type. However, two of the "$C_{16:1}$-reversible" strains had noticeable desaturase activity, indicating a degree of heterogeneity among that category of mutants.

Analysis of the flanking region of transposon insertion was conducted on one of the "$C_{16:1}$-reversible" strains which was deficient in desaturase activity. No sequence similarity to the fatty acid desaturase or its components has yet been found, but the insertion site has been localized to a position 4.2 kb from the *spo*0F gene of *B. subtilis*. Continued analysis using this and other insertional strains may aid in clarifying the genetic basis of protonophore resistance in *B. subtilis*.

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ABBREVIATIONS

CO	carbon monoxide
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CL	cardiolipin
CoA	coenzyme A
$C_{16:0}$	palmitic acid
$C_{16:1}$	palmitoleic acid
$C_{18:0}$	stearic acid
$C_{18:1}$	oleic acid
DNA	deoxyribonucleic acid
KCN	potassium cyanide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
PMSF	phenylmethylsulfonylfluoride
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
TLC	thin-layer chromatography

FOREWORD

Parts of this thesis have appeared in the following publications:

Dunkley, E.A. Jr., S. Clejan, A.A. Guffanti and T.A. Krulwich. 1988. Large decreases in membrane phosphatidylethanolamine and diphosphatidylglycerol upon mutation to duramycin resistance do not change the protonophore resistance of *Bacillus subtilis*. Biochim. Biophys. Acta **943**:13-18. Reprinted by permission of the publisher.

Dunkley, E.A. Jr., A.A. Guffanti, S. Clejan and T.A. Krulwich. 1991. Facultative alkaliphiles lack fatty acid desaturase activity and lose the ability to grow at near-neutral pH when supplemented with an unsaturated fatty acid. J. Bacteriol. **173**:1331-1334. Reprinted by permission of the publisher.

Chapter One

Literature Review

Introduction and Objectives

Earlier studies from the laboratory in which my dissertation work has been conducted indicated that mutations of *Bacillus subtilis* and *Bacillus megaterium* that caused a reduction in the unsaturated fatty acid content of the membrane phospholipids resulted in a protonophore-resistant phenotype. The phenotype was of bioenergetic interest because the mutants neither excluded nor inactivated the protonophore (6,7,20-22). The chemiosmotic hypothesis holds that an electrochemical gradient of protons, the $\Delta\bar{\mu}_{H+}$, is the sole, obligatory driving force of oxidative phosphorylation (36). Results from experiments utilizing either starved whole cells or membrane vesicles (7,20,21) that were re-energized with an electron donor indicate that the mutants synthesize more ATP than the wild-type parent at sub-maximal levels of the $\Delta\bar{\mu}_{H+}$ when electron donors were used for energization, but not when artificial gradients were used; in fact, the protonophore-resistant strains synthesized less ATP than the parent strains when re-energized by artificial gradients (7,21).

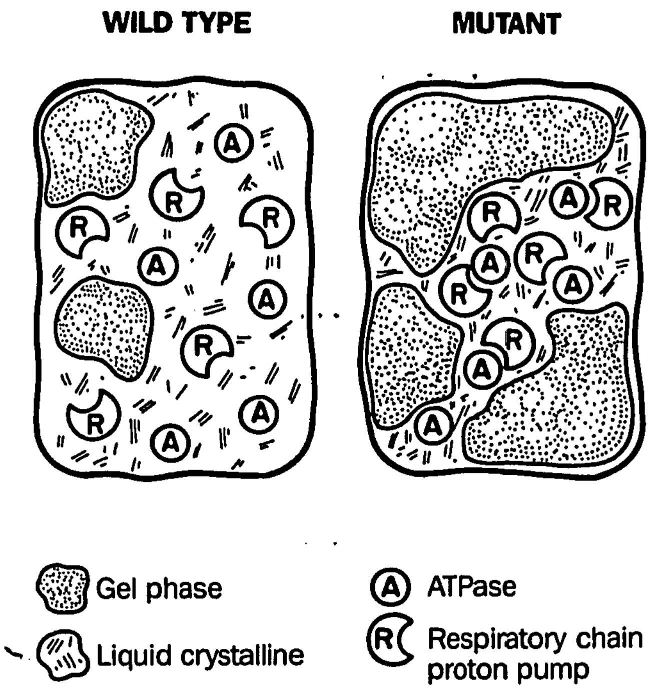
Detailed characterizations of the protonophore-resistant strains and revertants led to the recognition of a correlation between protonophore resistance and a decreased level of unsaturated fatty acids in the membrane phospholipids (24). Addition of an unsaturated fatty acid supplement to the growth medium, which restored wild-type levels of unsaturated fatty acid, greatly enhanced the protonophore sensitivity of malate-energized growth and oxidative phosphorylation by the mutants (24).

Krulwich, Quirk, and Guffanti (25) have presented a speculative model for how an increase in the saturation of the phospholipids of the coupling membrane might lead to protonophore resistance. As shown in the cartoon in Figure 1, this model involves the possibility that the overall increase in saturation of the membrane phospholipids results in an increase in the proportion of gel state relative to liquid crystalline membrane. If the proteins are preferentially localized in the latter phase, then a given number of respiratory chain proton pumps and ATP synthases would now be confined to a smaller area. They might, in closer average proximity, more frequently exchange a proton directly, without release into the bulk. These direct transfers might, in turn, be somewhat more CCCP-resistant, producing the observed phenotype, including the difference between respiration- and artificially-generated gradients. Several colleagues in the laboratory are currently endeavoring to test aspects of this model. My own work on the protonophore-resistant mutants has focused on the central biochemical questions.

The first question I addressed emerged from the observation that when palmitoleic acid was added to the growth medium of protonophore-resistant strains of *B. subtilis*, the unsaturated fatty acid supplement was preferentially incorporated into phosphatidylethanolamine (PE) and cardiolipin (CL) over phosphatidylglycerol (PG). PE and CL represent 25% and 5%, respectively, of the phospholipid content of the membrane (8,24). The converse pattern of incorporation was observed for the saturated counterpart, palmitic acid, which was preferentially incorporated into PG, which represents 70% of the membrane phospholipid (24). It was conceivable that a mutation which resulted in greatly reduced levels of PE and CL may render strains of *B. subtilis* incapable of incorporation of exogenous palmitoleic acid. If so, would there be a loss

Figure 1. A diagrammatic representation of a hypothesis by Krulwich *et al.* (10) explaining how an increase in the saturation of membrane phospholipids might result in a CCCP-resistant phenotype.

HOW DOES THE HIGHER SATURATED/UNSATURATED FATTY ACID RATIO OF MEMBRANE PHOSPHOLIPIDS RESULT IN HIGHER $\Delta Gp/\Delta p$ AT REDUCED Δp?



HYPOTHESIS

Higher saturated/unsaturated fatty acids increase the proportion of the membrane lipid that is in the gel state.

Even the gel state of *Bacillus* species, with branched chain fatty acids that preclude dramatic phase separation, may exclude proteins relative to the liquid crystalline phase.

The respiratory chain proton pumps and the F_1F_0 ATPase are brought into closer average contact, with a higher random collision rate, by their concentration in the smaller liquid crystalline phase membrane of the mutant.

The mutant thus enhances its capacity to carry out non-chemiosmotic direct transfer of some protons from respiratory pump to ATPase, resulting in a higher $\Delta Gp/\Delta p$ ratio at submaximal values of the bulk Δp.

of the modulatory affect of exogenous palmitoleic acid on protonophore resistance? These questions were addressed by the isolation of duramycin-resistant strains of *B. subtilis*. The antibiotic duramycin apparently requires PE for binding to the cell (38); resistantmutants have greatly reduced PE and CL. My results showed that introduction of such a mutation into a protonophore-resistant strain did not change the resistance to protonophore or its reversibility by exogenous palmitoleic acid.

The most extensive question addressed in my work was whether a deficiency in fatty acid desaturase was the biochemical basis of the mutation. When an affirmative answer to that question had been convincingly developed, an effort was initiated to use transposon mutagenesis to a CCCP-resistant phenotype to identify relevant gene(s). Moreover, this effort should indicate whether other null mutations, not involving the desaturase or any regulators thereof, can result in protonophore resistance. Thus far, no other apparent types have emerged, and an interesting heterogeneity has been found in insertional mutants that are desaturase-deficient and CCCP-resistant.

Background

Protonophore-Resistant Bacteria

The chemiosmotic hypothesis (35,36) posits that an electrochemical proton gradient, the $\Delta\bar{\mu}_{H+}$, across the coupling membrane is the primary driving force for bioenergetic work. Since the coupling membrane is somewhat impermeable to protons, primary proton pumping events due to respiration or photosynthesis result in a $\Delta\bar{\mu}_{H+}$. This bulk force, in turn, is proposed to be the sole, obligatory driving force for ATP synthesis, solute transport and other bioenergetic work. The $\Delta\bar{\mu}_{H+}$ can be dissipated by uncoupling agents such as protonophores, which are lipophilic weak acids that allow protons to rapidly equilibrate across the membrane. This collapse of the $\Delta\bar{\mu}_{H+}$ allows the primary proton pumping events to proceed without bioenergetic work occurring.

An organism growing aerobically on a non-fermentative carbon source might exhibit protonophore resistance if it could inactivate or exclude the uncoupler so that its $\Delta\bar{\mu}_{H+}$ remained relatively uncompromised. To date, for example, the protonophore-resistant mutant strains isolated from *Escherichia coli* have all turned out to have some alteration in the outer membrane that apparently renders protonophores less able to affect the inner, coupling membrane (22,25,42). On the other hand, protonophore-resistant mutants of *Bacillus subtilis* and *Bacillus megaterium* (6,21) pose a challenge to the chemiosmotic hypothesis. They grow in the presence of concentrations of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) that are inhibitory to the wild type. Furthermore, they are capable of synthesizing more ATP than the wild type at sub-maximal levels of the $\Delta\bar{\mu}_{H+}$ (21), though they maintain sensitivity to the dissipative effect of protonophores on the $\Delta\bar{\mu}_{H+}$. The feature of cell physiology that correlated with the phenotype was first elucidated from studies of membrane composition. The most striking aspect of this analysis was that the ratio of unsaturated fatty acids to saturated fatty acids esterified in the phospholipid was markedly reduced in the mutants in comparison to the wild type (21). The enzyme responsible for the biosynthesis of unsaturated fatty acids was, for reasons summarized below, expected to be a multicomponent fatty acid desaturase. The aim of this work was to characterize the fatty acid desaturase of *B. subtilis* by biochemical and genetic means and to compare this activity to that of protonophore-resistant mutants of *B. subtilis*. Fatty acid desaturases of a number of other organisms are discussed further.

Fatty acid desaturases

Animals

Fatty acid desaturases are of importance in animals because of the essential role polyunsaturated fatty acids play in cellular membrane composition and fluidity (3,23,32,34,37,59). The O_2-, NADH-dependent conversion of stearyl-CoA to oleoyl CoA involves electron transport mediated by cytochrome b_5, cytochrome b_5 reductase, and the fatty acid desaturase (33,44,48,52). The activity is inhibited by cyanide and azide, but not by carbon monoxide, distinguishing it from P450-catalyzed desaturation (48). The $\Delta 9$ acyl-CoA desaturase was first extracted from rat liver microsomes and purified to homogeneity in 1974 by Strittmatter and colleagues (48), and reconstituted into proteoliposomes by detergent extraction and addition of purified cytochrome b_5 and cytochrome b_5 reductase (47,48). The fatty acid desaturase is a single polypeptide of 53,000 daltons containing 62% hydrophobic amino acids and one non-heme iron (48). The cytochrome b_5 reductase is 43,000 daltons, largely hydrophilic with an anchoring "tail" of 99 amino acids (47). Cytochrome b_5 is a 16,700 dalton heme protein, 11,000 of which constitute the soluble catalytic activity (47). The transcription of the DNA coding for the fatty acid desaturase was induced by a cycle of starvation and refeeding of the animals; the mRNA was used as a probe of the genomic DNA to isolate the cDNA clone encoding the Δ9 desaturase (53,54). A truncated cDNA lacking the coding sequence for the first 26 amino acid residues was demonstrated to be capable of encoding an active fatty acid desaturase (49). The Δ6 acyl-CoA desaturase has also been purified to homogeneity (19) and uses the same components as the Δ9 desaturase (40). It catalyzes the conversion of linoleoyl-CoA ($C_{18:2}$) to linolenoyl-CoA ($C_{18:3}$). The Δ5 acyl-CoA desaturase converts eicosatrienoyl-CoA ($C_{20:3}$) to arachidonoyl acid ($C_{20:4}$) (4), but has not been purified to date. The effects of dietary and hormonal manipulation have been studied on each of these systems.

Effect of diet. Several studies in rats have shown (27,28) that cholesterol incorporation has a profound effect on the desaturases. In particular, *in vivo* cholesterol incorporation into liver cells increases Δ9 desaturation while decreasing Δ6 and Δ5 activities. Conversely, removal of cholesterol from the diet led to a decrease in the Δ9 activity in whole cells with a concomitant increase in Δ6 and Δ5 activities. *In vitro* addition of cholesterol to microsomes led to an increase in the activities of all three desaturases (27). Cholesterol incorporation increased the rigidity of the membrane (27) and altered the distribution of the phospholipids (PL) (28). These results indicate that the Δ9 desaturase activity was modulated by the level of fluidity of the membrane, whereas Δ5 and Δ6 activities were modulated by both fluidity and the cholesterol/phospholipid ratio (28). The altered PL ratio may in fact be due to the enhanced synthesis of phosphatidylcholine (PC) due to regulation by cholesterol of the CTP:phosphocholinecytidylyltransferase (55).

Diets high in saturated fat have been reported to increase Δ9 activity while decreasing Δ6 and Δ5 activity, while diets high in polyunsaturated fatty acids decrease both Δ9 and Δ6 activities; Δ5 activity appears unaffected (3). Trans-monoenoic fatty acids are potent inhibitors of both Δ6 and Δ5 (3).

Effect of hormones. Several studies (3,39,41,59) have shown that Δ9, Δ6 and Δ5 desaturase activities are all lower in liver microsomes of diabetic rats. Insulin treatment restores activities to those found in non-diabetic rats (3); pretreatment with Actinomycin D blocked the effect of insulin. Administration of glycogenolytic hormones such as glucagon or adrenalin (3) inhibits Δ6 and Δ5 activities, probably due to increase in the second messenger cAMP (3). Testosterone administration to rats *in vivo* leads to an increase in Δ9 and decrease in Δ6 and Δ5 activities (31). This modulation of desaturase activity is mediated by a soluble factor (3). The effect of testosterone is significant because changes in polyunsaturated fatty acid metabolism are believed to play a role in the higher occurrence of atherosclerosis in human males (31).

Plants

In plants, chloroplasts and plastids are the site of fatty acid biosynthesis. Plant fatty acid desaturases utilize either coenzyme A (CoA) or acyl-carrier-protein (ACP) activated fatty acids. They are distinct genetically and functionally (43) from their animal and fungal counterparts.

The plant acyl-CoA desaturases are unusual in that the site of desaturation is the phospholipid (34,40,45). The activated fatty acid is first transferred to the phospholipid. After desaturation, the newly formed unsaturated fatty acid is then unesterified from the phospholipid and re-activated, so that the overall reaction results in the conversion of a long-chained saturated fatty acid to a long-chained unsaturated fatty acid. An important activity is Δ12 desaturation, responsible for the formation of linoleoyl-CoA (45). The electron transport components involved in this activity have recently been identified as cytochrome b_5, cytochrome b_5 reductase, and the Δ12 desaturase (Figure 2); all are associated with the microsomal membrane (45). The presence of high levels of unsaturated fatty acids is important in the posttranslational insertion of proteins into chloroplast membranes at low temperatures (46). In *Synechocystis* PCC6803, deficiency in Δ6 activity has been associated with decreased cold tolerance (56). Introduction of the gene encoding Δ6 desaturase into the cold-sensitive species *Anacystis nidulans* led to increased cold tolerance in this species (57). Studies of *fad*D mutants of higher plants indicated the presence of a desaturase which is expressed only at cold temperatures (46). On the other hand, decreased unsaturation has been associated with increased thermal stability of chloroplast membranes (46).

The stearyl-acyl-carrier-protein (ACP) desaturase from higher plants is the only soluble desaturase identified at present (43). The activity converting stearyl-ACP to oleoyl-ACP requires, in addition to the desaturase, ferredoxin, ferredoxin NADPH reductase, and O_2 (43). Studies of both the purified protein and cDNA clones encoding stearyl-ACP desaturase from *Ricinus communis* show a lack of structural similarity to the stearyl-CoA desaturase of animals and fungi (43). However, significant homology in the C terminus does exist between the stearyl-ACP desaturase and the yeast fatty acid synthase (43).

Yeast

Bloomfield and Bloch (1) first characterized the formation of palmitoleic ($C_{16:1}$) and oleic ($C_{18:1}$) acids from the saturated fatty acid counterpart in yeast as an O_2-, NADH-dependent activity (2). Early *in vitro* work with *Saccharomyces cerevisiae* suggested that both microsomal and soluble fractions were required for activity, but that the soluble fraction was dispensable if the fatty acid substrate had been activated with coenzyme A (2). Under anaerobic conditions, growth of *S. cerevisiae* required the presence of unsaturated fatty acid in the medium; this auxotrophy was lost when the cells were grown aerobically, suggesting that fatty acid desaturation is an oxygen-dependent activity (2). The components of the enzyme complex which catalyzes this activity in yeast, as in animals, are the cytochrome b_5, NADH-cytochrome b_5 reductase, and the $\Delta 9$ acyl-CoA desaturase (11). There are no polyunsaturated fatty acids in *S. cerevisiae* (50). Yeast mutants deficient in $\Delta 9$ activity are auxotrophic for unsaturated fatty acids. The gene encoding the Δ9 desaturase, *OLE1*, has recently been cloned (50) and sequenced (51). The gene encodes a protein of 510 amino acids, 251 of which are hydrophobic. Sequence analysis shows a 36% amino acid identity with the rat stearyl-CoA desaturase; conserved regions have greater than 70% identity. There are four putative transmembrane spanning regions, yet the regions of high sequence similarity with the rat liver desaturase are in the loops connecting the transmembrane regions on the cytosolic side (50). Of significance is the fact that the rat liver desaturase can functionally replace the *OLE1* gene in yeast desaturase mutants, meaning that the yeast cytochrome b_5 reductase can recognize the rat liver desaturase (51).

Bacteria

Not much is known about bacterial fatty acid desaturases and the O_2-dependent conversion of saturated to unsaturated fatty acids in bacteria. Few *in vitro* studies have been conducted, let alone purification of the protein or gene cloning. Extensive studies of the biosynthesis of unsaturated fatty acids in bacteria have been conducted in *Escherichia coli* (5); however, this pathway is oxygen-independent (5). Oxygen-dependent unsaturated fatty acid biosynthesis has been demonstrated in *Bacillus megaterium* (14). *In vivo* studies of the fatty acid desaturase by Fulco and colleagues (12,13,16) showed convincing evidence that the growth temperature has a profound effect on fatty acid desaturase activity. Cultures grown at 20°C had a substantially higher level of unsaturated fatty acids in the membrane compared to a similar culture grown at 35°C (14). Furthermore, the increase in activity at 20°C appeared to be due to increased synthesis of the desaturase itself, since inhibitors of protein synthesis abolished the increased activity (14). Cultures initially grown at 35°C exhibited hyperinduction of desaturase activity when suddenly exposed to 20°C; the resulting activity was greater than that of a culture initially grown and maintained at 20°C (14). This phenomenon was attributed to the presence of a temperature-sensitive soluble protein involved in transcriptional regulation; this regulator was proposed to negatively regulate the synthesis of desaturase at 20°C, but to be inactive at 35°C. Its apparent absence upon "shift-down" from 35°C to 20°C could then lead to the hyperinduction (14). Subsequent to Fulco's studies of the fatty acid desaturase of *B. megaterium*, his attention focused on an enzyme-catalyzed hydrogenation and epoxidation of unsaturated fatty acids; this activity was shown to be due to the presence of a phenobarbital-inducible cytochrome P450 (15-17).

In vitro assays of the prokaryotic fatty acyl-CoA desaturase have been previously reported for *Alcaligenes* (26), *Mycobacterium* (18), *Pseudomonas* (58), and *Bacillus firmus* (9). All of these activities characteristically show O_2-, NADH (NADPH)-dependence and cyanide sensitivity; however, the components involved in the activity have not yet been elucidated. Though the activity is membrane-associated, a cytosolic factor is required for full activity in *Mycobacterium* (18). The *in vitro* fatty acid desaturase activities of aerobic prokaryotes are not sensitive to carbon monoxide, indicating that desaturation is not due to cytochrome P450 (18,26,58). Substrates for desaturation are either palmitoyl-CoA or stearoyl-CoA; no ACP-activated fatty acid or phospholipid has been demonstrated to be a substrate. The site of the double bond in *Bacillus* sp. is between carbons 5 and 6 (14); the other bacteria insert the double bond between carbons 9 and 10 (18).

Protonophore-resistant mutants of *B. subtilis* and *B. megaterium* (6,21) are deficient in fatty acid desaturase activity (10). They are not, however, auxotrophic for unsaturated fatty acids, probably because of the high percentage of branched-chained fatty acids esterified in the phospholipid (8,24); branched chain fatty acids apparently confer the requisite fluidity upon the membrane. The actual role that unsaturated fatty acids play in maintaining membrane fluidity and ultrastructure therefore is not completely clear in these bacteria. They may be, however, involved in "fine-tuning" the optimal membrane environment for integral membrane proteins, since only a 50% reduction in the unsaturated fatty acid level confers uncoupler resistance in *B. subtilis* (24).

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Chapter Two

Large Decreases in Membrane Phosphatidylethanolamine and Diphosphatidylglycerol upon Mutation to Duramycin Resistance Do not Change the Protonophore Resistance of *Bacillus subtilis*

Abstract

Duramycin-resistant mutant strains were selected from wild-type *Bacillus subtilis* (BD99) and its protonophore-resistant mutant derivative, strain AG1A3. Analyses of the membranes of the duramycin-resistant mutants showed that they had little or no phosphatidylethanolamine and diphosphatidylglycerol as determined by chemical detection after thin-layer chromatography. Small amounts of these phospholipids must remain in the mutant strains, however, because during studies of incorporation of exogenous, radioactive fatty acids, label associated with palmitoleic acid was found in chromatographic positions that corresponded to the expected positions of phosphatidylethanolamine and diphosphatidylglycerol. The duramycin-resistant strains both showed elevated levels of phosphatidylglycerol and aminoacylphosphatidylglycerol. The duramycin-resistant derivative of protonophore-resistant AG1A3 (AG1A3-DR4), but not that of the wild type, also showed a decreased content of neutral relative to polar lipid in the membrane. The composition of neutral lipid in that strain was higher in free fatty acids and lower in 1,2-diacylglycerol than its parent strain. AG1A3-DR4 also contained appreciable levels of lysophosphatidylethanolamine and somewhat elevated diglycosyldiacylglycerol relative to the other strains in the study. The protonophore resistance of AG1A3 was unaltered by mutation to duramycin resistance. Nor was there any change in the efficacy of exogenous palmitoleic acid in diminishing the protonophore resistance of AG1A3-DR4. This phenomenon persists upon dramatic reduction in the content of phosphatidylethanolamine and diphosphatidylglycerol even though those

phospholipids are normally the preferred sites in incorporation of the exogenous unsaturated fatty acids that mediate the effect.

Introduction

Uncoupler-resistant mutants of *Bacillus subtilis* (1) and *Bacillus megaterium* (2) have recently been found to possess reduced levels of monoenoic fatty acids in their membrane lipids. The addition of palmitoleic acid to the growth medium of the mutants restores wild-type levels of unsaturated fatty acids in the membrane and concomitantly reduces their resistance to protonophores (2,3). The exogenous unsaturated fatty acids are preferentially incorporated into phosphatidylethanolamine and diphosphatidylglycerol, whereas exogenous saturated fatty acids are incorporated more substantially into phosphatidylglycerol (2,3). It thus became of interest to examine the possibility that uncoupler resistance or sensitivity would be modulated not only by monoenoic fatty acid levels but also by major changes in the phospholipids that most appreciably incorporate those fatty acids. Navarro *et al.* (4) reported that duramycin-resistant mutants of *B. subtilis* have greatly reduced levels of membrane phosphatidylethanolamine and diphosphatidylglycerol. As reported here, we selected duramycin-resistant derivatives of one of the uncoupler-resistant *B. subtilis* mutants and its wild-type parent so that the effect of resulting alterations in the membrane phospholipids could be examined with respect to protonophore resistance and its diminution by exogenous palmitoleic acid.

Materials and Methods

Strains, growth conditions and isolation of mutants. The wild type *B. subtilis* strain (BD99) and its uncoupler-resistant derivative, strain AG1A3 (1), were the starting point for all studies. For growth experiments, routine maintenance in liquid culture, and growth of cells for lipid characterization, cells were grown on DL-malate in Spizizen salts (5) supplemented with 0.1% (w/v) yeast extract and required amino acids as previously described (1). Additions of CCCP (2 μM), palmitoleic acid (10 μM), or duramycin (25 μg/ml) were made from separate solutions. Cells that were grown in the presence of radioactive fatty acids were prepared as described previously (3). All cultures were incubated, with aeration, at 30°C. Growth experiments were conducted in sidearm flasks; growth was monitored by following turbidity using a Klett-Summerson colorimeter (No. 42 filter).

Spontaneously arising duramycin-resistant mutants of the two strains were isolated on petri plates containing tryptose blood agar medium (Difco) to which duramycin had been added to a final concentration of 25 μg/ml. Colonies that developed on these plates were restreaked several times and were checked with respect to morphology and appropriate genetic markers. The duramycin-resistant strains derived from wild-type BD99 and AG1A3, respectively, were designated BD99-DR4 and AG1A3-DR4.

Isolation and characterization of membrane lipids. Right-side-out membrane vesicles were prepared from washed, late logarithmic phase cells by the lysozyme method of Kaback (6). Lipids were extracted from these vesicles by the method of Bligh and

Dyer (7) and were characterized by procedures that were recently summarized (8).

Materials. CCCP and non-radioactive palmitoleic acid were purchased from Sigma Chemical Company. Radioactive palmitic and palmitoleic acids were purchased from Amersham Corporation. Duramycin was generously provided by Drs. E. Racker (Cornell University) and O. Shotwell (Northern Regional Research Center).

Results

As shown in Fig. 1, the duramycin-resistant derivatives of wild-type (BD99) and uncoupler-resistant (AG1A3) *B. subtilis* were completely resistant to growth inhibition by 25 μg/ml of duramycin in liquid media. Interestingly, while BD99-DR4 grew indistinguishably from the wild type, strain AG1A3-DR4 exhibited the same initial growth rate as its parent strain but attained lower final levels of growth. Consistent with this observation, the changes in membrane lipids found in the two duramycin-resistant strains showed some common features, but also showed differences. Mutation of both the wild-type and uncoupler-resistant strains of *B. subtilis* to duramycin-resistance resulted in a marked reduction in phosphatidylethanolamine, such that this phospholipid was undetectable by standard thin-layer chromatography analysis (Table 1). Both duramycin-resistant strains also exhibited marked decreases in diphosphatidylglycerol and increases in phosphatidylglycerol and aminophosphatidylglycerol relative to their parent strains. Whereas the parent strains contained less than three percent of their membrane fatty acid as branched chain $C_{17:1}$, the duramycin-resistant strains contained 8-9% of such fatty acid. In BD99-DR4, 8% *anteiso*$C_{17:1}$ was found, and in AG1A3-DR4, 9% *iso*$C_{17:1}$ was found. This difference in the type of branched chain fatty acid utilized by the protonophore-sensitive vs. protonophore-resistant mutants is consistent with the pattern previously found in their parent strains (1). Together with the appearance of greater amounts of $C_{17:1}$ in AG1A3-DR4, but not in BD99-DR4, there was a decrease in the level of the only other monoenoic fatty acid, $C_{16:1}$. Thus the overall decrease in monoenoic

Fig. 1. The effect of duramycin on the growth of *B. subtilis* wild-type and CCCP-resistant strains and their derivatives selected for duramycin resistance. Growth on DL-malate-containing medium was followed in the absence (closed symbols) or presence (open symbols) of 25 μg/ml duramycin. Panel A shows the data for wild-type BD99 (■,□) and BD99-DR4 (●,○), and Panel B shows the data for AG1A3 (■,□) and AG1A3-DR4 (●,○).

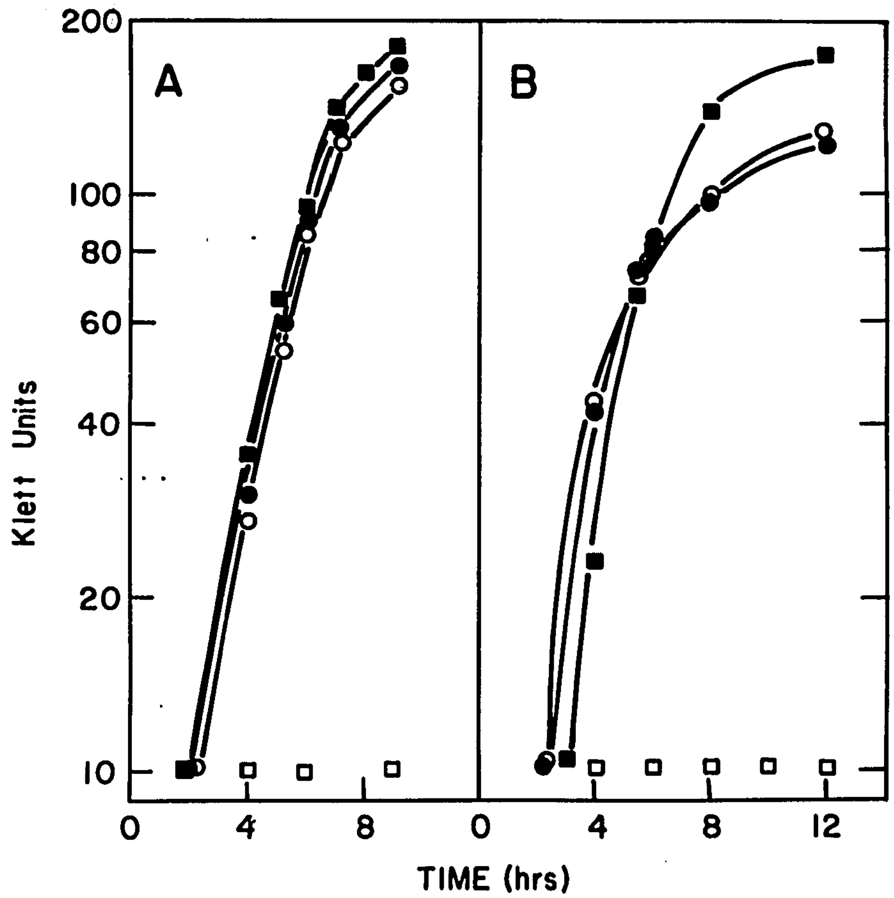

A
B
200
100
80
60
40
20
10
Klett Units
0
4
8
0
4
8
12
TIME (hrs)

TABLE I

MEMBRANE LIPIDS FROM DURAMYCIN-RESISTANT MUTANTS OF WILD-TYPE (BD99-DR4) AND PROTONOPHORE-RESISTANT (AG1A3-DR4) *B. SUBTILIS* [a]

Membrane composition	BD99-DR4	Significant change from BD99 [b]	AG1A3-DR4	Significant change from AG1A3 [b]
Lipid/protein (mg/mg)	0.66	-	0.72	-
Neutral lipid/polar lipid (%/%)	25/75	-	10/90	Decrease
Neutral lipid (% of total)				
1,2-Diacylglycerol	90	-	78	Decrease
Free fatty acids	10	-	22	Increase
Polar lipids (% of total)				
Phosphatidylethanolamine	0	Decrease	0	Decrease
Phosphatidylglycerol	80	Increase	74	Increase
Diphosphatidylglycerol	1	Decrease	0	Decrease
Monoglycosyldiacylglycerol	4	-	5	-
Diglycosyldiacylglycerol	6	-	8	Increase
Aminoacylphosphatidylglycerol	6	Increase	3	Increase
Phosphoglycolipid	3	-	5	-
Lysophosphatidylethanolamine	0	-	5	Increase
Fatty acids (% of total)				
iso$C_{15:0}$	15	-	30	-
anteiso$C_{15:0}$	38	-	21	-
iso$C_{17:1}$	1	-	9	Increase
anteiso$C_{17:1}$	8	Increase	2	-
n$C_{16:0}$	10	-	16	-
n$C_{16:1}$	11	-	3	Decrease
iso$C_{17:0}$	12	-	12	-

[a] Values are averages of three determinations of at least two independent samples.

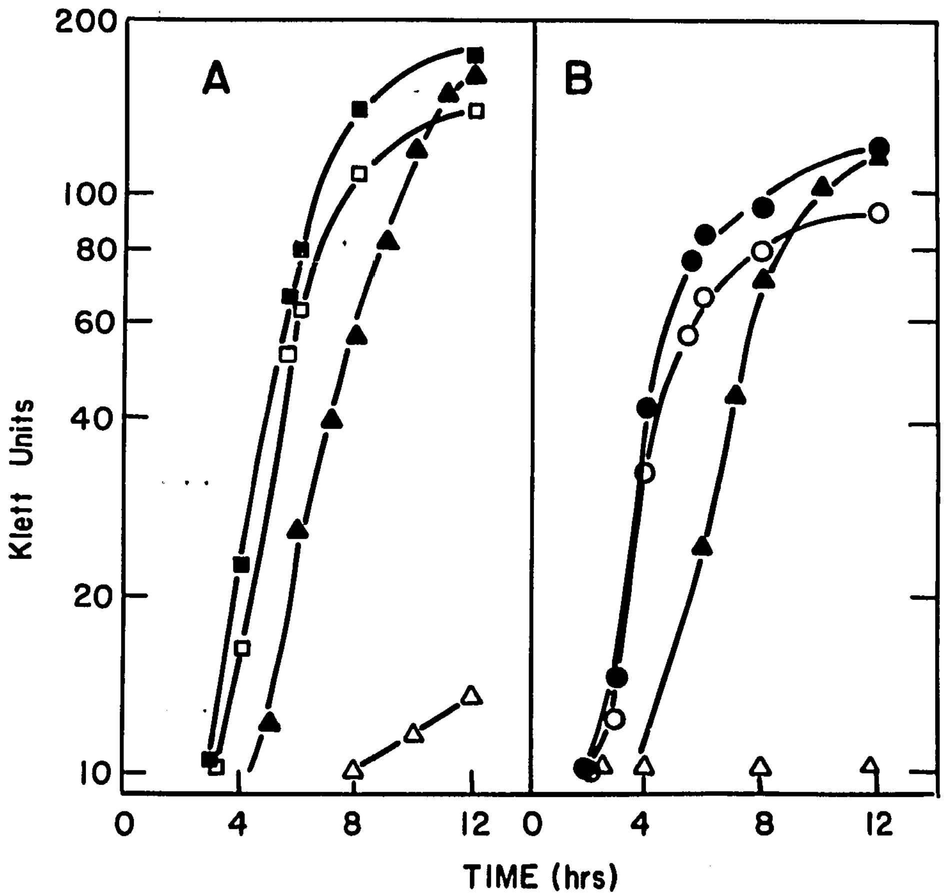
[b] Increases or decreases beyond two standard deviations from values for parental strains in Ref. 1.

fatty acids of the membrane phospholipids that characterized the protonophore-resistant strains of *B. subtilis* was reinforced.

Several other changes in membrane lipids were found in the AG1A3 derivative that were not found in the derivative of the wild type upon mutation to duramycin resistance. These included: a pronounced decrease in the ratio of total neutral lipid to total polar lipid in the membrane; a marked increase in the free fatty acid component of the neutral lipid relative to the 1,2-diacylglycerol; a modest increase in the content of diglycosyl-diacylglycerol; and the presence of 5% lysophosphatidylethanolamine (Table 1).

AG1A3-DR4 retained resistance to growth inhibition by low concentrations of CCCP, as shown in Fig. 2; although data are not shown, BD99-DR4 was also unaltered in its sensitivity to CCCP, i.e., it retained sensitivity to low concentrations of CCCP. Levels of exogenous palmitoleic acid, added as a growth supplement, modestly inhibited growth, and abolished the apparent protonophore-resistance in AG1A3-DR4 at least as well as it did in AG1A3 (Fig. 2). Since previous studies of AG1A3 and other CCCP-resistant strains had indicated that in this type of experiment the exogenous palmitoleic acid was preferentially incorporated into phosphatidylethanolamine and diphosphatidylglycerol (3), it was of interest to determine where incorporation of the unsaturated fatty acid occurred in the double mutant that had negligible levels of these phospholipids. BD99-DR4 and AG1A3-DR4 were analyzed with respect to the incorporation of radioactive growth supplements of either palmitic or palmitoleic acid. As shown in Table II, traces of phosphatidylethanolamine and diphosphatidylglycerol in the mutant membranes are made evident by these incorporation experiments, in which

Fig. 2. The resistance of AG1A3 and AG1A3-DR4 to inhibition by CCCP and modulation of that inhibition by exogenous palmitoleic acid. AG1A3 (Panel A) and AG1A3-DR4 (Panel B) were grown on DL-malate-containing media in the absence of additions (■,●) in the presence of 2 μM CCCP (□ ,○), in the presence of 10 μM palmitoleic acid (▲), or in the presence of both 2 μM CCCP and 10 μM palmitoleic acid (△).



200
100
80
60
40
20
10
Klett Units
A
B
0
4
8
12
TIME (hrs)

TABLE II

DISTRIBUTION OF LABEL FROM RADIOACTIVE FATTY ACID GROWTH SUPPLEMENTS AMONG MAJOR MEMBRANE LIPID FRACTIONS OF DURAMYCIN-RESISTANT *B. SUBTILIS* STRAINS [a]

Strain	Radioactive supplement	Radioactive fatty acid incorporation into fraction (nmol/mg total lipid) [b]				
		1,2-Diacylglycerol	Free fatty acids	Phosphatidylethanolamine	Phosphatidylglycerol	Diphosphatidylglycerol
BD99-DR4	Palmitic acid	9.6	0.8	0.2	14.8	0.2
	Palmitoleic acid	12.2	0.8	0.4	16.4	0.4
AG1A3-DR4	Palmitic acid	8.8	0.9	0	15.2	0
	Palmitoleic acid	14.4	1.0	0.6	20.4	0.4

[a] Cultures of BD99-DR4 and AG1A3-DR4 were grown in the presence of 10 μM [^{14}C]palmitic or 10 μM [^{14}C]palmitoleic acid and lipids were prepared as described under Materials and Methods.

[b] In all experiments, the fatty acid recovered was 98–99% unchanged from the radiolabeled substrate added. The values are means of two determinations from each of two independent preparations with standard deviations within 10% of the experimental values

a radioactive spot was found in the appropriate place although no chemically detectable phospholipid had been found. Thus, palmitoleic acid was still incorporated, albeit in very small amounts, into both phosphatidylethanolamine and diphosphatidylglycerol in both strains. In BD99-DR4, palmitic acid was also incorporated very modestly into those phospholipids. Relative to the earlier experiments with the duramycin-sensitive parent strains, incorporation of palmitoleic acid into phosphatidylglycerol was greatly elevated. Also, incorporation of both palmitic and palmitoleic acid into the neutral lipid fraction was markedly increased.

Discussion

Duramycin is a polypeptide with antibiotic activity against several Gram-positive bacteria and fungi (9). Racker and his colleagues (4,10,11) have shown that duramycin inhibits a number of membrane-associated ion pumps in eukaryotes, proton pumping by bacteriorhodopsin in phosphatidylethanolamine-containing proteoliposomes, and both proton secretion and Ca^{2+} uptake in *B. subtilis*. In a duramycin-resistant mutant of *B. subtilis*, the inhibitory effects on cation translocation were greatly diminished (4). In the current study we sought to take advantage of the observation made in the latter report that mutation to duramycin resistance in *B. subtilis* is accompanied by loss of membrane phosphatidylethanolamine and diphosphatidylglycerol. The analyses of the mutants selected here confirm those observations, although it is clear from the studies of incorporation of exogenous fatty acids that the loss of those phospholipids is not total. Indeed, as found in numerous studies of membrane phospholipid mutants of *Escherichia coli*, e.g. (12,13), mutational loss of all but a tiny fraction of a particular membrane phospholipid seems to be without evident phenotypic consequence even though total loss of the phospholipid might be lethal for the organism.

The patterns of change in the membrane lipids of BD99-DR4 and AG1A3-DR4 relative to their parent strains are notable in their complexity as well as in their differences from one another. The increases in phosphatidylglycerol and aminoacylphosphatidylglycerol are compensatory, but the increase in monoenoic, branched C_{17} fatty acids that was found in both duramycin-resistant strains is not easily interpreted. We have observed a modest increase in the average chain length of the fatty acids upon mutation to duramycin resistance in an unrelated *Bacillus* species (Clejan, S., unpublished data), and are thus inclined to suppose that this trend represents an adaptation to the changes in polar head groups in such mutants Similarly the appearance of a significant amount of lysophosphatidylethanolamine seems to be common to this type of mutant; presumably lysophosphatidylethanolamine does not allow binding of duramycin but may fulfill some of the normal role(s) of phosphatidylethanolamine in the membrane. Among the dramatic changes observed here, the rather profound changes in the amount and composition of the neutral lipid fraction found only in the duramycin-resistant derivative of AG1A3 are intriguing but impossible to rationalize at present.

Notably, although AG1A3-DR4 was selected on duramycin-containing plates that did not also contain CCCP, this double mutant retained CCCP-resistance. Moreover, in spite of the tremendous reduction in the amounts of phosphatidylethanolamine and diphosphatidylglycerol in the membranes of AG1A3-DR4, exogenous palmitoleic acid was just as effective as in AG1A3 in abolishing the protonophore resistance. The effectiveness of the exogenous palmitoleic acid may not depend upon its preferential incorporation into phosphatidylethanolamine and diphosphatidylglycerol, but depends only upon a significant increase in the level of monoenoic fatty acids, i.e. restoration of wild-type levels. Alternatively, incorporation may have to be into specific phospholipids but even the very tiny amounts of phosphatidylethanolamine and/or diphosphatidylglycerol remaining in this strain might be sufficient to mediate the effect.

Acknowledgment

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Chapter Three

Mutants of *Bacillus* species Isolated on the Basis of Protonophore-Resistance are Deficient in Fatty Acid Desaturase Activity

Abstract

The fatty acid desaturase activity in cell extracts of *Bacillus subtilis* was characterized and found to be O_2- and NADH-dependent and CN-sensitive. In cell fractionation studies, only 10% of the desaturase activity was recovered in the membrane fraction; addition of cytosolic factors, which by themselves were devoid of activity, restored membrane activity to the level found in the unfractionated cell extracts. NADH was preferred over NADPH as an electron donor, and palmitoyl-CoA was used preferentially over stearoyl-CoA as the straight-chain fatty acid substrate. An increase in desaturase activity was observed when either the growth or assay temperature was lowered from 37°C to 20°C, though the assay temperature appeared to be the more important parameter. Three protonophore-resistant mutants of *B. subtilis* and a comparable mutant of *Bacillus megaterium* had been found to possess reduced levels of unsaturated fatty acids in their membrane phospholipids; their protonophore resistance was abolished when grown in the presence of an unsaturated fatty acid supplement. All of these strains were found to be either significantly deficient in or totally lacking desaturase activity in comparison to their wild-type parent strains. Full, protonophore-sensitive revertants of the mutants had levels of desaturase activity comparable to the wild-type. Temperature-sensitive revertants of two of the mutants, which grew at 32°C, but not 26°C, in the presence of protonophore, exhibited desaturase activity comparable to the wild type at 26°C, but lacked activity at 32°C. These results indicate that the biochemical basis for protonophore resistance in these *Bacillus* mutants is a fatty acid desaturase deficiency.

Introduction

Three protonophore-resistant strains of *Bacillus subtilis* that were isolated in our laboratory grew on non-fermentable carbon sources in the presence of protonophore concentrations that completely inhibited growth of the wild-type (14). The mutants did not, however, inactivate or exclude the protonophore (14). Like a similar mutant strain of *Bacillus megaterium* described by Decker and Lang (3,4), these strains synthesized more ATP than the wild-type at sub-maximal levels of the proton motive force (4,14). All the mutants had reduced unsaturated fatty acid content in their membrane phospholipids relative to wild-type *B. subtilis* and *B. megaterium*. In the *B. subtilis* mutants, the sole monounsaturated fatty acid, $C_{16:1}$ was reduced by approximately 50% in each mutant. More dramatically, the entire monounsaturated fatty acid content drops from 12% of the total fatty acid in wild-type *B. megaterium* phospholipids to zero in the protonophore-resistant mutant C8 (17). We therefore sought to examine whether the primary mutational event in the protonophore-resistant mutants of *Bacillus* species was in the fatty acid desaturase.

The most extensive studies of the biosynthesis of unsaturated fatty acids in bacteria have been conducted in *Escherichia coli*. However, this pathway is oxygen-independent (5). By contrast, studies using whole cells of *Bacillus megaterium*, conducted by Fulco and colleagues (11), indicated that the activity involved an oxygen-dependent pathway (11,12). Detailed *in vitro* and molecular studies of the eukaryotic O_2-dependent desaturase had been conducted in mammals (25,26,28,29), plants (23), and yeast (21,27), from which a well-characterized pattern has emerged. The fatty acid desaturase system of eukaryotes is membrane associated, is O_2-, NADH-dependent and cyanide sensitive and is composed of cytochrome b_5, cytochrome b_5 reductase, and the cyanide-sensitive desaturase (9,15). *In vitro* studies of a comparable activity in prokaryotes of the O_2-dependent pathway have been less extensive, with work reported for *Alcaligenes* (16), *Mycobacterium* (13), *Pseudomonas* (33), and a cyanobacterium (30-32). In this work, we characterize the O_2-dependent pathway in cell extracts of *B. subtilis*, and then compare this activity in the wild-type strains of both *B. subtilis* and *B. megaterium* to that of their protonophore-resistant counterparts.

Materials and Methods

Strains and growth conditions. Wild-type *B. subtilis* BD99, protonophore-resistant AG1A3, AG2A, and AG3A, full revertants AG1A3rev8 and AG2Arev35, and temperature-sensitive revertants AG1A3Ts47 and AG2ATs1 were grown on 50 mM DL-malate, pH 7, in Spizizen salts (24) supplemented with 0.1% yeast extract and 5 μg/ml of tryptophan, threonine, and histidine at 30°C to late logarithmic phase. Wild-type *B. megaterium* ATCC19213 and its uncoupler-resistant mutant derivative C8 (3,4) were grown in the same medium without the amino acid supplements. In some experiments, wild type *B. subtilis* BD99 was grown on the above media at temperatures of 20°C or 37°C. Temperature-sensitive revertants were selected by growth on plates at either 26°C or 32°C in the presence and absence of 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP).

Preparation of cell extracts and membranes. One liter of cells was harvested and resuspended in 40 ml of 0.1 M potassium phosphate buffer, pH 7.2, containing 0.5 M sucrose. The protease inhibitors phenylmethylsulfonylfluoride (PMSF) and *p*-aminobenzamidine were added to final concentrations of 1 and 5 mM, respectively. After French pressure cell lysis at 20,000 psi, the mixture was centrifuged for 15 minutes at 12,000 x g to remove debris and unbroken cells. The supernatant was the crude extract used in most of the assays. In fractionation studies, the crude extract was centrifuged for 60 minutes at 200,000 x g to obtain the membrane fraction (pellet) and the cytosol fraction (supernatant).

Assay of the fatty acid desaturase. The conversion of radioactive palmitoyl CoA to a monounsaturated product has been used previously for assays of the fatty acid desaturase of alkaliphilic *Bacillus* extracts (7). The assay contained 60 mM potassium phosphate buffer pH 7.2, 1.25 mM NADH, 150 nmoles [1-C^{14}] palmitoyl CoA and 0.25-2 mg of the enzyme source in a total volume of 0.5 ml (7) unless otherwise indicated. The protein content was determined by the method of Lowry (19), using lysozyme as the standard. The assay components were preincubated for 5 minutes at the assay temperature. The reaction was initiated by the addition of crude extract and stopped at time points, between 8-30 seconds, by the addition of 0.25 ml 3M KOH/25% methanol. The mixtures were then saponified by incubation under nitrogen for 20 minutes at 80°C. The extraction of the fatty acids followed a modified procedure of Bligh and Dyer (1). After cooling, the mixtures were titrated to pH 4 by addition of concentrated HCl. Then, 3 ml of chloroform/methanol (1:2) were added followed by 1 ml chloroform and 1 ml water. After the phase separation, the lower (chloroform) layer was taken and dried under nitrogen. Methyl esters of the fatty acids were made by the addition of 1 ml boron trifluoride, under N_2, for one hour at 90°C and extracted with 1 ml chloroform and 1 ml H_2O. After phase separation, the lower (chloroform) layer was taken, dried under N_2, and resuspended in 100 μl chloroform. The fatty acid methyl esters were then plated on Analtech TLC plates containing either 5% $AgNO_3$ or Silica gel G (1000 microns) and separated by development in benzene:hexane (60:40), or acetone:water (90:10).

Commercial fatty acid methyl esters from Sigma were used as the standards, and the R_f values of the methyl esters were as described by others (16,33). Plates were developed by iodine vapors and the visible spots were scraped off with a scraping efficiency of 90%. Radioactivity was measured by suspension of the scrapings in a toluene cocktail and counted in a liquid scintillation counter.

Isolation of temperature-sensitive revertants. *B. subtilis* strains AG1A3 and AG2A were grown to late log phase and 10 ml of these cultures were subjected to ethyl methanesulfonate mutagenesis (14) in standard growth medium (24). After 90 minutes the cells were washed with Spizizen salts, allowed to grow for one generation, plated onto solid standard medium containing 5 μM CCCP and incubated at 32°C overnight. Colonies which grew overnight were replica plated on identical plates which were incubated at 26°C. Temperature-sensitive revertants were chosen on the ability to grow at 32°C and inability to grow at 26°C in the presence of 5 μM CCCP. Phenotypic rescreening was performed in broth medium identical to that of the plates.

Locant of the double bond. ^{13}C-NMR and gas chromatography-mass spectroscopy was used to determine the double bond positions of the monounsaturated fatty acids (10). ^{13}C-NMR proton-decoupled spectra were obtained using a computer controlled system based on a Varian XL-100 spectrometer operating at 25.2 MHz, with data memory size 16K for free induction decay. The spectra were obtained in $CDCl_3$ solutions. The table of Bus *et al.* (2) was used for assignment of carbonyl carbon atoms. GC-MS analysis was carried out on a JEOL JCG-20K gas chromatograph coupled directly to a JEOL JMS D-100 mass spectrometer operating at an electron bombardment energy of 20 eV. The mass spectra were obtained with the machine operating at 70 eV. The column used for chromatography was made of stainless steel, 6 ft. x 1/8 in. I.D. packed with 10% SE-30 on Chromosorb WAW, with helium as the carrier gas. Fatty acids were converted to the methyl derivative by addition of 6 mmol dimethyl disulphide and 0.05 mmol iodine (Sigma) and stirring under nitrogen for 24 hours at room temperature. Standard methyl (Z)-9-hexadecenoate was obtained from Supelco Inc.

Results

Characterization of the fatty acid desaturase of *B. subtilis*. The assay conditions described in Materials and Methods were optimized with respect to components of the reaction mixture, protein concentration, and time. Under optimal conditions, the rate of formation of unsaturated fatty acid from fatty acyl CoA was linear from 0 to 30 seconds at protein concentrations between 0.25 to 2 mg/ml protein; after 30 seconds, the rate of desaturation decreased dramatically. The fatty acid desaturase activity of *B. subtilis* BD99, as shown in Table 1, was O_2-, NADH-dependent and CN^- sensitive. The reaction was also inhibited by EDTA, suggesting the involvement of a divalent cation in the reaction. Dialyzed cell extracts were devoid of desaturase activity. The activity was not inhibited by carbon monoxide, which was added 1:1 v/v with O_2. Addition of FAD or FMN had an inhibitory effect on undialyzed samples and no stimulatory effect on dialyzed samples (data not shown). The requirement for reduced pyridine nucleotide was studied in detail. As shown in Figure 1, activity increased from 0 to 1.25 mM NADH, then steadily decreased until the reaction was completely inhibited at 5 mM NADH. No activity was found when NADPH was substituted for NADH at a concentration of 1.25 mM. Equimolar addition of NADH + NADPH to a final concentration of 1.25 mM resulted in an activity which was less than that with 0.62 mM NADH alone. The activity seen when no NADH was added to the assay was probably due to the presence of residual NADH or another endogenous reductant in the preparations.

Palmitoyl-CoA was preferred to stearoyl-CoA as a substrate. Under the standard

Table 1. Effect of various inhibitors and cofactors on the fatty acid desaturase activity of *B.subtilis*

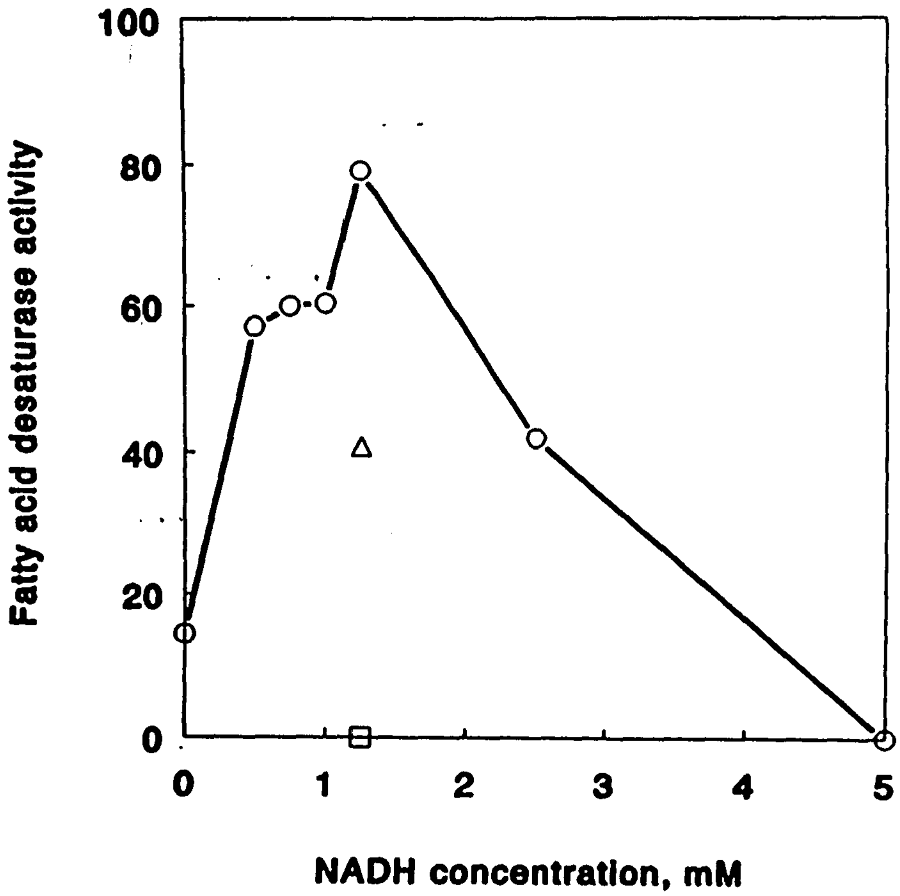
Assay system	% activity of the complete system
Complete[a]	100
-NADH	11
$-O_2$	0[b]
+50 mM NaCN	0[b]
+2 mM EDTA	0[b]
+CO[c]	145

[a]The complete assay contains 60 mM potassium phosphate pH 7.2, 1.25 mM NADH, 0.5 mg crude extract, and 150 μM C^{14} palmitoyl CoA in a total reaction volume of 0.5 ml.

[b]Activity below limits of detection.

[c]In carbon monoxide studies, reaction tubes were purged with CO for 1 min and 50% of the atmosphere replaced with O_2. Control experiments were purged with N_2 instead of CO.

Fig. 1. The effect of increasing NADH concentration on the activity of the fatty acid desaturase. Crude extracts were prepared as described in Materials and Methods from wild type *B. subtilis* BD99 and assayed at 30°C in the standard assay mixture in the presence of 0 to 5 mM NADH and 0.5 mg crude extract (○). In one assay, NADPH was substituted for NADH at a concentration of 1.25 mM (□), and NADH + NADPH were combined in another assay in an equimolar ratio to a final concentration of 1.25 mM (Δ).


100
80
60
40
20
0
Fatty acid desaturase activity
0
1
2
3
4
5
NADH concentration, mM

assay conditions, the rate of product formation using palmitoyl CoA was 9.5 nmoles/min/mg protein, and using stearyl CoA was 4.0 nmoles/min/mg protein. Phosphatidylethanolamine, a major phospholipid in *B. subtilis* and the site of esterification of the major portion of monounsaturated fatty acid (6), was not utilized as a substrate. With palmitoyl CoA as the substrate, the activity of the desaturase appeared to approach a maximal velocity at about 1 mM (Fig. 2). At substrate concentrations below 50 μM, activity was not linear with concentration but the assays were not sufficiently reproducible at these concentrations to define the kinetic properties more definitively. Cells of *B. subtilis* were fractionated and the fractions were assayed for desaturase activity. The activity was associated with the membrane fraction, but the amount was less than 10% of the total activity measured in the crude extract (Table 2). Recombination of the membrane with untreated cytosol restored the activity to that of the unfractionated extracts. Dialyzed or boiled cytosol added separately to the membrane fraction restored only 12% and 5%, respectively, of the activity, but together restored 40% of the activity.

The effects of growth and assay temperature on desaturase activity were examined (Table 3). Raising the assay temperature led to lower desaturase activity in cultures grown at any of the three temperatures. Raising the growth temperature also led to lower desaturase activity. However, cultures grown at 37°C had substantial activity when assayed at 20°C, indicating that a functional desaturase system was present in these cells.

Fig. 2. The effect of increasing palmitoyl-CoA concentration on the activity of the fatty acid desaturase. Crude extracts were prepared as described in Materials and Methods from wild type *B. subtilis* BD99 and assayed at 30°C in the standard assay mixture in the presence of 0.05 to 1 mM palmitoyl-CoA and 0.5 mg crude extract.

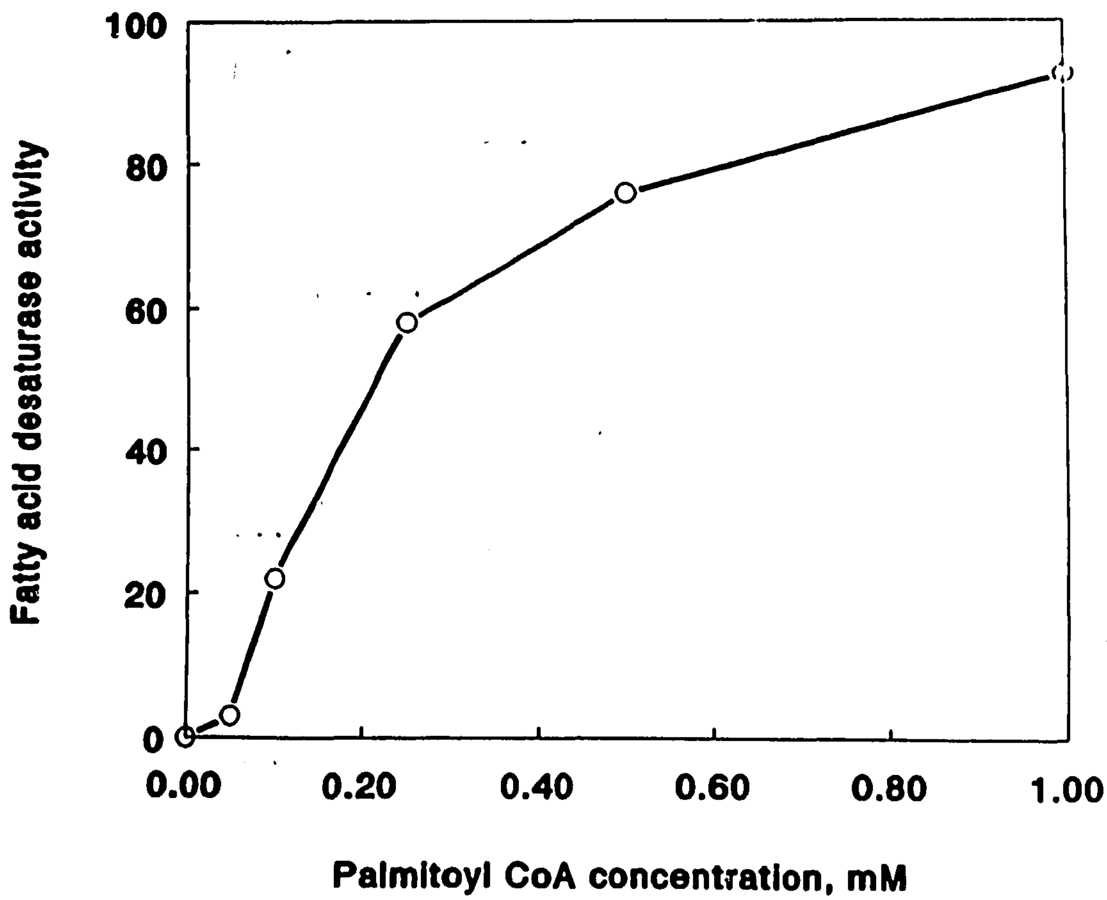

100
80
60
40
20
0
0.00
0.20
0.40
0.60
0.80
1.00
Fatty acid desaturase activity
Palmitoyl CoA concentration, mM

Table 2. Fatty acid desaturase activity of *Bacillus subtilis* BD99

Fraction	Total units[a] (nmoles/min)	Specific activity[b] (nmoles/min/mg protein)
Crude extract[d]	1858	10.6
Membrane[d]	177	16.3
Cytosol[d]	ND[c]	ND[c]
Membrane[d] +		
No Cytosol	ND[c]	ND[c]
Untreated Cytosol[d]	2142	22.1
Dialyzed Cytosol[d]	258	3.9
Boiled Cytosol[d]	116	4.6
Boiled Cytosol + Dialyzed Cytosol[d]	819	22.0

[a]Values are the means of five independent experiments, with standard deviations less than 30%. Numbers represent conversion of palmitoyl-CoA to a monounsaturated derivative, calculated by the total amount of protein in the preparation.

[b]Values are the means of at least five independent experiments, with standard deviations less than 30%. Numbers represent conversion of palmitoyl-CoA to a monounsaturated derivative.

[c]Not detectable.

[d]Cells were grown at 30°C to late log phase, harvested and lysed in a French pressure cell at 20,000 psi. The supernatant resulting from low speed (12,000 x g) centrifugation is defined as the crude extract. Membrane and cytosol fractions are the pellet and supernatant, respectively, of high speed (100,000 x g) centrifugation. Cell fractions at a concentration of 1 mg/ml were assayed from zero to twenty seconds. In recombination studies, the total protein concentration was 1 mg/ml with a membrane/cytosol ratio of 1:9, which is representative of the unfractionated crude extract.

Table 3. Effect of growth and assay temperatures on the fatty acid desaturase activity of crude extracts of *B. subtilis* wild-type

Growth temperature[a]	Specific activity (nmoles/min/mg protein) Assay temperature[b] 20°C	30°C	37°C
20°C	28.6	13.3	7.8
30°C	14.3	11.1	1.7
37°C	18.1	4.2	<0.7[c]

[a]Cells of wild type *B. subtilis* BD99 were grown at the temperature indicated to late log phase.

[b]All components of the reaction were incubated for 5 minutes at the assay temperature indicated prior to the assay.

[c]Activity below the limits of detection.

Table 4. Comparison of the fatty acid desaturase activities of wild-type *Bacillus* and uncoupler-resistant strains.

Strain[a]	Specific activity[b] (nmoles/min/mg protein)	
	Crude extract	Membranes
B. subtilis BD99, wild type	11.4	15.2
AG1A3	1.8	2.7
AG2A	<0.7[c]	<0.7[c]
AG3A	<0.7[c]	<0.7[c]
B. megaterium, wild type	7.4	7.6
C8	<0.7[c]	<0.7[c]

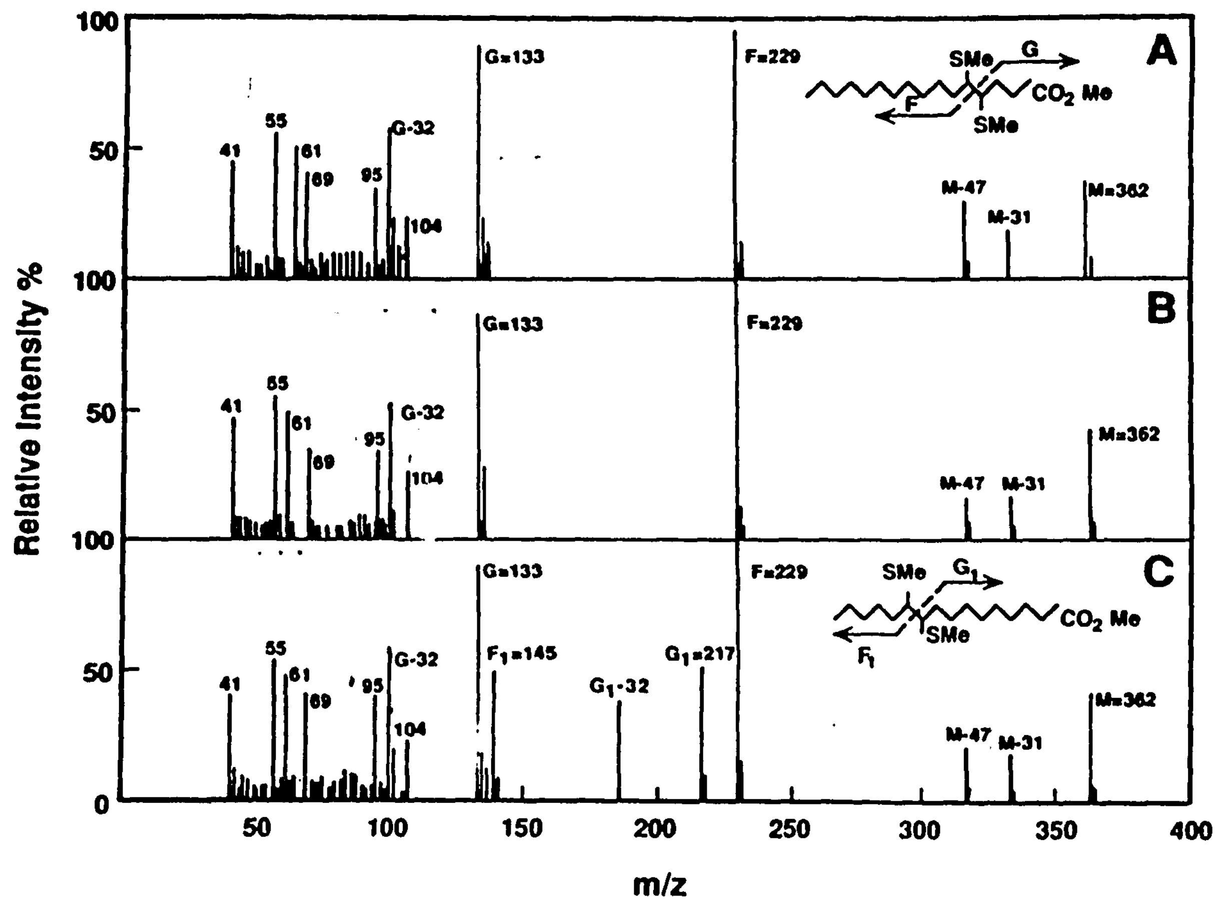
[a]All strains were grown at 30°C to late log phase.

[b]Values are the means of at least three independent experiments with standard deviations less than 20%.

[c]Activity below the limits of detection.

Comparison of the wild-type activity to that of the protonophore resistant mutants. All the protonophore-resistant mutants exhibited a substantial reduction in fatty acid desaturase activity in both membrane and crude fractions when compared to their wild type parents (Table 4). *B. subtilis* mutant AG1A3 exhibited approximately 10% of the wild type activity, whereas AG2Aand AG3A exhibited no detectable activity. Similarly, strain C8, the protonophore-resistant mutant of *B. megaterium*, was totally deficient in desaturase activity. Since prior studies (17) had shown that all three of the protonophore-resistant strains of *B. subtilis*, but not strain C8 of *B. megaterium* (17), had residual $C_{16:1}$ in the membranes, it was of interest to determine the position of the double bond in these strains. Studies of the locant of the double bond (Fig. 3 and Table 5) indicated that wild-type, AG2A and AG3A only had the double bond in the 5-6 position in $C_{16:1}$. Interestingly, AG1A3 had the double bond in the 5-6 position and in the 9-10 position in its $C_{16:1}$. Quantitatively, it appeared that the $\Delta 9$ fatty acid was a minor component (data not shown).

Fig. 3. Mass spectra for locant of the double bond for wild type *B. subtilis* BD99 and uncoupler-resistant strains AG1A3, AG2A, and AG3A. Total lipids were prepared by the procedure of Bligh and Dyer (1) and the monounsaturated fatty acids derivatized by alkylthiolation (10) for purposes of the spectra. Panel A-wild type *B. subtilis* BD99. Panel B-AG2A and AG3A. Panel C-AG1A3.



Relative Intensity %
m/z
A
B
C
G=133
F=229
SMe
G
CO2 Me
F
G1
F1
M-47
M-31
M=362
G-32
104
F1=145
G1-32
G1=217

Table 5. Carbon Chemical Shifts (PPM) of Palmitoleic Acid Methyl Ester (in $CDCl_3$) from *B. subtilis* Wild Type and Protonophore - Resistant Mutant Strains[a]

Lipid Sample	Locant of C Atom																
	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	(OCH_3)
Standard (Δ9)	14.05	22.65	31.60	29.40	27.30	27.80	120.00	129.50	28.50	26.80	26 50	26 00	25.10	24 95	34 10	174.25	51.60
Wild Type	14.05	22.60	31.60	30.80	27.30	27.00	26.80	26.50	26.40	27.0	129 0	130.0	27.0	24.95	34 10	174.25	51.60
AG2A	14.04	22.60	31.50	29.60	27.30	27.20	26.80	26.50	26.40	27.0	129 0	130 0	27 0	24 95	34 10	174.25	51.60
AG3A	14.05	22.60	31.50	29.60	27.30	27.20	26.80	26.50	26.40	27.0	129 0	130.0	27 0	24 95	34 10	174.25	51.60
AG1A3	14.05	22.60	31.50	29.60	27.30	28.40	125.80	130.50	28.40	27.0	128 40	128.60	27 0	24 95	34 10	174.25	51.60

[a] Internal Reference TMS

Table 6. Effect of Temperature on Growth in the Presence of CCCP and Fatty Acid Desaturase Activity of *B. subtilis* Wild Type, Uncoupler-resistant Mutants, Full and Temperature-sensitive Revertants

Strain	Growth on 5 μM CCCP at 25°C	Growth on 5 μM CCCP at 32°C
Wild type	No	No
<u>CCCP-resistant</u>		
AG1A3	Yes	Yes
AG2A	Yes	Yes
<u>Full-revertant</u>		
AG1A3rev8	No	No
AG2Arev35	No	No
<u>Temperature-sensitive revertant</u>		
Ts47AG1A3	No	Yes
Ts1AG2A	No	Yes

Strain	Specific activity (nmoles/min/mg protein) when assayed at[a] 25°C	Specific activity (nmoles/min/mg protein) when assayed at[a] 32°C
Wild type	13.9	11.6
<u>CCCP-resistant</u>		
AG1A3	1.6	<0.7[b]
AG2A	<0.7[b]	<0.7[b]
<u>Full-revertant</u>		
AG1A3rev8	18.7	3.4
AG2Arev35	12.6	8.1
<u>Temperature-sensitive revertant</u>		
Ts47AG1A3	5.8	<0.7[b]
Ts1AG2A	8.6	<0.7[b]

[a]Values are the means of at least two independent experiments with standard deviations less than 20%.

[b]Activity below the limits of detection.

Assays of wild-type *B. subtilis*, two protonophore-resistant derivatives, two temperature-sensitive and two full revertants were conducted. When assayed at 26°C, both AG1A3Ts47 and AG2ATs1 exhibited substantial desaturase activity which was comparable to that of the wild type (Table 6). At 32°C, however, the temperature sensitive revertants had markedly reduced desaturase activity approximating that of the resistant strains AG1A3 and AG2A. These temperature sensitive, protonophore-resistant strains thus showed temperature sensitive desaturase activity that correlated inversely with their pattern of protonophore resistance. Full revertants exhibited restored levels of desaturase activity at both temperatures and had lost the ability to grow at either temperature in the presence of the protonophore.

Discussion

The fatty acid desaturase of *B. subtilis* exhibited many properties that were characteristic of previously described aerobic desaturase systems, e.g. membrane-associated, oxygen-dependent and cyanide sensitive. The specific activity was higher than that observed in *Mycobacterium* (13), *Alcaligenes* (16), and *Pseudomonas* (33). However, the linear time frame for activity was 30 seconds, considerably shorter than that of the other prokaryotic systems. This could be the result of rapid turnover of the enzyme, or of cytochrome P450-mediated degradation of the monounsaturated product. Evidence for the latter possibility came from the observation that addition of CO, an inhibitor of cytochrome P450, modestly increased the activity (Table 1). Cytochrome P450s are known to be capable of desaturation (15) as well as epoxidation of double bonds. Since carbon monoxide did not inhibit the desaturase activity and in fact gave some stimulation, insertion of a double bond into palmitoyl-CoA was probably not due to P450 activity, but rather to fatty acid desaturase activity. The preference for NADH over NADPH had been noted in the insect system (34), though NADPH was preferred in the rat liver system (25). The potent inhibition by EDTA pointed to a possible requirement for divalent cations. Ferrous iron and flavins were presumably required for desaturase activity (13); however, these requirements were not directly demonstrable, probably because of bound cation and cofactor.

Fatty acid desaturases of eukaryotes are membrane-bound systems composed of cytochrome b_5, cytochrome b_5 reductase, and the desaturase (8,9). However, the fractionation experiments indicated that soluble factors are required in the *Bacillus* system. The fractionation experiments showed that although the activity was confined to the membrane, both dialyzable and non-dialyzable cytosolic factors were required to restore the membrane activity to a level comparable to that observed in the crude extract. This result is similar to results in *Mycobacterium* (15), in which both a heat-labile factor and a dialyzable factor were found to be required for activity. Palmitoyl CoA was preferred to stearoyl CoA as the substrate for the fatty acid desaturase of *B. subtilis*. Consistently, the only monounsaturated fatty acid esterified in the membrane phospholipids is $C_{16:1}$ (17). This contrasts with the observation of the stearyl-CoA desaturase of eukaryotes (25), for which stearyl-CoA is the preferred substrate. The fact that fatty acid desaturation did not take place on the phospholipid, as has been observed in plants (23), indicated the requirement for an activated fatty acid substrate. Assays using the free fatty acid as a substrate were not feasible due to the insolubility of palmitic acid in an aqueous environment.

In vivo studies of the effect of temperature on the activity of the fatty acid desaturase of *B. megaterium* by Fulco and colleagues (11) indicated that elevated growth temperatures inhibited the synthesis of the enzyme, and implicated the involvement of a protein modulator (11). The current results with *B. subtilis* suggest that the desaturase activity is also directly affected by lowering or raising the temperature. Desaturase activity of cultures grown at 20°C was significantly higher than that of cultures grown at 37°C. However, increasing the assay temperature led to a reduction in activity regardless of the growth temperature, and substantial activity was observed from a culture grown at 37°C when assayed at 20°C. These results indicated that while growth temperature modulated desaturase activity, the *in vitro* desaturase activity was also directly sensitive to temperature. It would be of interest to determine whether this property would persist in a solubilized preparation. A major finding in the current study was the absence of O_2-dependent fatty acid desaturase activity in two protonophore-resistant strains AG2A and AG3A of *B. subtilis* and strain C8 of *B. megaterium*, and the presence of low desaturase activity in protonophore-resistant AG1A3. However, while membrane composition studies of the wild type and mutant strains showed that strain C8 of *B. megaterium* totally lacked unsaturated fatty acids, the three *B. subtilis* mutants retained 50% of the wild-type percentage of monounsaturated fatty acid esterified into the phospholipid. There must be an alternate pathway for the biosynthesis of unsaturated fatty acids in *B. subtilis* which is active in AG2A and AG3A in the absence of the O_2-dependent fatty acid desaturase activity assayed here. The mutation in AG1A3 probably leaves that alternate pathway intact and changes both the activity of the O_2-dependent enzyme and its specificity with respect to the placement of the double bond. In contrast, C8, a protonophore-resistant mutant of *B. megaterium*, had no detectable levels of unsaturated fatty acids; this species may thus have no alternate pathway for the biosynthesis of unsaturated fatty acids.

The inverse correlation between desaturase activity and uncoupler resistance was most clearly shown in the assays of the temperature sensitive revertants. At a temperature which allowed growth of the temperature-sensitive strains in the presence of the uncoupler the desaturase activity was like that of the parent uncoupler-resistant strains. At the non-permissive temperature, the activity of both temperature-sensitive strains was significantly higher, indicating that temperature-sensitive desaturase activity correlated inversely with temperature-sensitive uncoupler resistance. Full revertants, which had desaturase activity resembling that of the wild type, were sensitive to the uncoupler at both temperatures.

Acknowledgements

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Chapter Four

Isolation and Analysis of Tn917-Mediated Insertional Mutants of *Bacillus subtilis* that are Protonophore-Resistant

Abstract

Protonophore resistance in mutants of *Bacillus subtilis* has previously been shown to result from a deficiency in a fatty acyl-CoA desaturase; the resulting increase in the saturated/unsaturated fatty acid ratio in membrane phospholipids is associated with a bioenergetic phenotype that is reversible by exogenous unsaturated fatty acid. In the current study, libraries of insertional mutants of *B. subtilis* were prepared by transformation with the Tn917-containing plasmid pLTV1 and screened for protonophore resistance. Of the twenty-five protonophore-resistant strains isolated from four libraries, nineteen were rendered sensitive to the protonophore upon inclusion of palmitoleic acid in the medium. Nine of the twenty five strains were assayed for fatty acid desaturase activity, including six mutant strains that were thus "reversible" in phenotype and three mutant strains that were not reversible. All proved to be markedly deficient in fatty acid desaturase activity relative to the wild type, with only two of the "reversible" mutants showing any detectable activity. The region of chromosomal DNA flanking the Tn917 insertion was cloned from one of the "reversible", desaturase-deficient, strains. Sequence analysis indicated that the insertion site was approximately 4 kb from the *spo*0F gene.

Introduction

Protonophore-resistant mutants AG1A3, AG2A, and AG3A, previously isolated in our laboratory from *B. subtilis* (5,7), have been shown to be capable of growth on malate in the presence of 5 μM carbonyl cyanide *m*-chloro-phenylhydrazone (CCCP), which is inhibitory to wild-type growth (5,7). The three mutants had reduced levels of unsaturated fatty acids esterified into the membrane phospholipids compared to the wild-type (7). Assays of the fatty acyl-CoA desaturase showed that the mutants were markedly reduced in the activity compared to the wild type (3). Temperature-sensitive CCCP-resistant mutants exhibited desaturase activity that was inversely related to their capacity for growth in the presence of protonophore (3,8), supporting the conclusion that a fatty acid desaturase deficiency is the biochemical basis for the protonophore-resistant phenotype. It was of interest to ascertain whether any other null mutation could result in this phenotype and also to use the phenotype as a means of identifying bacterial genes that encode the desaturase components or crucial positive regulators thereof. Towards these ends, wild type *B. subtilis* was transformed with pLTV1, a plasmid containing a highly engineered Tn917 transposon (2,14). This transposon has been demonstrated to insert into the chromosome of *B. subtilis* with a high degree of randomness, and the insertional mutants thus generated are very stable (14,20,21). Transposition libraries have been prepared and screened for CCCP-resistant insertional mutants in order to determine if all such strains were desaturase deficient and to initiate a characterization of the insertion site(s).

Materials and Methods

Bacterial strains and plasmids. *B. subtilis* BD99 (5) was the parent strain for the transposition libraries. All mutant strains were grown in enriched medium containing veal extract (10g/l) and yeast extract (5g/l). *Escherichia coli* DH5αMCR (Gibco-BRL) was the host cell used for cloning. All *E. coli* strains and transformants were grown in Luria Bertoni (LB) medium (10g tryptone, 10g sodium chloride, and 5g yeast extract per liter) supplemented with ampicillin (100 μg/ml). The plasmid pLTV1 (2) was obtained from Dr. Philip Youngman, University of Georgia. The plasmid contains Tn917 as well as genes for ampicillin, tetracycline, and chloramphenicol resistance.

Growth of bacterial cells to competence. *B. subtilis* BD99 was grown to competence (1,10) by first growing cells in Spizizen salts supplemented with 0.1% yeast extract, 0.02% casamino acids, 0.5% glucose and 10 μg/ml histidine, threonine, and tryptophan at 30°C to stationary phase. Growth was followed by measuring the turbidity. Then, 5 ml of the cells were inoculated into fresh medium of the same composition at 37°C. When the cells reached stationary phase, 5 ml of cells was inoculated into fresh medium of the same composition, but containing 2 mM EGTA, for 90 minutes, after which the cells were harvested and frozen in 10% glycerol for future use.

E. coli DH5αMCR was grown to competence by a modified procedure of Hanahan (6). Cells (200 μl) were inoculated from a starter culture grown to stationary phase into 300 ml LB supplemented with 0.1% glucose. The cells were harvested after growth to an A_{550} reading of 0.5, resuspended in 30 ml TFB1 (30 mM potassium acetate, 50 mM $MnCl_2$, 10 mM $CaCl_2$, 100mM KCl, and 15% glycerol (w/v)), pH 5.8, and placed on ice for two hours. After incubation, the cells were harvested, resuspended in 6 ml TFB2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM KCl, and 15% glycerol (w/v)), pH 7, aliquoted and stored at -70°C.

Transformation of *B. subtilis*. Competent cells of *B. subtilis* were incubated with 2.5 μg/ml pLTV1 for 30 minutes at 37°C (10). Two ml of VYE medium containing 10g/l veal infusion broth (Difco), 5g/l yeast extract, and 0.1 ml of 50% glucose were added, and the incubation was continued for 60 minutes. After centrifugation, the supernatant was decanted and the cells were resuspended in liquid VYE medium and plated on VYE plates containing 5 μM chloramphenicol (Boehringer Mannheim) at 30°C. Colonies which appeared following overnight growth were tested for tetracycline resistance by streaking them onto VYE plates containing 12 μg/ml tetracycline (Boehringer Mannheim).

Preparation of transposition libraries of B. subtilis BD99. Single colonies that had been transformed with pLTV1 to chloramphenicol and tetracycline resistance (2) were grown in 2 ml VYE broth containing 1 μg/ml erythromycin (Boehringer Mannheim), 25 μg/ml lincomycin, and 12.5 μg/ml tetracycline at 30°C to stationary phase. These cultures were inoculated into 1 liter of VYE medium containing 1 μg/ml erythromycin and 25 μg/ml lincomycin and grown at 48°C to stationary phase. The cells were harvested by centrifugation at 12,000 x g for 10 minutes and frozen in 10% glycerol for subsequent screenings. Each independent preparation was designated as a library; five such libraries were prepared.

Frequency of transposition. Transformed colonies were streaked to plates containing 1 μg/ml erythromycin, 25 μg/ml lincomycin, and 12.5 μg/ml tetracycline and incubated at 30°C for sixteen to twenty hours (2). Single colonies were picked and inoculated in 10 ml of VYE broth and grown at 30°C to an A_{600} reading of 0.4. The cultures were plated on solid VYE medium containing 1 μg/ml erythromycin and 25 μg/ml lincomycin at both 30°C and 48°C. The frequency of transposition was measured by the number of colonies grown at 48°C that were erythromycin resistant and tetracycline sensitive divided by the number of colonies grown at 30°C that were erythromycin and tetracycline resistant. The average frequency of transposition in two independent determinations was 5.5×10^{-3}, a frequency very close to that of 5×10^{-3} found by Youngman and colleagues (2).

Selection of CCCP-resistant mutants. Libraries were screened for CCCP-resistant mutants by plating 100 μl of cells from concentrated frozen aliquots on solid media containing VYE supplemented with 5 μM or 10 μM CCCP without antibiotics. The plates were incubated for three to five days at 30°C. Colonies that grew above the background were picked and rescreened for chloramphenicol resistance and tetracycline sensitivity as well as protonophore resistance. The protonophore resistance of the insertional mutants was comparable to that of the protonophore-resistant mutants of *B. subtilis* previously isolated in our laboratory by conventional mutagenesis (5), as assessed by their capacity to grow in the presence of various concentrations of CCCP.

Screening for reversibility of the CCCP-resistant phenotype by exogenous palmitoleic acid. CCCP-resistant insertional mutants were screened for the loss of protonophore resistance in the presence of 5 μM palmitoleic acid by inoculating 0.5 ml of a starter culture grown in medium without CCCP to stationary phase into flasks containing 50 ml Spizizen salts, 50 mM malate pH 7, 0.1% yeast extract, 10 μg/ml of histidine, threonine, and tryptophan and 3 μM CCCP with or without 5 μM palmitoleic acid ($C_{16:1}$). Strains which grew overnight in the presence of 3 μM CCCP without $C_{16:1}$ but did not grow in the presence of 3 μM CCCP and $C_{16:1}$ were categorized as $C_{16:1}$-reversible.

Assay of the fatty acid desaturase of *Bacillus subtilis*. Wild-type BD99 and CCCP-resistant insertional mutants from four independent libraries were assayed for fatty acyl-CoA desaturase activity. Cells were grown in Spizizen salts, 50 mM malate pH 7, 0.1% yeast extract, and 10 μg/ml histidine, threonine and tryptophan to late logarithmic phase in Spizizen salts at 30°C. Extracts were prepared by French pressure cell lysis. Desaturase activity was assayed as described (3).

Preparation of plasmids, chromosomal DNA, and sequencing. Chromosomal DNA from CCCP-resistant insertional mutant strains was isolated and purified by the method of Marmur (11). Briefly, cells were lysed by lysozyme/SDS and the proteins removed by proteolysis and phenol extraction. The DNA was precipitated by ethanol and removed by spooling. DNA thus obtained was digested with *Eco*RI; self-ligation of the diluted DNA yielded a plasmid which contained the coding region for ampicillin resistance and the ColE1 origin of replication of Tn917 in addition to an insert of *B. subtilis* chromosomal DNA (2,14) (Figure 1). The resulting plasmid was used to transform competent *E. coli* DH5αMCR cells (10) to ampicillin resistance. Plasmids from transformed *E. coli* were prepared by large-scale alkaline lysis and purified on a CsCl gradient (10). Sequencing of the chromosomal DNA insert was performed using an Applied Biosystems 373A DNA sequencer in the DNA Core Facility of the Brookdale Center for Molecular Biology at Mount Sinai School of Medicine. Oligonucleotide primers used for sequencing were synthesized using an Applied Biosystems 380B DNA synthesizer in the same Core Facility.

Results

As shown in Table 1, the screening of five transposition libraries of *B. subtilis* for CCCP-resistant strains resulted in the isolation of twenty five such strains. These were presumed to be the result of transpositional rather than spontaneous mutagenesis because they were chloramphenicol resistant and tetracycline sensitive, indicating that the plasmid encoding the tetracycline resistance had been lost and the transposon encoding the chloramphenicol resistance had been retained by a stable insertion into the chromosomal DNA (2). Of the twenty five CCCP-resistant insertional mutants, nineteen were unable to grow in the presence of CCCP when the medium was supplemented with 5 μM $C_{16:1}$, i.e. were $C_{16:1}$-reversible. Notably, the six strains whose CCCP-resistant phenotype was not reversed had all been isolated from the same library.

Nine of the twenty five CCCP-resistant insertional mutants were assayed for the fatty acid desaturase activity of crude cell extracts. Six of these strains had the $C_{16:1}$-reversible CCCP-resistant phenotype and three retained CCCP resistance in the presence of exogenous $C_{16:1}$. As shown in Table 2, all of the CCCP-resistant insertional strains were either totally lacking or notably deficient in fatty acid desaturase activity in comparison to the wild type. Interestingly, two of the six mutants with a $C_{16:1}$-reversible phenotype had some residual activity, indicating a degree of heterogeneity in this group of mutants.

The region flanking the site of the Tn917 insertion was cloned from mutant I-33. One plasmid, containing a fragment generated as shown in Figure 1, was purified and studied. Analysis of this plasmid, designated pED133, indicated that it contained 4.2 kb of DNA derived from the *B. subtilis* chromosome. A restriction analysis of the insert was conducted,

Table 1. CCCP-resistant strains isolated from Tn917-insertional libraries of *Bacillus subtilis*.

Library number	CCCP-resistant strains reversed by 5 μM $C_{16:1}$ YES	NO	TOTAL
I	9	6	15
II	4	0	4
III	5	0	5
IV	1	0	1
V	0	0	0
Total	19	6	25

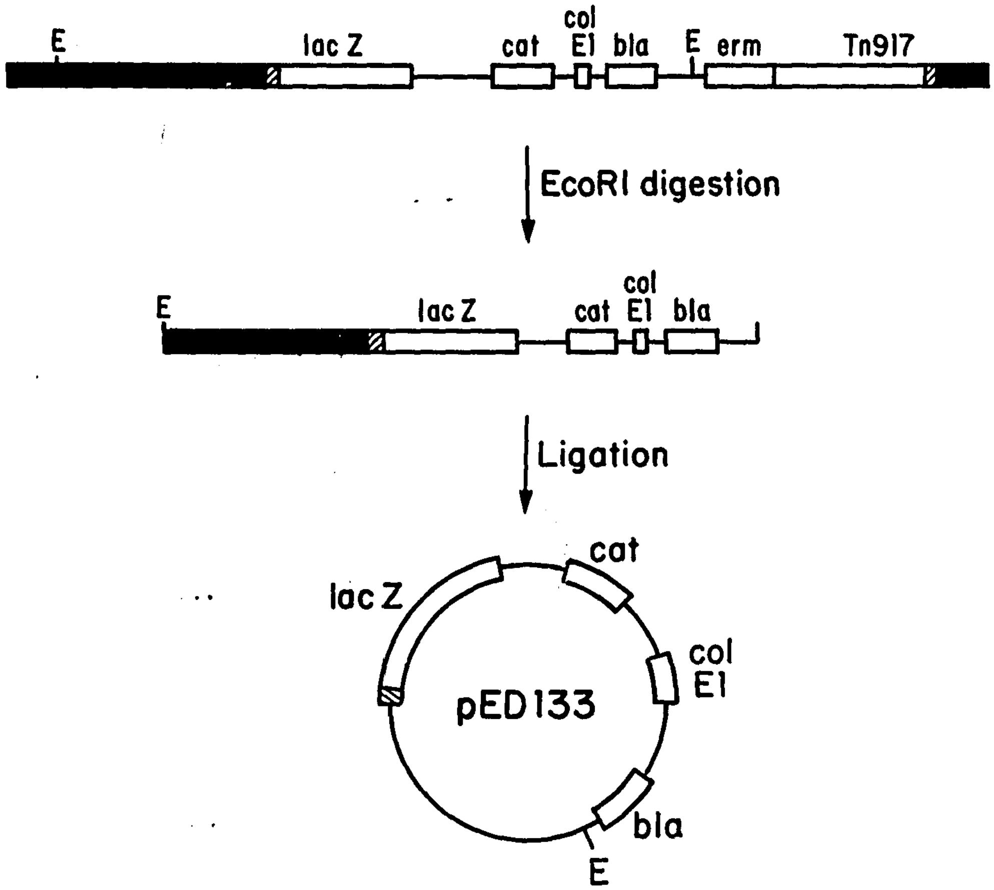
Table 2. Fatty acid desaturase activity of *Bacillus subtilis* BD99 and CCCP-resistant insertional mutants.

Strain	Fatty acid desaturase activity[a] (nmoles/min/mg protein)
Wild type BD99	25.2
Insertional mutant, CCCP-resistance reversed by 5μM $C_{16:1}$ in growth medium	
I-1	8.3
I-29	6.1
I-33	<0.7[b]
II-1	<0.7[b]
II-2	<0.7[b]
III-4	<0.7[b]
Insertional mutant, CCCP-resistance not reversed by 5 μM $C_{16:1}$ in growth medium	
I-9	<0.7[b]
I-19	<0.7[b]
III-7	<0.7[b]

[a]Values are the means of two determinations of the fatty acid desaturase activity of crude cell extracts. The standard deviations were less than 30%.

[b]Activity below the limits of detectability.

Figure 1. Strategy for cloning the flanking region of Tn917-insertional mutants produced subsequent to transposition with pLTV1. E-*Eco*R1 restriction site; lacZ-coding region for β-galactosidase; cat-coding region for chloramphenicol acyltransferase; colE1-colE1 origin of replication; bla-coding region for ampicillin resistance; erm-coding region for erythromycin resistance. The thick darkened segment is chromosomal DNA flanking the inserted transposon in the upper diagram and cloned in the lower diagram.


E
lac Z
cat
col
E1
bla
E
erm
Tn917
EcoRI digestion
E
lac Z
cat
col
E1
bla
Ligation
lac Z
cat
col
E1
pED133
bla
E

and sequencing of the *B. subtilis* DNA was initiated from both ends. Thus far, only 613 bases have been sequenced from the end of the clone immediately flanking the insertion; this sequence is shown in Figure 2. No open reading frame has been found to date; translation of the sequence in all three reading frames in both directions indicated a number of stop codons. By contrast, the sequence obtained from the end of the clone that represents the location of the first *Eco*RI site in the *B. subtilis* DNA past the insertion on the lacZ end of Tn917 was more extensive and is recognizable from the published work of others. The most distal sequence was identical to that reported by Hoch and colleagues (19) for the downstream region of the *spo*0F gene. Hoch and colleagues (19) had sequenced the open reading frame downstream of *spo*0F, the *tsr* gene, an open reading frame coding for a protein of unknown function designated *orf*U, and part of the next downstream open reading frame designated *orf*R. The sequence in pED133 began at nucleotide 4304 of their clone, just downstream of the start of the *orf*U coding region, and was confirmed identical to the published sequence as far as nucleotide 4642. In Figure 3, a diagram of the clone is shown, indicating restriction sites mapped and the location of the sequenced and unsequenced regions to scale. Thus far, using oligonucleotide primers based on the sequence of the 3' end of *orf*R, a further 305 bases of sequence have been obtained beyond the published sequence, containing the rest of the *orf*R reading frame and the start of another possible open reading frame (Figure 4).

Figure 2. DNA sequence of the chromosomal DNA flanking the site of Tn917 insertion of Tn917 in pED133. Position 1 of the nucleotide sequence indicates the first base of the chromosomal DNA of *B. subtilis* immediately adjacent to the 3'end of the *lac*Z coding region of the vector. Sequence of one strand is shown.

1 AAAGAGGAAATAGATACGTCTTTCATAAAAACACCACGCTTTTCATAGTCAATATCTTACTCTGAACAT

70 GTCATCTGCTGCTTTGGCACAACATCCGCCAAAGCACATTCTTTTTTAACATAAAGGAACTTCTATCG

139 CAACACTTCCACCCATGATCGGCGCTTTTGTTAAAAGGATTGTCTTAATGAGTTTTGAAGGAAGCGAGG

208 ACAAAAACATGAGAAGATCAATACTTATATCGTCTGTATGCGGTAGAAATTGAAACTTTCCTAGTGCTT

277 AAAAGAAGGTATTACGCAGTAAAATTCGCAGCACTCTTTATTTTTACCTTATTTCAAAAAATTATTCAA

346 TTGAAATAAGGTTTTGACACGGAATTGATAGAGTTGACGCTTTCTGTAAAAGTTAAAGCGGGATCACAC

415 CCGCTTTTTCTGTTTGTTATGGACGGATTACAAGATTTGGTTCTTTTCAGCTGTGTCTGCGTCAACGAA

484 ACGAACAGTGCCCCGATTTTGGACGGATAACTAAGCTTTCAGTTGTACCGACAGAGCCTTTGAATTGAA

554 CTCTTTTAACAGCTCGCCGTCAGTAACCCTGTAGCTGAAATCGGTCACTATTTCCTCTTT

Figure 3. Restriction map and diagrams of sequenced regions of plasmid pED133 derived from *B. subtilis* insertional mutant I-33. E-*Eco*RI; P-*Pst*I; Hp-*Hpa*I; H-*Hin*dIII. Regions of the clone that have been sequenced by Hoch and colleagues (19) or in this work are covered with a solid line.

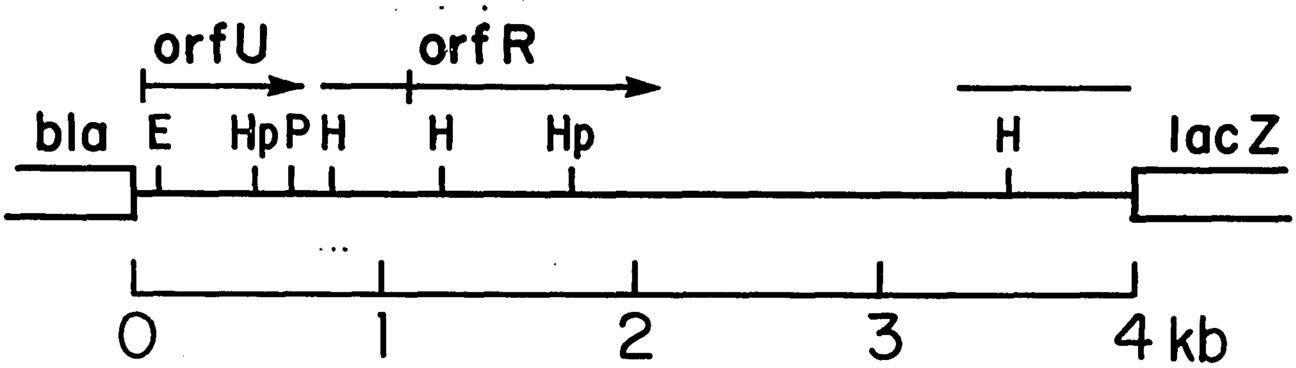

orfU
orfR
bla
E
Hp
P
H
H
Hp
H
lacZ
0
1
2
3
4 kb

Figure 4. Nucleotide and deduced amino acid sequence of the *orf*R coding region from the chromosomal DNA insert of pEDI33. Nucleotide 897 corresponds to that number of bases from the beginning of our clone. The sequence reported by Hoch and colleagues (19) extends to nucleotide 6314 of the *orf*R, corresponding to nucleotide 2011 of our clone. Extending from that is new sequence.

(orfR) M E
897 AGACAGAATAAAATGACATTTTATTTAGCTCCGCAGCTATCTTCTTATGGAAGGAGACTATCATGGAAA

K L N I A G G D S L N G T V H I S G A K N S A
966 AGTTGAATATTGCCGGCGGTGACTCGTTAAACGGTACAGTACATATCAGCGGCGCTAAAAACAGCGCTG

V A L I P A T I L A N S E V T I E G L P E I S
1035 TTGCGTTAATACCTGCAACCATTTTGGCAAATTCCGAGGTGACAATTGAAGGGCTTCCAGAGATTTCAG

D I E T L R D L L K E I G G N V H F E N G E M
1104 ATATTGAAACGCTGCGTGACCTGTTAAAGGAAATCGGCGGCAACGTGCATTTTGAGAATGGGGAAATGG

V V D P T S M I S M P L P N G K V K K L R A S
1173 TTGTTGACCCTACGTCGATGATCAGCATGCCGCTTCCTAACGGGAAAGTAAAAAAGCTTCGCGCGTCAT

Y Y L M G A M L G R F K Q A V I G L P G G C H
1242 ATTATTTAATGGGGGCGATGCTCGGCCGCTTCAAGCAGGCGGTCATTGGATTGCCTGGCGGCTGTCACT

L G P R P I D Q H I K G F E A L G A E V T N E
1311 TAGGGCCCCGTCCGATTGATCAGCATATCAAAGGCTTTGAAGCACTCGGAGCTGAAGTAACCAATGAAC

Q G A I Y L R A E R L R G A R I Y L D V V S V
1380 AAGGCGCCATTTATTTGCGAGCTGAAAGGCTGAGAGGCGCACGGATTTATTTAGATGTCGTAAGCGTTG

G A T I N I M L A A V L A E G K T I I E N A A
1449 GGGCAACGATTAACATTATGCTCGCCGCTGTTTTGGCAGAAGGGAAAACGATCATCGAAAACGCTGCCA

K E P E I I D V A T L L T S M G A K I K G A G
1518 AGGAGCCTGAGATCATTGACGTCGCGACATTGCTTACCAGCATGGGCGCCAAAATCAAAGGTGCGGGCA

T N V I R I D G V K E L H G C K H T I I P D R
1587 CCAATGTGATTCGAATCGACGGCGTGAAGGAACTGCACGGCTGCAAGCATACGATCATTCCGGACAGAA

I E A G T F M I A G A A M G K E V I I D N V I
1656 TTGAAGCCGGGACATTTATGATTGCAGGGGCTGCAATGGGCAAGGAAGTCATTATCGATAACGTCATCC

P T H L E S L T A K L R E M G Y H I E T S D D
1725 CTACTCATCTTGAGTCGTTAACGGCAAAGCTGAGAGAAATGGGCTATCATATCGAAACAAGCGACGACC

Q L L I V G G Q K N L K P V D V K T L V Y P G
1794 AGCTCCTCATTGTCGGCGGGCAGAAGAACTTAAAGCCGGTTGACGTCAAAACCCTCGTATACCCGGGGT

F P T D L Q Q P M T A L L T R A K G T S V V T
1863 TTCCGACTGATTTACAGCAGCCGATGACGGCGCTCCTGACAAGGGCGAAAGGGACGAGTGTCGTCACAG

D T I Y S A R F K H I D E L R R M G A N M K V
1932 ACACCATCTACTCGGCAAGATTCAAGCACATTGATGAGCTGAGACGAATGGGTGCCAATATGAAAGTAG

E G R S A I I T G P V E L N G A K V K A S D L
2001 AAGGCAGATCTGCCATCATCACAGGTCCTGTCGAGCTTCAAGGCGCAAAAGTGAAGGCGAGTGATCTGC

R V G A C L A V A G L M A D G V T E I T V L E
2070 GTGTCGGAGCCTGCTTGGCGGTAGCCGGACTGATGGCTGATGGCGTCACGGAAATTACGGTACTGGAGC

H I D R G Y S S L E Y Y F E G F G V T I C R E
2139 ATATTGACCGAGGATACAGTAGCCTCGAGAAGAGTTTGAGGGGTTTGGCGTGACAATTTGCCGTGAAA

R M T D E E I E N F Y I H N G F L *
2208 GAATGACTGACGAAGAAATAGAACAGTTCAAAATTCATAATGGTTTCTTGTGAGAGGAGATCGCTCTGA

M E R S L S M A L A R
2277 AATGGAAAGAAGTTTATCAATGGCATTGGCACGCT

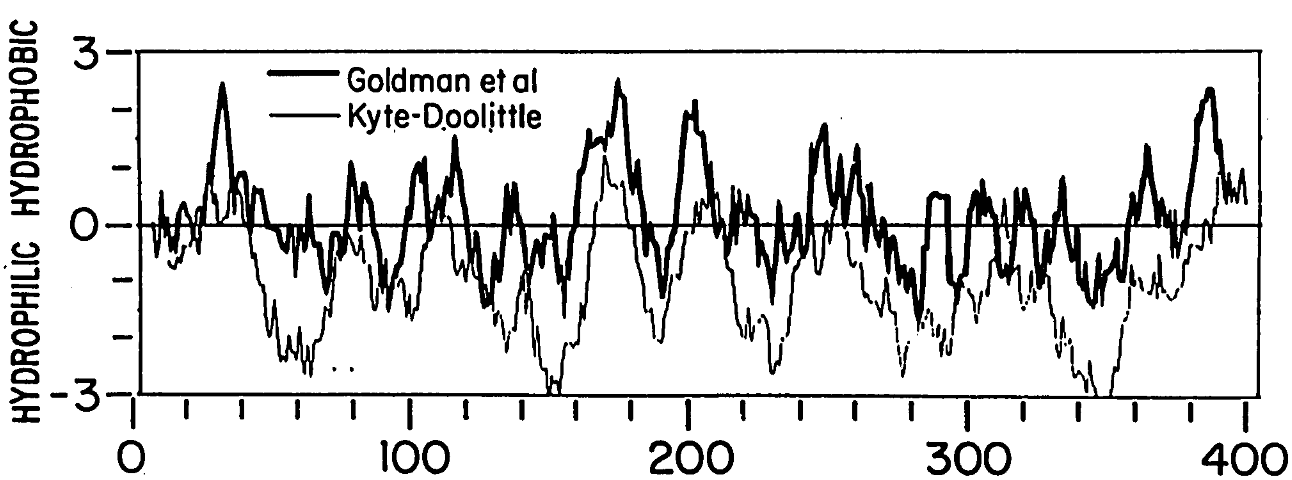
Discussion

All nine CCCP-resistant insertional mutants assayed so far proved to be deficient in fatty acid desaturase activity (Table 2). This study gives no indication of an alternative mutation to that previously reported for generating protonophore resistance in *B. subtilis* (3,7) that can be achieved by a gene inactivation that results in this phenotype. Interestingly, there was still heterogeneity among the isolates. First, two of the strains whose CCCP-resistance was reversed by exogenous $C_{16:1}$ had residual desaturase activity. Perhaps there is one desaturase component whose function can be partially maintained through substitution of a different gene product. This mutational event which yields partial activity is apparently rarer than insertion(s) yielding complete inactivation. Second, three mutant strains had no desaturase activity but were not susceptible to reversal of CCCP-resistance by exogenous $C_{16:1}$. A possible interpretation of this finding is that the insertion in those strains inactivated a positive regulatory element controlling both desaturase expression and expression of either the fatty acid uptake and/or activating enzyme systems. Such an insertion could lead to a loss of desaturase activity and an inability to incorporate exogenous unsaturated fatty acid which could reverse the protonophore-resistant phenotype. It will be of interest to compare the flanking sequences, and ultimately the complete genes which resulted in the three different types of CCCP-resistant strains.

My initial effort focused on strain I-33, one of the strains that completely lacked desaturase activity and whose phenotype was reversed by exogenous $C_{16:1}$. This phenotype is characteristic of the protonophore resistant strains AG2A, and AG3A of *B. subtilis* isolated previously (5,7), which were deficient in fatty acid desaturase activity (3). The insertion in I-33 is most likely to be into one of the structural genes encoding the fatty acid desaturase components. The fatty acyl-CoA desaturase of eukaryotes (12,16-18), which is O_2-dependent, CN^--sensitive, CO-insensitive and of the same type that is found in *B. subtilis* (3), has three protein components. The data obtained from the cloned flanking region of the insertional site in strain I-33 indicate that the location of insertion was approximately 4 kb from the end of *spo*0F, which is position 324 (15) on the *B. subtilis* chromosomal map. The initial sequence analysis of the flanking region nearest the insertion site has revealed no open reading frames as yet. It is quite possible that the insertion is close to the end of the coding region of the interrupted gene. Cloning of the flanking region of other "reversible" strains may be more informative. If not, the region on the other side of the transposon could be cloned using libraries prepared from DNA of the insertional mutants and screening for chloramphenicol resistance.

Hoch and colleagues (19) described the *orf*U gene product as a 20kD protein of unknown function cotranscribed with *tsr*. Inactivation of the gene by insertion of a chloramphenicol resistance determinant had no effect on growth or sporulation. *Orf*R was proposed by Hoch and colleagues to be an allele for *rev-4*, a pleiotropic suppressor of the sporulation defect resulting from ribosomal mutations induced by erythromycin resistance (19). Insertional inactivation of *orf*R likewise had no effect on growth or sporulation. Pearson analysis of the completed amino acid sequence of *orf*R (13) revealed no highly significant or informative sequence similarities. A Kyte and Doolittle hydropathy profile indicated that *orf*R (Figure 5) is largely hydrophilic, but contains some regions of hydrophobicity. It is impossible to assess whether *orf*R or the next downstream reading frame will be related to the desaturase until further analyses are completed.

Figure 5. Hydropathy plot of the deduced amino acid sequence of the *orf*R gene product. Profiles generated by the Kyte and Doolittle algorithm (9) are shown with shaded lines, while profiles generated by the algorithm of Goldman and colleagues (4) are shown with bold lines.



Goldman et al
Kyte-Doolittle
HYDROPHILIC HYDROPHOBIC
3
0
-3
0
100
200
300
400

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Appendix

Facultative Alkaliphiles Lack Fatty Acid Desaturase Activity and Lose the Ability to Grow at Near-Neutral pH when Supplemented with an Unsaturated Fatty Acid

This study took advantage of the effort that had been expended in developing the in vitro assay for the fatty acid desaturase in *Bacillus* to address a question of interest in relation to another ongoing project area in Dr. Krulwich's laboratory, namely the difference between obligate and facultative alkaliphiles. I would like to credit Dr. Arthur Guffanti for his work with the chemostat and growth curves of the duramycin-resistant mutants and Dr. Sanda Clejan for all the membrane lipid composition analyses and determination of the location of the double bond in the unsaturated fatty acids of *B. subtilis*.

Abstract

Two obligate alkaliphiles were found to have high levels of fatty acid desaturase, whereas two facultative alkaliphiles had no detectable activity. Supplementation of the growth medium of one facultative strain with palmitoleic acid, but not palmitic acid, at pH 7.5 inhibited growth. The obligate strain outgrows the facultative strain in a chemostat at a very high pH, whereas the converse is true at a pH of 7.5, and the two strains grow equally well at pH 9.0. Thus, the obligate strain is compromised at a near-neutral pH but is better adapted than a related facultative alkaliphile to an extremely alkaline pH.

Introduction

Extremely alkaliphilic *Bacillus* species grow optimally at pH 10.5 or higher (11). Some of these species are obligate alkaliphiles. They are unable to grow well at pHs below 9.0 and fail to grow at all at pHs of 7 to 7.5. Others are facultative alkaliphiles that grow at a broad range of pH values from near neutral to very alkaline (11). We have advanced and tested a number of hypotheses with respect to the basis for obligate versus facultative alkaliphily over the years (12), with current evidence pointing to an important role for the integrity of the cytoplasmic membrane. Obligately alkaliphilic strains appear to have compromised membrane integrity at near-neutral pH values, resulting in maintenance of only low electrochemical ion gradients (9) and a tendency to lyse (10). Compositional studies of the membrane lipids from to obligate and two facultative alkaliphiles (3) revealed that 20% of the total fatty acids in the phospholipids of the obligate strains were unsaturated fatty acids (with $nC_{16:1}$ being the most common) and at least 90% of the total, including unsaturated fatty acids, were various branched-chain fatty acids. By contrast, the facultative strains had 0 to 2% unsaturated fatty acids and 66 to 76% branched-chain fatty acids. In addition, the average chain length of the fatty acids was smaller in the facultative strains than in the obligate strains. Subsequently, direct measurements were made of passive solute efflux from vesicles prepared from membrane lipids of either obligately alkaliphilic *Bacillus firmus* RAB or closely related facultatively alkaliphilic *B. firmus* OF4 (2). At pH 8.0, the vesicles from lipids of the obligate strain were more permeable to several solutes than were those from lipids of the facultative strain. Moreover, the efflux rate of one solute from the facultative strain was unchanged or slightly increased at pH 9.0, whereas the efflux rate from the obligate strain was slightly decreased. These observations led to the hypothesis that obligately alkaliphiic strains could not grow at near-neutral pH values at least in part because the membrane composition of highly unsaturated and branched fatty acids resulted in a membrane that was functional at pH values of 9 or higher but whose integrity was compromised at near-neutral pH values. In the present study, we have sought to further examine and test elements of this hypothesis.

First, the fatty acid desaturase activity in the membranes from two obligate alkaliphiles, *B. firmus* RAB (5) and *Bacillus alcalophilus* (ATCC 27647), and two facultative alkaliphiles, *B. firmus* OF4 and *Bacillus* sp. strain OF1 (6), was assayed. All of the bacteria were grown at pH 10.5 in the malate-containing medium described previously (7), except that the concentration of buffer was four times as high as that used in the original formulation; use of the higher concentration of the carbonate buffer assures maintenance of the starting pH well into the logarithmic phase of growth, with only modest deviations from the starting pH throughout the growth curve. In some experiments, other buffers were used at the same concentration as in the high-pH media, and with no other changes in composition, to adjust the pH to the values indicated (6). Cultures were routinely incubated with aeration at 30°C. Late-log-phase cells were ruptured in a French pressure cell at 20,000 lbs/in^2. Unbroken cells were removed by centrifugation at 12,000 x g, and the resulting supernatant was subsequently centrifuged at 100,000 x g for 1 h to pellet the membrane vesicles (8). The assays monitored conversion of radioactively labeled palmitoyl coenzyme A to palmitoleyl coenzyme A.

The reaction mixture contained 60 mM potassium phosphate (pH 7.2), 1.25 mM NADH, 100 μM [1-C^{14}] palmitoyl coenzyme A (5 μCi/μmol), and membranes in a total volume of 1 ml. Fatty acids were extracted with chloroform-methanol, converted to methyl esters by using boron trifluoride-methanol (13), and separated on the basis of unsaturation by thin-layer chromatography with benzene-hexane (60:40, vol/vol) as the developing solvent (4). The membranes from two obligately alkaliphilic strains had substantial fatty acid desaturase activity, whereas those from two facultative strains had no detectable activity (Table 1). The failure to detect activity in *B. firmus* OF4 was apparently not due to the presence of an inhibitor, since mixing the membrane fractions before the assay or processing mixed batches of facultative *B. firmus* OF4 and obligate *B. firmus* RAB through the entire preparation and assay did not reduce the activity of the obligate strain; indeed, the mixtures had slightly higher activities than the *B. firmus* RAB preparations alone (Table 1). The activity in the membranes of the obligate alkaliphiles was inhibited by 100 mM KCN, consistent with the idea that the activity was of the oxygen-requiring type of desaturase. No activity was found in any of the cytosol fractions.

Facultative *B. firmus* OF4 and obligate *B. firmus* RAB were studied further, as these two strains are related and bioenergetically characterized. As shown in Figure 1, supplementation of the growth medium with palmitoleic acid to a final concentration of 5 or 10 μM inhibited the growth of *B. firmus* OF4 at pH 7.5; supplementation of the cultures with palmitic instead of palmitoleic acid failed to cause the growth inhibition. These results are consistent with the hypothesis that a substantial content of unsaturated

Table 1. Fatty acid desaturase activities of two obligate and two facultative alkaliphiles.

Strain	Specific activity[a] (nmoles/min/mg protein)
B. alcalophilus	4.8[b]
B. firmus RAB	5.0[b]
B. firmus OF4	<0.7
Bacillus sp. OF1	<0.7
Mixed cells of *B. firmus* RAB + OF4	10.0[c]
Mixed extracts of *B. firmus* RAB and OF4	9.0[c]

[a]Results are the means of two to five independent experiments, which had standard deviations of less than 10% of the means.

[b]Activity was inhibited by 100 mM KCN (specific activity <0.7 nmoles/min/mg protein)

[c]In one mixing experiment, cells of *B. firmus* RAB and OF4 were mixed in equal volume and membrane vesicles were prepared and assayed as described. In a second experiment, membrane extracts of *B. firmus* RAB and OF4 were prepared separately, mixed in equal amount, and assayed as described.

fatty acid in the alkaliphile membrane renders the membrane nonfunctional at near-neutral pH values.

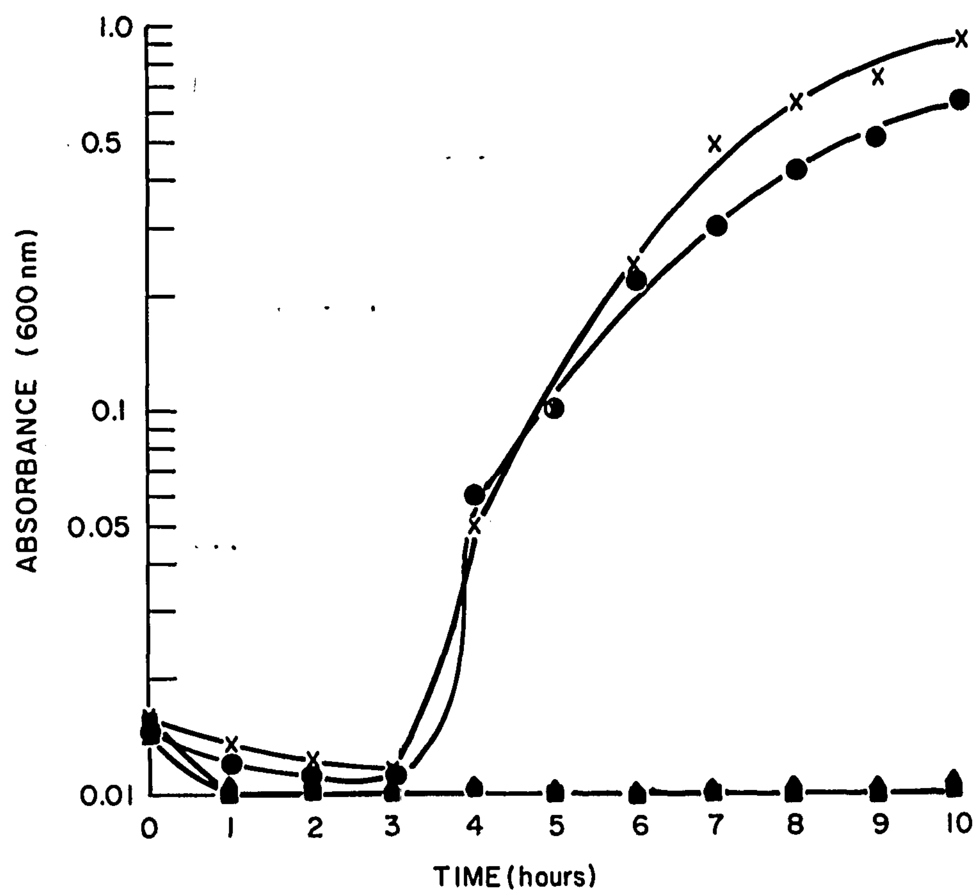
When cultures of *B. firmus* OF4 were similarly supplemented with palmitoleic acid at pH 10.5, no growth inhibition was observed. This failure of the unsaturated fatty acid to inhibit growth of the facultative alkaliphile at a very alkaline pH was not due to breakdown of the fatty acid. In control experiments, the supernatants of cultures supplemented with palmitoleic acid at pH 10.5 were found to retain inhibitory activity against growth of the organism at pH 7.5. However, analyses of membrane lipids, conducted as described previously (3) on lipids extracted from cells that had been grown at pH 10.5 with palmitoleic acid, indicated no increase in unsaturated fatty acid compared with that in controls that were not supplemented (data not shown). Perhaps transport, activation, or incorporation of exogenous fatty acids is impaired at very high pH values.

An alternative approach was taken to determine whether, as predicted by our hypothesis, cells of the facultative strain that incorporated palmitoleic acid would be able to grow at very alkaline but not at near-neutral pH values. *B. firmus* OF4 was grown to the early logarithmic phase at pH 7.5. Palmitoleic acid was then added to a final concentration of 10 μM. After growth began to show the inhibitory effect of palmitoleic acid, one of the cultures to which palmitoleic acid had been added was shifted to pH 10.5. Growth resumed in the culture shifted to pH 10.5 but not in the one that remained at pH 7.5 (Fig. 2). Membrane lipids were prepared from cells to which palmitoleic acid had been added; the cells were harvested 15 min after the addition. Parallel control cells were harvested at the same time. Analyses of the membrane fatty acid compositions of the two samples showed that whereas 1% of the fatty acids in the control membranes were unsaturated (all $C_{16:1}$), in the membranes from the cells that had been supplemented with palmitoleic acid for 15 min, 17% of the total fatty acids were unsaturated fatty acids. More than 70% of the latter were $C_{16:1}$, with the remainder distributed as small amounts of three other unsaturated fatty acids which might have been formed from trace contaminants of the palmitoleic acid supplement. Thus, the failure of *B. firmus* OF4 to grow at pH 7.5 was correlated with an increase in the unsaturated fatty acid content of the membrane, but this increase did not preclude growth of this strain at pH 10.5.

As an additional control in the pH shift experiment, the effect of lauric acid on parallel cultures at pH 7.5 was determined. This fatty acid, whose solubility properties are closer to those of palmitoleic acid than those of the palmitic acid control used in the initial experiments, did not inhibit growth. It is likely, therefore, that the effect of palmitoleic acid indeed depends upon its incorporation and is not a result of its ability to simply act as a surfactant.

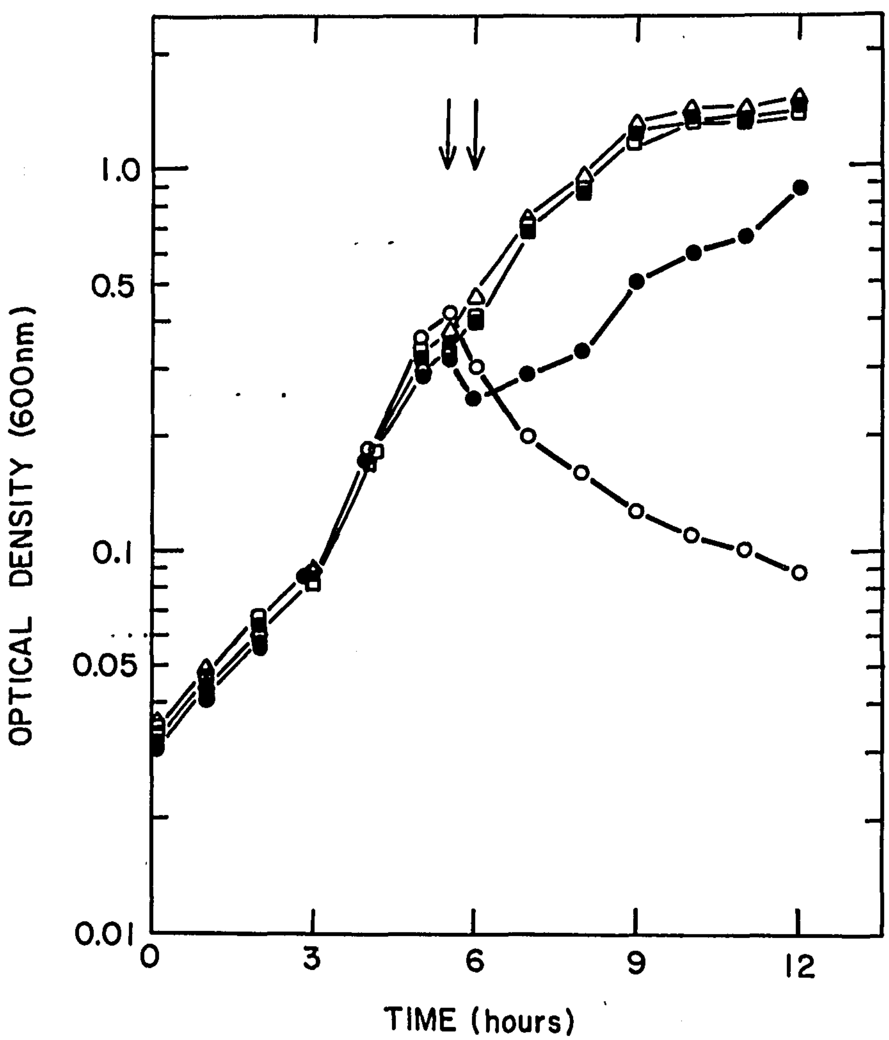
Obligate alkaliphiles can be isolated from natural environments in which the pH is not consistently very high (11). Thus, their possession of a membrane that loses integrity at near-neutral pHs would seem maladaptive unless a compensatory advantage were conferred at optimal pH values. In order to probe this possibility, mixed-continuous-culture experiments were conducted with a New Brunswick BioFlo model C30 chemostat. A strain of *B. firmus* RAB carrying a chromosomal marker conferring chloramphenicol resistance and *B. firmus* OF4-811M (a methionine auxotroph) were employed. The chemostat cultures contained the same medium that was used to grow the batch cultures,

Fig. 1. Inhibition of growth of facultatively alkaliphilic *B. firmus* OF4 by palmitoleic acid supplementation at pH 7.5. Growth on L-malate-containing medium buffered to pH 7.5 with 100 mM phosphate buffer and supplemented with nothing (○), 5 μM palmitoleic acid (■), 10 μM palmitoleic acid (Δ), or 10 μM palmitic acid (X) was monitored turbidimetrically as described previously (5).



1.0
0.5
0.1
0.05
0.01
ABSORBANCE (600 nm)
0
1
2
3
4
5
6
7
8
9
10
TIME (hours)

Fig. 2. Effect of laurate on growth of *B. firmus* OF4 and on the ability of this strain to grow at pH 10.5 after incubation with palmitoleic acid at pH 7.5. Cultures of *B. firmus* OF4 growing on L-malate-containing medium at pH 7.5 (buffered with 100 mM phosphate buffer) were monitored turbidimetrically and supplemented at the time indicated by the left arrow with nothing (Δ and ■), 10 μM lauric acid (□), or 10 μM palmitoleic acid (○ and ○). At the time indicated by the right arrow, control cells (■) and cells incubated with 10 μM palmitoleic acid (○) were shifted to pH 10.5.

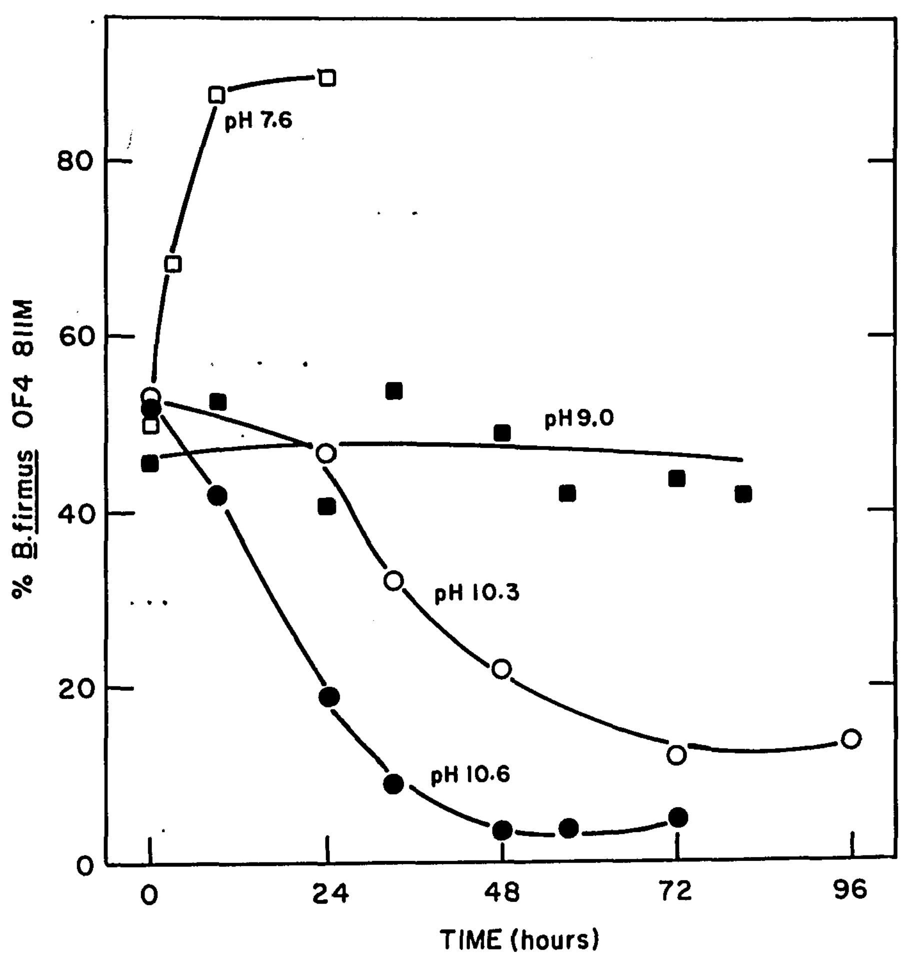


OPTICAL DENSITY (600nm)
1.0
0.5
0.1
0.05
0.01
0
3
6
9
12
TIME (hours)

buffered at pH 9.0, 10.3, and 10.6 with 100 mM Na_2CO_3-$NaHCO_3$ and at pH 7.6 with 100 mM Na_2HPO_4-NaH_2PO4. The medium was supplemented with 100 μg of L-methionine per ml to ensure full growth of the auxotrophic strain. This medium gave indistinguishable growth rates and yields of the two strains in batch cultures and on plates. The chemostat was inoculated with equal proportions (as assessed by turbidimetric readings) to 150 Klett units (+5%) and run at 30°C and a constant pH. The actual pH of the culture was carefully monitored throughout the experiment. Medium was pumped into the 300-ml chemostat chamber at a rate that maintained the culture at 150 Klett units, equivalent to the mid-logarithmic phase of a batch culture; this flow rate was generally about 150 ml/h. Vigorous aeration was maintained by forced pumping of air together with stirring at 300 rpm. At zero time and at intervals thereafter, samples were removed for total and differential plate counts, taking advantage of the resistance and auxotrophic markers. The two strains grew at the same rate, as assessed by a constant proportion of the two strains, in continuous culture at pH 9.0 (Figure 3). At pH values above 10, the proportion of the facultative strain dropped rapidly. Conversely, at pH 7.5, the proportion of the facultative strain rapidly rose to 90%. Separate control chemostat runs for *B. firmus* OF4 and *B. firmus* RAB confirmed that each of these strains grew logarithmically (i.e., could be maintained at 150 Klett units) in the chemostat at a constant pH of 10.5 but that *B. firmus* OF4 required a slightly lower flow rate.

Taken together, these studies support the hypothesis that the fatty acid composition of the obligate alkaliphile membrane is a factor in precluding growth at near-neutral pH

Fig. 3. Cultivation of *B. firmus* RAB19 (chloramphenicol resistant) and *B. firmus* OF4-811M (a methionine-auxotroph) in a chemostat at various pH values. Logarithmic-phase batch cultures of the two organisms, grown at pH 9.0, were harvested by centrifugation and separately resuspended in buffer plus nutrients at pH 7.6 (□), 9.0 (□), 10.3 (○), or 10.6 (○). The two organisms were mixed in equal proportions and used to inoculate the chemostat, which was incubated under aerobic conditions so as to maintain the culture in logarithmic growth at a constant pH, as described in the text.



pH 7.6
pH 9.0
pH 10.3
pH 10.6
80
60
40
20
0
% B.firmus OF4 811M
0
24
48
72
96
TIME (hours)

values. Specifically, the combination of a high concentration of branched-chain fatty acids and a substantial fraction of unsaturated fatty acid may make the membrane leaky at a near-neutral pH, although it is functional at a highly alkaline pH. In fact, this membrane composition may give the obligate strain an advantage in the very high pH range for reasons that are not established but which we have considered in connection with the energy-coupling problems of this organism (11). Another unresolved question of interest is the physical chemical basis for greaterinstability of that membrane at near-neutral pH values relative to that at highly alkaline pH values.

Acknowledgments

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