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Palmitoylation of Membrane Proteins in *Spiroplasma floricola*

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Abstract. Covalent modification of *Spiroplasma floricola* membrane proteins by fatty acids was determined by in vivo labeling of the cells with radioactive fatty acids followed by separation on one-dimensional SDS–polyacrylamide gels and visualization by autoradiography. Approximately 25 different proteins were found to be labeled with [^{3}H]-palmitate, whereas almost none were labeled with [^{3}H]-oleate. The radioactivity could not be removed from the palmitoylated membrane proteins by boiling in SDS or by exhaustive extraction with chloroform–methanol (2 : 1). Nevertheless, treating the palmitoylated proteins with a 0.1 *N* KOH solution removed approximately 70% of the bound [^{3}H]-palmitate. The major protein-bound fatty acid species were identified, following their release from the protein by chemical cleavage, as palmitic acid and stearic acid (83% and 7.5%, respectively).

Mycoplasmas are among the simplest and most useful microorganisms known for membrane studies [11]. These organisms lack a rigid cell wall and are bound by a single membrane, the cytoplasmic membrane [11]. Unlike other microorganisms, cholesterol constitutes a major component of the membrane of *Mycoplasma*, *Spiroplasma*, and *Ureaplasma* species [13], and it is generally accepted that its presence gives the membrane tensile strength and structural integrity [12]. Recently, it has been demonstrated that in several sterol-requiring *Mycoplasma* species, numerous membrane proteins are modified by covalent binding of both saturated and unsaturated fatty acids [2, 3, 17, 18], whereas in the sterol nonrequiring *Acholeplasma laidlawii*, membrane proteins were found to be covalently modified only with saturated acyl chain [4, 6, 9]. It has been suggested that the introduction of saturated fatty acyl groups facilitates the anchorage of proteins to the membrane, ensuring its structural integrity [4, 6, 9], whereas the function of protein modified by unsaturated fatty acids may be of a more regulatory nature related to the sterol requirement of the organism [4].

The data presented in this study demonstrate that in the helical sterol-requiring *Spiroplasma floricola*, numerous membrane proteins are modified with palmitoyl, but not with oleoyl chains. The pattern of the palmitoylated proteins was almost identical in all the strains tested and was not affected upon aging of the culture.

Materials and Methods

Spiroplasma floricola strains (BNR1, from *Magnolia grandiflora;* OBMG, from the tulip poplar; CD262, from the gut of a caddis fly; Odonata 53, from a dragonfly; MB73, from the gut of a milkweed bug; and BNR1 III, from a tulip blossom) and *S. melliferum* (strain BC3) were kindly provided by R. F. Whitcomb (Beltsville, Maryland). The organisms were grown for 24–48 h at 32°C (2% inoculum) in the medium of Saglio et al. [14] supplemented with 2–8% (by vol) of horse serum. In some experiments, the horse serum was replaced with 1.0% bovine serum albumin and a mixture of cholesterol (10 μg/ml, Sigma), oleic and palmitic acids (0.5 μg/ml of each, Sigma). To label membranes, 0.05–1 μCi of either [^{3}H]-palmitic acid (60 Ci/mmole), [^{3}H]-oleic acid (15 Ci/mmole) or [^{14}C]-palmitic acid (200–400 mCi/mmole) per ml (all products of the Radiochemical Center, Amersham, UK) were added to the medium. The cells were harvested by centrifugation at 12,000 *g* for 15 min, washed twice, and resuspended in 0.25 *M* NaCl in 25 m*M* Tris-HCl buffer, pH 7.5. The cells were osmotically lysed in deionized water, and membranes were collected by centrifugation at 34,000 *g* for 30 min. The membranes were washed once and resuspended in 25 m*M* Tris-HCl buffer, pH 7.5. Protein was determined in the membrane preparations by the method of Lowry et al. [8]. Pellets of cells or membrane preparations (1 mg) were extracted four times with 1.0 ml of chloroform : methanol (2 : 1 by vol). The protein pellets were solubilized by boiling for 5 min in a cracking buffer containing 2.3% sodium dodecylsulfate (SDS), 5% β-mercaptoethanol, 20% glycerol, 0.01% bromphenol blue in 63 m*M* Tris-HCl buffer, pH 6.3. The samples were then analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [7]. The gels

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Table 1. Incorporation of labeled fatty acids into *Spiroplasma floricola* (BNR1)

Preparation	[^{3}H]-Palmitate (cpm/mg protein)	[^{3}H]-Oleate
Intact cells	797,860	746,000
Isolated membranes	1,526,600	1,423,500
Lipid preextracted membranes	26,700	660

Cells were grown in a medium containing 1% BSA, cholesterol (10 μg/ml), palmitic acid and oleic acids (0.5 μg/ml each), and 0.065 μCi of the radioactive fatty acid. Membrane isolation and lipid extraction were performed as described in the text.

were strained with Coomassie blue R-250 in methanol–acetic acid–water (12.5 : 2.5 : 10 by vol) at room temperature and destained for 4 h with frequent changes of the solvent. The gels were then incubated at room temperature with gentle shaking in an Amplify solution (Amersham, UK) for 20 min, dried, and exposed to X-Omat film (Eastman Kodak Co.) at −70°C for autoradiography. Fatty acid methyl esters of the total lipid fraction and of the acylated membrane protein fraction were prepared by heating the lipids in 14% boron trifluoride in methanol (Sigma) at 72°C for 15 min. The extraction of the methyl esters and their analysis was performed as previously described [5].

Results and Discussion

We have identified acylated membrane proteins of *Spiroplasma floricola* by in vivo labeling experiments, followed by separation of the proteins by SDS–polyacrylamide gels and autoradiography. The cells were labeled by growing them in a medium in which the horse serum was replaced by 1% bovine serum albumin plus cholesterol (10 μg/ml), oleic and palmitic acids (0.5 μg/ml of each). In order for a protein to be considered as an acylated protein, it should be, first of all, resistant to exhaustive extraction by organic solvents. Table 1 shows that with [^{3}H]-palmitate-labeled *S. floricola* membranes, about 3.3% of the label remained tightly protein bound after four consecutive extractions with chloroform–methanol. When [^{3}H]-oleate-labeled membranes were utilized, <0.1% of the label remained protein bound. The protein-associated radioactivity was found exclusively in the membrane fraction. Because *Spiroplasma* species cannot modify exogenous fatty acids or metabolically convert them into other intermediates [13], it is apparent that the labeled protein represents acylated proteins. The protein-bound radioactive palmitate could not be removed by boiling in SDS or treatment with hydroxylamine (1 *M*) at neutral pH (1 h at 22°C), whereas treatment with 0.1 *N* KOH in 90% methanol for 2 h at 45°C removed approximately 66% of the radioactivity. These findings suggest that membrane proteins are palmitoylated through an ester linkage rather than through an amide linkage or a thioester linkage to a cysteine residue. Thioester linkages are extremely labile and easily hydrolyzed by hydroxylamine, whereas amide linkages are insensitive to both hydroxylamine and KOH treatments [10].

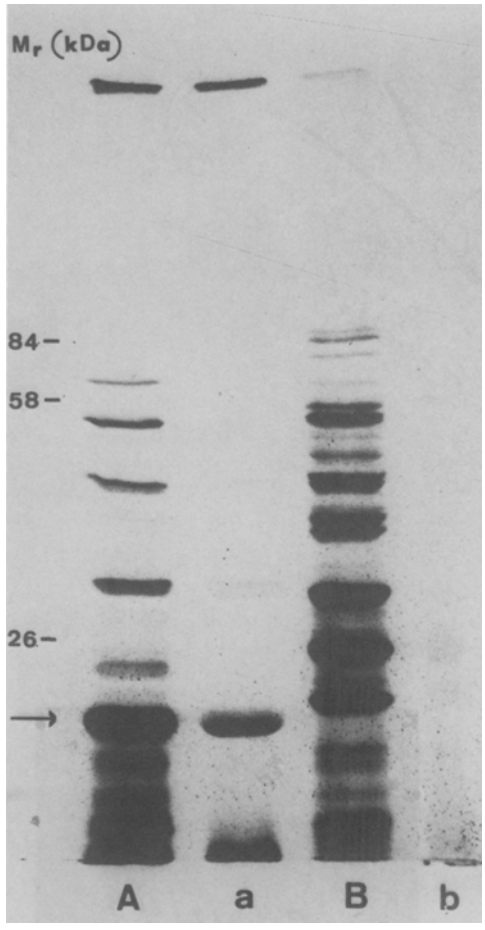

Fig. 1. Identification of acylated membrane proteins of *Spiroplasma floricola* and *S. melliferum*. *S. floricola* (BNR1) cells were grown in a medium supplemented with [^{3}H]-fatty acids. Membrane isolation, chloroform : methanol extraction, SDS–PAGE chromatography, and autoradiography were performed as described in Materials and Methods. Capital letters, [^{3}H]-palmitate labeling pattern; small letters, [^{3}H]-oleate labeling pattern. (A,a), *S. melliferum* (BC3); (B,b), *S. floricola* (BNR1)

Analysis of the lipid pre-extracted *S. floricola* membranes by one-dimensional SDS–PAGE followed by autoradiography revealed that out of about 50 protein bands identified by Coomassie blue staining, about 25 bands were labeled by [^{3}H]-palmitate, whereas none were labeled with [^{3}H]oleate (Fig 1, B and b). When lipid pre-extracted membrane prepara-

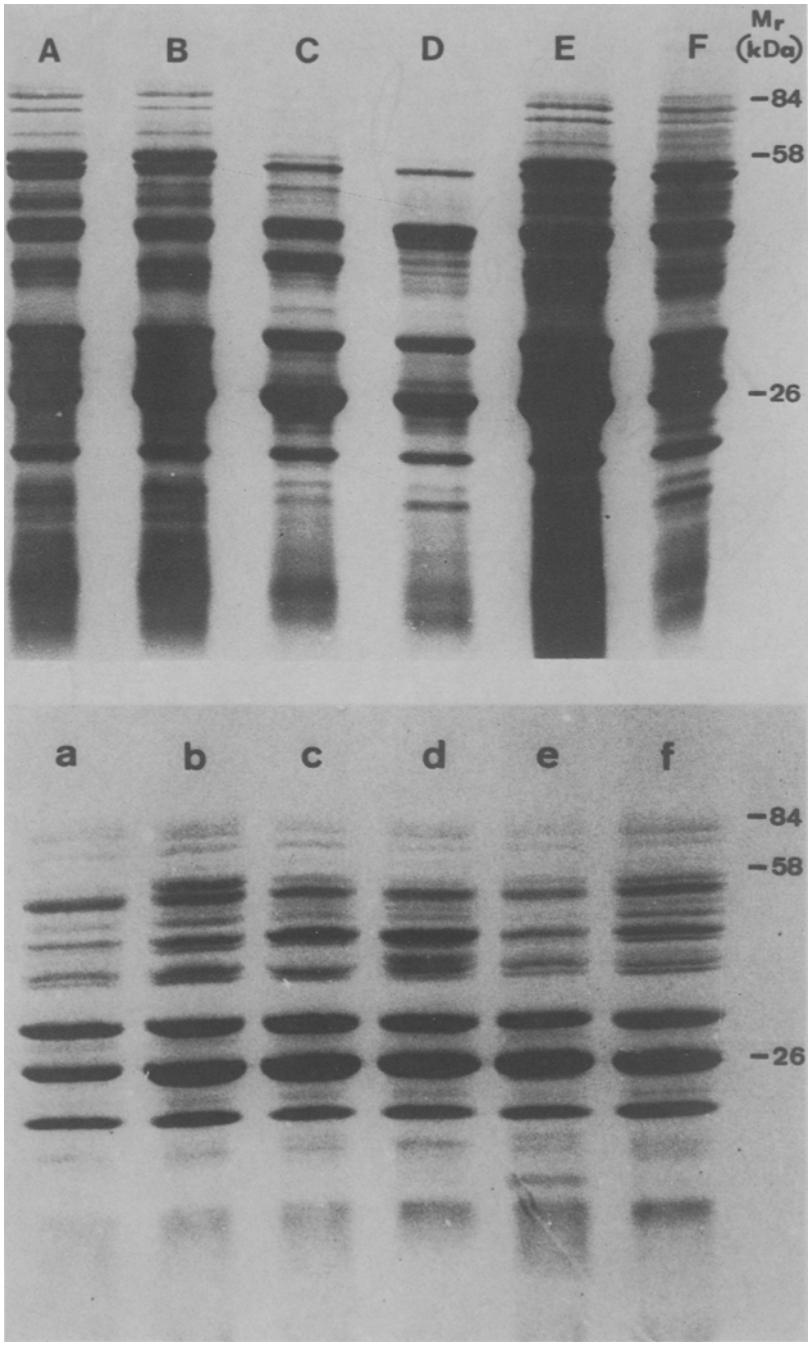

Fig. 2. Identification of palmitoylated membrane proteins of *S. floricola* strains. *S. floricola* strains were grown in a medium supplemented with [^{14}C]palmitic acid. Membrane isolation, chloroform : methanol extraction, SDS–PAGE chromatography, and autoradiography were performed as described in Materials and Methods. Capital letters, Coomasie-blue stained proteins; small letters, autoradiogram of membrane protein. (A, a), BNR1 strain; (B,b), OBMG strain; (C,c), CD262 strain; (D,d), BNR1 III strain; (E,e), Odonata 53 strain; (F,f), MB 73 strain.

tions of *S. melliferum* cells grown with radioactive palmitate or oleate were analyzed, 16 protein bands were labeled by [^{3}H]-palmitate, and four protein bands were labeled by [^{3}H]-oleate (Fig 1, A and a). The pattern of the palmitoylated protein bands of *S. melliferum* was completely different from the pattern of *S. floricola* (Fig. 1a and [20]). These two organisms are genomically and serologically distinct [16]. Among the labeled protein bands of *S. melliferum* the most intensively labeled was a major membrane protein with an apparent molecular weight of 23 kDa. This protein band was labeled by both [^{3}H]-palmitate and [^{3}H]-oleate and interacted specifically with polyclonal antibodies generated against the *S.*

Table 2. Fatty acid composition of the lipid-preextracted membranes and the total lipid extract of *S. floricola* (BNR1) membranes

Preparation	Fatty acid content (mol %)				
	14:0	16:0	18:0	18:1	18:2
	(Chain length: no. of double bonds)				
Lipid preextracted membranes	1.5	83.0	7.5	6.5	<1.0
Lipid extract	1.0	24.0	5.0	49.0	15.0

Cells were grown in a medium containing 1% BSA, cholesterol (10 μg/ml) palmitic and oleic acids (0.5 μg/ml of each). Lipid extraction and analysis were performed as described in the text.

melliferum spiralin-like protein (not shown). These results strongly suggest that this protein is the spiralin-like membrane protein of *S. melliferum*. The Spiralin-like protein of *S. melliferum* and the spiralin of *S. citri* are the most abundant membrane proteins of these organisms [15]. These proteins share many properties, but are serologically different [1]. It was suggested that in their dimer form these proteins span the spiroplasma membrane, but their function is yet unknown [19].

The pattern of the palmitoylated membrane proteins of *S. floricola* was highly reproducible and was not affected by the age of the culture. Thus, harvesting the cells at the early exponential, late exponential, or stationary phase of growth revealed almost identical patterns. Furthermore, the pattern of palmitoylated protein was very similar among six *S. floricola* strains isolated from various plant and insect tissues, although some changes were apparent in the pattern of the Coomassie blue-stained protein bands of these strains (Fig. 2).

Table 2 shows the results of gas liquid chromatography analysis of the fatty acid methyl esters of the total membrane lipid fraction and the acylated protein fraction from *S. floricola* cells grown with 4% horse serum. The data revealed that, whereas in the total membrane lipid fraction saturated fatty acids comprise only one-third of the total fatty acids, in the acylated protein fraction over 90% of the fatty acyl chain were saturated fatty acids. The predominant fatty acid covalently bound to membrane proteins was palmitic acid (83% of the total). Thus, fatty acid incorporation into *S. floricola* membrane proteins seemed to favor palmitic acid, even in the media in which free palmitic acid forms less than 20% of the total free fatty acid fraction [13].

Determining the function of palmitoylation in the *Mollicutes* seems to be a more difficult task than defining the structural nature of the modification. The fact that a major fraction of *S. floricola* membrane proteins are covalently modified by fatty acids indicates that this modification plays a major role in the physiology of spiroplasmal membranes. It has been previously suggested that protein acylation by the *Mollicutes* aids in both the insertion and anchoring of membrane proteins, enhancing membrane integrity in organisms that lack a rigid cell wall [2, 3, 6, 9]. Nonetheless, further conclusions on the role of protein acylation in the cell membranes of the *Mollicutes* will have to await the isolation or construction of proteins bearing mutations at or surrounding the site for palmitate attachment [10].

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