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Large decreases in membrane phosphatidylethanolamine and diphosphatidylglycerol upon mutation to duramycin resistance do not change the protonophore resistance of *Bacillus subtilis*

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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 aminoacyl(lysyl)phosphatidylglycerol. 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 of 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

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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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 could 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 those 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).

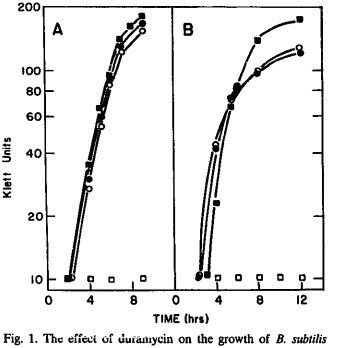

Fig. 1. The effect of duramycin on the growth of *B. subtilis* wild-type and CCCP-resistant strains and their derivatives selected for duramycin resistance. Growth on DL-malate-containing medium was followed in the absence (closed symbols) or presence (open symbols) of 25 μg/ml duramycin. Panel A shows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

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 chromatographic analysis (Table I). 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 mutant 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

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 diglycosyldiacylglycerol; and the presence of 5% lysophosphatidylethanolamine (Table I).

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., 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 a radioactive spot was found in the appropriate place although no chemically detectable phospholipid had been found. Thus,

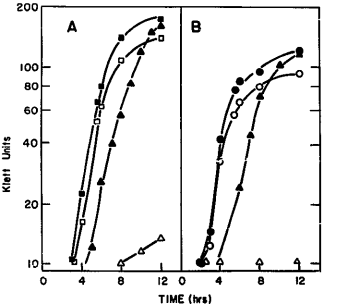

Fig. 2. The resistance of AG1A3 and AG1A3-DR4 to inhibition by CCCP and modulation of that inhibition by exogenous palmitoleic acid. AG1A3 (Panel A) and AG1A3-DR4 (Panel B) were grown on DL-malate-containing media in the absence of additions (■, ●), in the presence of 2 μM CCCP (□, ○), in the presence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

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.

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 were found in both duramycin-resistant strains are 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 depend 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.

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