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Mutation of *Bacillus firmus* OF4 to Duramycin Resistance Results in Substantial Replacement of Membrane Lipid Phosphatidylethanolamine by Its Plasmalogen Form

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Mutant strains of alkalophilic *Bacillus firmus* OF4 that were selected for resistance to duramycin had greatly reduced levels of membrane diacylphosphatidylethanolamine, as had been found in studies of such mutants of *Bacillus subtilis*. In the *B. firmus* strains, however, substantial levels of plasmenylethanolamine were found. This is an unusual membrane component for an aerobic eubacterium, but the presence of trace amounts even in the wild type was confirmed in experiments with $^{32}P_i$-labeled growth medium. The membrane lipid composition of the duramycin-resistant strains had several other changes that also left alkalophilic growth unimpaired.

Duramycin is a peptide antibiotic (12) that has been shown to affect several disparate ion-translocating processes that are membrane associated (9, 10, 13). Navarro et al. (10) have shown that sensitivity of a reconstituted system to duramycin depends on the presence of phosphatidylethanolamine and that duramycin-resistant mutants of *Bacillus subtilis* have greatly reduced levels of phosphatidylethanolamine and diphosphatidylglycerol in their membrane lipids; this latter finding was confirmed in a subsequent study of the duramycin resistance of *B. subtilis* and an uncoupler-resistant strain of *B. subtilis* (2a). Each of the duramycin-resistant strains had only trace amounts of phosphatidylethanolamine and diphosphatidylglycerol.

These observations led us to attempt to isolate duramycin-resistant strains of the extremely alkalophilic organism *Bacillus firmus*. The interest in this endeavor arose from the observation that all the extremely alkalophilic bacilli examined thus far possess certain common features with respect to their membrane lipids (2, 8, 8a). These include the presence of a very high content of diphosphatidylglycerol and substantial amounts of phosphatidylethanolamine, in addition to the major phospholipid phosphatidylglycerol, and the presence of squalene, reduced squalenes, and C_{40} and C_{50} isoprenoids in the neutral lipid fraction. It is not yet known whether any of these compositional features is essential for alkalophily. Thus, the possibility that mutation to duramycin resistance might lead to a dramatic reduction in two of the major polar lipids presented an opportunity to examine the importance of those two components. A facultatively alkalophilic strain, *B. firmus* OF4 (5), was used for the study so that mutations to duramycin resistance could be isolated at pH 7.5 as well as at pH 10.5.

A double mutant of *B. firmus* OF4 8 11M was used in this study; this strain was resistant to streptomycin and required methionine for growth. The cells were grown at either pH 7.5 or 10.5, with aeration at 30°C, in the malate-containing medium described previously (5), except that 10 μg of L-methionine per ml was routinely included in the medium unless the methionine marker was checked and 50 μg of streptomycin per ml was added when the streptomycin marker was checked. Duramycin was added from separate sterile solutions to a final concentration of 10 μg/ml. Duramycin was generously provided by E. Racker (Cornell University, Ithaca, N.Y.) and O. Shotwell (Northern Regional Research Laboratories). When radioactive lipids were prepared, $^{32}P_i$ was added to the growth medium as described previously (2). Growth curves were conducted as described elsewhere (5). Membrane lipids were isolated from right-side-out membrane vesicles (7) by the method of Bligh and Dyer (1) and were analyzed by procedures that have been summarized recently (2). Additional methods were used, however, to identify an apparent plasmalogen component. The presence of the plasmalogen was initially considered to be possible because of the color of the fractions that were developed on thin-layer chromatography plates and detected with dinitrophenylhydrazine. We therefore examined aldehyde production on acid hydrolysis and then, having detected aldehyde production, used two different methods to quantitate the ether phospholipids, as follows. Parallel samples before and after acid hydrolysis were used, in addition to mild alkaline deacylation of the polar lipids. The polar lipids were treated with acetic acid at 30°C overnight as described by Johnston and Goldfine (6). After removal of the acetic acid by lyophilization, the aldehydes and phospholipids were separated on silicic acid columns. Chloroform was used to elute the aldehydes, and chloroform-methanol (1:2 [vol/vol]) was used to elute the phospholipids. The aldehydes were separated on thin-layer chromatography plates in a system containing hexane-chloroform-methanol (27:12:1), as described by Gray (3), and esters were separated on thin-layer chromatography plates in the system containing petroleum ether-diethyl ether-acetic acid (90:10:1). Plates were sprayed with water or 0.4% dinitrophenylhydrazine in 2 M HCl to detect the plasmalogens; methods for the detection of other components have been described previously (2). The two methods used for the quantitation of ether phospholipids were the iodine method of Williams et al. (14) and the procedure of Wittenberg et al. (15). The results obtained by these two methods gave values that fell within 5% of each other.

Spontaneously arising duramycin-resistant mutants of *B. firmus* OF4 8 11M were readily isolated at pH 7.5 and 10.5.

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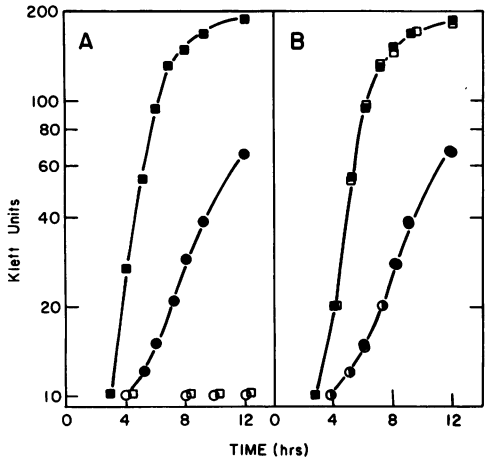

FIG. 1. Growth of *B. firmus* OF4 8 11M and its duramycin-resistant mutant derivative in the absence and presence of duramycin. Growth data for *B. firmus* OF4 8 11M (A) and one of the duramycin derivatives of *B. firmus* OF4 8 11M (B) during growth at pH 10.5 (squares) or pH 7.5 (circles) in the absence of duramycin (closed symbols) or in the presence of 10 μg of duramycin per ml (open symbols) are presented.

Plate counts established that the frequency of mutation was approximately 8 in 10^6 cells. Replica-plating further established that the isolates at pH 7.5 all retained the ability to grow at pH 10.5, and all the isolates at pH 10.5 retained the ability to grow at near neutral pH. For one duramycin-resistant strain, the growth profiles at the two pHs were identical to those of the parent strain, except for the resistance of growth to 10 μg of duramycin per ml (Fig. 1).

The membrane lipid composition of the doubly marked parent strain *B. firmus* OF4 8 11M was compared with those of two different duramycin-resistant strains, one of which was isolated at pH 10.5 and one of which was isolated at pH 7.5. Two to four independent lipid preparations of each strain were examined. The composition of *B. firmus* OF4 8 11M is presented in Table 1 and is essentially indistinguishable from that of *B. firmus* OF4 (2). The results for each duramycin-resistant strain were almost identical; data for one of the strains (that isolated at pH 7.5) are given in Table 1. All the values in Table 1 are averages of three to four determinations for each of the independent lipid preparations, in which the standard deviations were within 10% of the experimental values. While the expected reduction in phosphatidylethanolamine was observed in the mutants, as shown for one of them in Table 1, the mutants also contained, unexpectedly, plasmenylethanolamine and smaller amounts of lysophosphatidylethanolamine. Since phosphatidylethanolamine is implicated as a necessary component for duramycin sensitivity (10), it is likely that the plasmalogen and monodeacylated forms of the phospholipid do not substitute for that function while fulfilling other roles of phosphatidylethanolamine in the membranes. The mutants do not, then, offer us the opportunity to study alkalophily in the presence of greatly reduced amounts of phosphatidylethanolamine. Rather, they exhibit a change in the form of the phospholipid that is incorporated. Since the finding of the plasmalogen form is unexpected in an aerobic eubacterium (4), we examined the possibility that even the parent strain produced trace amounts that were unidentified in the earlier survey of alkalophile membrane lipids (2). Indeed, when $^{32}P_i$-labeled growth medium was used, the parent strain produced small amounts of the plasmalogen as well as small

TABLE 1. Membrane lipid composition of a duramycin-resistant strain of alkalophilic *B. firmus* OF4 8 11M

Membrane composition	*B. firmus* OF4 8 11M grown at:		*B. firmus* OF4 8 11M Dur^r grown at:	
	pH 10.5	pH 7.5	pH 10.5	pH 7.5
Lipid/protein (mg/mg)	0.95	1.06	0.90	0.96
Neutral lipid/polar lipid (%/%)	25/75	35/65	25/75	35/65
Neutral lipids (% of total)				
Free fatty acids	4	2	5	10
1,3-Diacylglycerol	23	21	29	23
1,2-Diacylglycerol	32	27	50	52
Squalene	11	9	3	5
Dihydro-, tetrahydrosqualene	10	12	4	8
C_{40} isoprenoid	17	25	5	ND[a]
C_{50} isoprenoid	3	4	5	2
Polar lipids (% of total)				
Phosphatidylglycerol	52	59	70	67
Phosphatidylethanolamine	22	18	ND	2
Plasmenylethanolamine	ND	ND	6	10
Diphosphatidylglycerol	25	20	5	9
Bismonoacyl glycerolphosphate	ND	ND	5	3
Phosphatidic acid	ND	1	2	4
Lysophosphatidylethanolamine	ND	3	7	3
Aminoacylphosphatidylglycerol	1	ND	5	2
Fatty acids (% of total) with chain lengths below C_{14}	3	10	ND	3
iso-$C_{14:0}$	10	10	12	12
n-$C_{14:0}$	9	9	8	6
iso-$C_{15:0}$	18	18	18	18
anteiso-$C_{15:0}$	33	26	35	30
n-$C_{16:0}$	8	6	10	8
n-$C_{16:1}$	2	1	4	7
iso-$C_{17:0}$	4	6	3	4
n-$C_{17:0}$	11	14	10	12

[a] ND, Not detected.

amounts of other minor polar lipids that had elevated levels in the duramycin-resistant mutants, i.e., lysophosphatidylethanolamine, aminoacylphosphatidylglycerol, and bismonoacylglycerolphosphate (Table 2). Bismonoacylglycerolphosphate has been found in several alkalophilic bacilli, but it is not invariably associated with the membranes of alkalophiles except, perhaps, in trace amounts (2, 11).

The other polar lipid that we expected to find in greatly reduced amounts, diphosphatidylglycerol, was indeed found in considerably lower amounts in the duramycin-resistant mutants than in the parent strain (Table 1). Unlike similar mutants of *B. subtilis* (2a, 10), however, the mutants of the alkalophile still retained substantial amounts of diphosphatidylglycerol. Thus, again, alkalophily was retained, but so were appreciable levels of diphosphatidylglycerol. It is possible that in *B. subtilis* the loss of diphosphatidylglycerol is secondary to a mutational impairment in phosphatidylethanolamine production in duramycin-resistant mutants. In the alkalophile mutants, the phosphatidylethanolamine was still present at appreciable levels, but in an altered form, so that a secondary effect on diphosphatidylglycerol might be much less pronounced.

The fatty acid composition of the mutant strain was altered by a modest shift to a generally longer chain length, presumably in response to the changes in polar lipid head groups. In addition, there were increases in the free fatty

TABLE 2. Distribution of label from $^{32}P_i$-supplemented growth medium among polar membrane lipids of *B. firmus* OF4 8 11M and its duramycin-resistant mutant derivative[a]

Radiolabeled polar lipid	pmol/mg of total membrane lipid in:			
	B. firmus OF4 8 11M grown at:		*B. firmus* OF4 8 11M Dur^r grown at:	
	pH 10.5	pH 7.5	pH 10.5	pH 7.5
Phosphatidylglycerol	23	30	34	30
Phosphatidylethanolamine	10	9	0.1	0.4
Plasmenylethanolamine	0.1	0.2	4	5
Diphosphatidylglycerol	11	9	3	4
Bismonoacylglycerolphosphate	0.1	0.1	0.3	0.3
Phosphatidic acid	0.2	0.6	0.4	0.4
Lysophosphatidylethanolamine	0.1	0.1	5.2	6.0
Aminoacylphosphatidylglycerol	0.1	0.1	3	0.3

[a] The growth medium, at either pH 10.5 or 7.5, was supplemented with 500 μCi of $^{32}P_i$ per liter. On harvesting in the late-logarithmic phase, approximately 20% of the label was incorporated into cells and about 10% was recovered in the membrane fractions. Values are averages obtained from two independent preparations.

acid content of mutant strains grown at pH 7.5 and increases in 1,2-diacylglycerol. The relationship of these observations to duramycin resistance or to the changes in polar lipids is unclear. Of interest with respect to the neutral lipids was the finding that whereas the squalenes and C_{40} isoprenoid contents were markedly lower in the duramycin-resistant mutants than in the parent strain, the C_{50} isoprenoids, which may function in rigidifying the membrane (S. Clejan and T. A. Krulwich, Biochim. Biophys. Acta, in press), were not reduced at pH 10.5. We have begun studies of pH-conditional mutants that may be helpful in clarifying which of the membrane lipid components, if any, are essential for alkalophily. Results of the present study indicate that there is latitude with respect to the composition of the membrane and alkalophily but that, at least in duramycin-resistant mutants, some of the common compositional features of the membrane lipids of extreme alkalophiles are retained.

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