

Respiration-Associated Components of *Mollicutes*

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No cytochrome pigments were detected by difference (reduced minus oxidized) spectroscopy at liquid nitrogen temperature in whole-cell preparations or membrane fractions of *Acholeplasma axanthum* S273, *Acholeplasma equifetale* N93, *Acholeplasma granularum* BTS39, *Acholeplasma laidlawii* B-PG9, *Acholeplasma modicum* PG49, *Acholeplasma oculi* 19L, *Mycoplasma arginini* G230, *Mycoplasma arthritidis* 07, *Mycoplasma pneumoniae* FH, and *Mycoplasma pulmonis* JB. All ten *Mollicutes* species examined contained iron of unknown function (3.0 to 15.3 nmol of iron per mg of protein). Relatively small amounts of acid-labile sulfide were found in all fractions (0.10 to 1.07 nmol of acid-labile sulfide per mg of protein). The data suggest that, as *Mollicutes* lack cytochrome pigments, they would synthesize most if not all adenosine triphosphate at the substrate level.

The fermentative *Mollicutes* have a "flavin-terminated respiratory chain" since they contain flavin and they lack quinones and apparently cytochromes (9-11, 20, 28). Other *Mollicutes*, excluding the *Thermoplasma* genus, which may not belong in this group (34), have been described as possessing quinones and cytochromes and are capable of oxidative phosphorylation (32). These latter reports supported our opinion that some *Mollicutes* might also contain iron-sulfur proteins, proteins where sulfur is a ligand of iron. There are many different iron-sulfur proteins. They are found in microbes, plants and animals, possibly in all cells, and are involved in biological oxidation-reduction, hydroxylation reactions, and nitrogen fixation (7, 18, 35). Iron-sulfur proteins are specifically identified by their characteristic electron paramagnetic resonance spectra, and most of them contain acid-labile sulfide, i.e., they release H₂S upon acidification (17). In those nonsulfur proteins which contain acid-labile sulfide, the molar iron/sulfide ratio is 1 (3, 17). Our preliminary experiments suggested that cytochromes and iron-sulfur proteins were absent in *Mollicutes* (20); and J. D. Pollack, R. L. Booth, and A. J. Merola, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, G5, p. 83; and J. D. Pollack, R. L. Booth, and A. J. Merola, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, G21, p. 80). These results required reexamination because the absence of cytochromes and iron-sulfur proteins would be a major taxonomic characteristic of the class and metabolically distinguish these organisms from many chemoheterotrophic bacteria. Our purpose was to determine

whether cytochromes and acid-labile sulfide are present in *Mollicutes* and to examine the respiration-associated components of these wall-less procaryotes.

MATERIALS AND METHODS

Organisms. *Acholeplasma axanthum* S743, *Acholeplasma equifetale* N93, *Acholeplasma granularum* BTS39, *Acholeplasma laidlawii* B-PG9, *Acholeplasma modicum* PG49, *Acholeplasma oculi* 19L, and *Mycoplasma arthritidis* 07 were received from J. G. Tully (National Institutes of Health). *Mycoplasma pneumoniae* FH, *Mycoplasma arginini* G230, and *Mycoplasma pulmonis* JB were obtained from N. L. Somerson (The Ohio State University, Columbus). *A. laidlawii* B-PS was obtained from P. Smith (University of South Dakota, Vermillion).

Media and growth conditions. *M. pneumoniae* FH was grown in SSR2 medium (21) with 2% (vol/vol) heat-inactivated (56°C for 1 h) horse serum (K. C. Biologicals Inc., Lenexa, Kan.) replacing PPLO-serum fraction. Cultures of *Mycoplasma pneumoniae* FH were grown in 200 ml of medium in 2-liter Povitsky bottles by the procedure of Somerson et al. (29).

All other organisms were grown in modified Edward medium (23). Heat-inactivated horse serum was used at 3% (vol/vol) in media for *M. pulmonis* JB, *M. arginini* G230, and *M. arthritidis* 07 and at 0.5% or 3% (vol/vol) for all *Acholeplasmas*. Media were dispensed in 175-ml quantities in 2-liter flasks. Temperature-equilibrated media were inoculated with 12 to 15 ml of a 24- to 48-h 37°C log-phase culture and incubated statically at 37°C. Thallium acetate was not used.

Preparation of cells, membranes, and soluble fractions. Cells were harvested between 20 and 72 h when they were in mid- to late log phase. The glass-attached *M. pneumoniae* FH cultures were harvested

by discarding the overlying culture medium, washing the glass-adherent growth with cold kappa buffer, and shaking the organisms off the glass with 4-mm glass beads in cold kappa buffer (22). The broth cultures were centrifuged at $10,400 \times g$ for 20 min. Pelleted cells were washed four times with 75 to 200 volumes of cold kappa buffer. The washed whole cells, membranes, and supernatant fractions were prepared as previously described (19) and either used as fresh, wet preparations (for spectroscopy) or lyophilized and stored in vacuo over phosphorus pentoxide at -60°C for future analyses (spectroscopy, iron, and acid-labile sulfide assays). All centrifugation was done in an RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.), using a GSA or SS-34 rotor head at 4°C .

Low-temperature spectroscopy. Turbid suspensions of cells or membranes (fresh or lyophilized) were suspended in glycerin-phosphate buffer (GPB) consisting of equal parts of glycerin and 0.1 M K_2HPO_4 buffer, pH 7.4 (13). Reduction of samples was accomplished with 5 to 10 mg of sodium dithionite (Fisher Scientific Co., Fair Lawn, N.J.), and in some experiments with two to six crystals of potassium cyanide (Fisher) per 0.85 ml of cell suspension, or by NADH (Sigma Chemical Co., St. Louis, Mo.) at 6 to $7 \mu\text{mol}$ per 0.5 ml of reaction mixture. Oxidation was facilitated by slowly bubbling gaseous O_2 into the samples at about 40 ml per min for 10 to 20 min. Samples in GPB, or GPB alone, were placed in a low-temperature cuvette accessory with a 0.1-cm light path (no. B35-69004, American Instrument Co., Inc., Silver Spring, Md.) and subsequently frozen in liquid nitrogen. After devitrification, the samples were scanned through 350 to 650 nm for the presence of cytochromes in an Aminco-Chance dual wavelength/split-beam recording spectrophotometer (American Instrument Co., Inc.), utilizing the dewar attachment (no. 4-8416, American Instrument Co.) for low-temperature analysis. Spectral scans were either difference spectra (reduced samples minus oxidized samples) or direct spectra (reduced or oxidized samples minus GPB). Beef heart mitochondria suspensions were used as a biological standard for comparison. Our examination of the mycoplasmal spectra for microbial-type cytochromes followed the procedures of Smith (27).

Chemical assays. (i) Total iron. Total iron was determined by a slight modification of the procedure of Brumby and Massey (2). We were able to detect 1.8 nmol of iron per assay.

(ii) Acid-labile sulfide. Acid-labile sulfide was determined by the procedure of King and Morris (14). The assay was modified by increasing the 1-min room temperature incubation in zinc acetate-NaOH to 5 to 30 min with frequent blending with a Vortex mixer. Absorbance was determined at 670 nm. Sodium sulfide (Fisher Scientific Co.) titrated by standard iodometric methods was used as a reference standard. Cysteine served as a negative control. We were able to detect 2.5 nmol of acid-labile sulfide per assay.

(iii) Protein assays. The protein content of beef heart mitochondrion suspensions was determined by the biuret procedure (5). All other protein assays were performed by the method of Lowry et al. (16).

Materials. Fresh beef heart mitochondrion suspensions were generously supplied by G. P. Brierley, The

Ohio State University, Columbus (12). Type V ferredoxin isolated from *Clostridium pasteurianum* (lot no. 78C-850 and 89C-6860) was purchased from Sigma Chemical Co.

RESULTS

Figure 1 illustrates typical direct spectra of *M. arthritidis* 07, *M. pulmonis* JB, *M. pneumoniae* FH, *M. arginini* G230, and *A. laidlawii* B-PG9 membrane fractions. The *M. arthritidis*, *M. pulmonis*, and *M. arginini* spectra are similar in that they possess absorption peaks at 444 to 446, 471 to 472, and 504 to 505 nm. The *M. pneumoniae* spectrum, at 0.9 or 1.8 mg of membrane protein per ml, did not reveal any prominent absorption peaks. *A. laidlawii* membranes have a distinctly different direct spectrum, with absorption peaks at 426, 452, 484, and 522 nm. The *A. laidlawii* spectrum has a significant trough at 470 nm, which is approximately the same wavelength as the maximum peak detected in *M. pulmonis*, *M. arthritidis*, and *M. arginini*. These findings were confirmed in three to six different membrane and whole-cell preparations obtained from each organism.

Examination of the difference spectra of dithionite-reduced minus O_2 -oxidizing membranes or whole-cell preparation of *M. arthritidis* 07, *M. pulmonis* JB, and *M. pneumoniae* FH indicated no evidence of any α -, β -, or Soret band cytochrome absorption peaks at maximum spectral sensitivity (data not shown).

The difference spectra (Fig. 2) of dithionite-reduced minus O_2 -oxidized membranes of six *Acholeplasma* species are similar. There is no evidence for any cytochrome pigments at maximum spectral sensitivity. All spectra had peaks at 403 to 410 and 462 to 469 nm, and all spectra but that of *A. granularum* had a peak at 432 to 434 nm. *A. laidlawii* B-PG9 spectra also had peaks at 491 and 510 nm. All spectra had troughs at 384 to 392 and 446 to 451 nm. The assignment of wavelengths is accurate to 2 nm. The difference spectra show "negative" absorbance between 380 to 480 nm that are relatable to flavins (26). The differential absorbances we observe are "negative" because flavins characteristically "bleach" upon dithionite reduction and are less absorbent than the oxidized reference samples. Similar results were obtained for whole-cell preparations of all of these *acholeplasmas* and when NADH was the reductant of membrane preparations of *A. laidlawii* B-PG9 (data not shown). The peak near 365 nm is due to dithionite.

Table 1 lists the content of iron and acid-labile sulfide in various *Mollicutes* fractions. Where there are sufficient data ($n > 3$), the molar iron/acid-labile sulfide ratios are also listed.

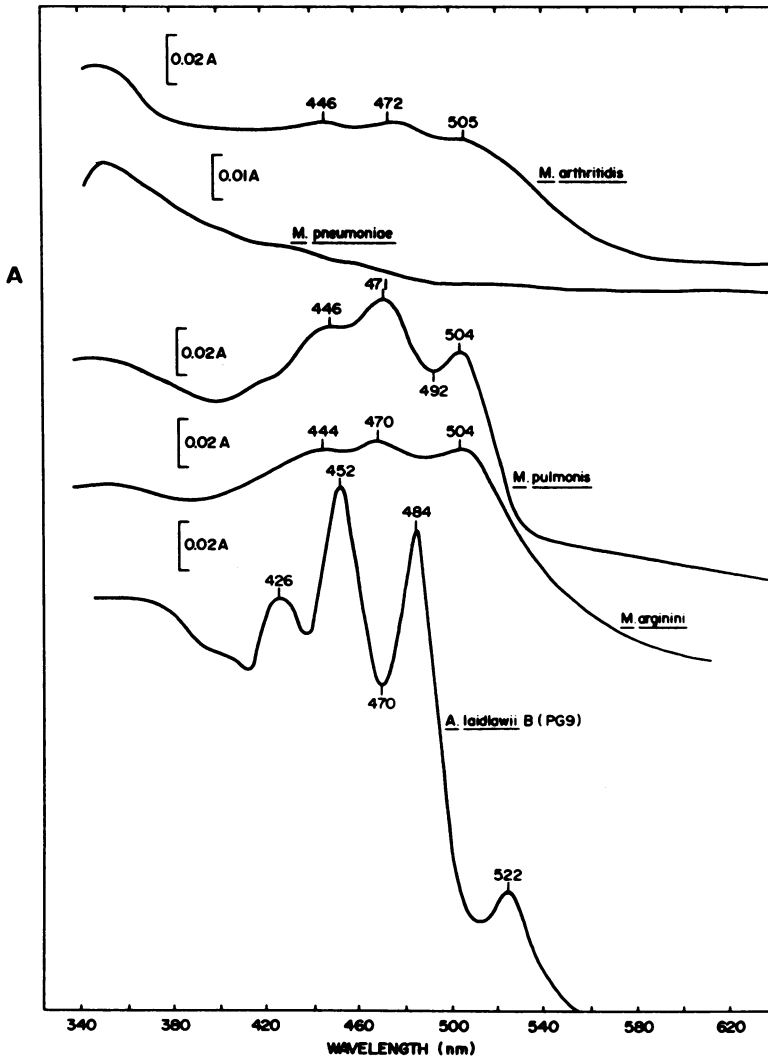


FIG. 1. Direct spectra of oxidized *Mollicutes* membranes. Suspensions of membranes in glycerin-phosphate buffer were oxidized with O_2 , frozen in liquid nitrogen, and compared with frozen buffer as described in the text. Concentrations of membrane suspensions in milligrams of protein per milliliter of buffer are: *M. arthritis*, 5.6; *M. pneumoniae*, 0.9; *M. pulmonis*, 1.6; *M. arginini*, 3.2; and *A. laidlawii* (B-PG9), 3.2.

Using the italicized values from Table 1, we averaged and compared the content of iron and acid-labile sulfide in some *Acholeplasma* and *Mycoplasma* spp. Our calculations showed that there is significantly more iron, almost double, in the membranes of the three *Mycoplasma* spp. than in the five *Acholeplasma* spp. ($P < 0.05$). We found no significant difference between the two genera in their whole cell or membrane content of acid-labile sulfide. There was a significant difference ($P < 0.05$), more than three-fold, in the iron/acid-labile sulfide ratio of membranes from the *Mycoplasma* compared with

membranes from the *Acholeplasma* spp. This was an apparent reflection of the differences in iron content.

The lowest molar iron/acid-labile sulfide ratio found in the *Mollicutes* was 5.0, whereas with the control preparations of beef heart mitochondria and *C. pasteurianum* ferredoxin, the ratios were about 1.07 (theoretical 1).

There was no significant difference in the iron content of whole cells and membranes of *A. laidlawii* B-PG9, *A. equifetale*, and *A. oculi* grown in modified Edward medium containing either 0.5 or 3.0% horse serum (data not shown).

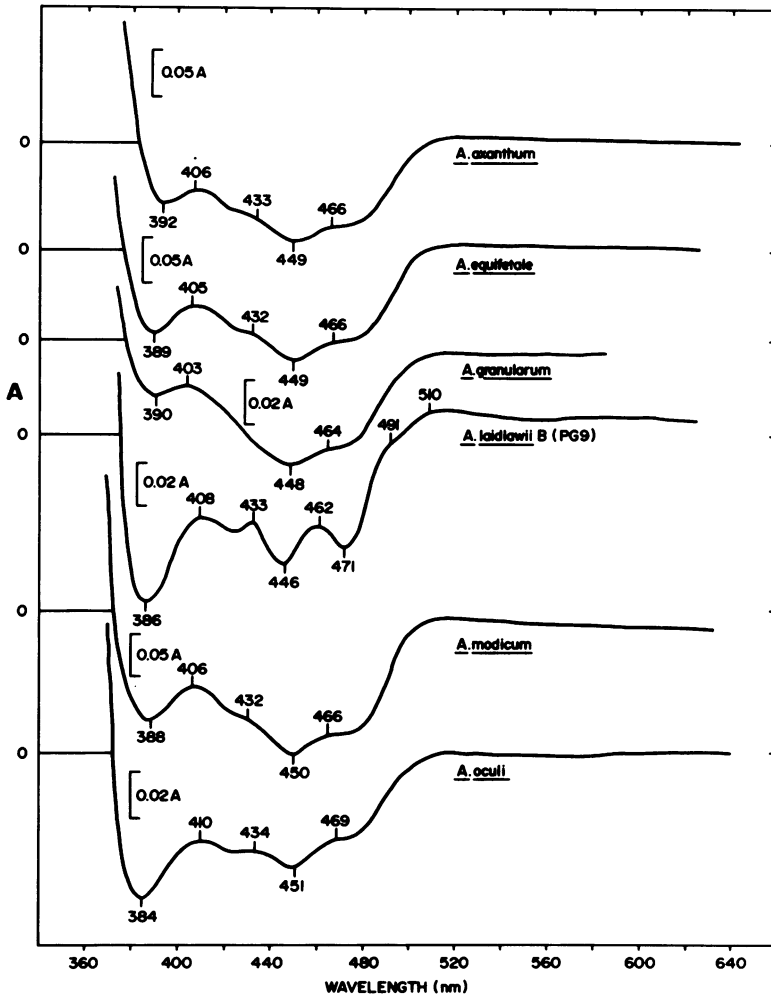


FIG. 2. Difference spectra of *Acholeplasma* membranes. Suspensions of each membrane preparation in glycerin-phosphate buffer were oxidized with O_2 , frozen in liquid nitrogen, and compared with frozen samples of the same suspension which were reduced with dithionite as described in the text. Concentrations of membrane suspensions in milligrams of protein per milliliter of buffer are: *A. axanthum*, 0.77; *A. equifetale*, 0.63; *A. granularum*, 2.19; *A. laidlawii* (B-PG9), 0.56; *A. modicum*, 1.65; and *A. oculi* 1.44.

A comparison of these data from three *Acholeplasma* spp. with data from three *Mycoplasma* spp. (Table 1), all grown with 3% horse serum, indicated that the amount of iron in whole cells did not differ significantly between the two genera, whereas the amount of iron in their membrane fractions was different ($P < 0.05$).

These data suggest that the amount of iron in the membranes of *Acholeplasma* spp. is greater than in the three *Mycoplasma* spp. regardless of whether the organisms were grown with 0.5 or 3.0% horse serum in the medium. The concentration of acid-labile sulfide detected in the *Mollicutes* fractions was less than one-fifth of their iron content.

DISCUSSION

Low-temperature difference spectroscopy was our method of choice for the detection of cytochrome pigments because of the sensitivity of the assay and the pronounced sharpening and intensification of the absorption bands at liquid nitrogen temperature (4). Glycerol was used as the solvent because it is nondestructive to hemoproteins (13).

The absence of detectable cytochrome pigments may be related to the composition of the growth medium or due to a nonoptimal pO_2 (25). However, *M. arthritidis* 07 or *A. laidlawii* B-PG9 did not produce detectable cytochrome

TABLE 1. Iron (Fe) and acid-labile sulfide (S) content of *Mollicutes* fractions^a

Strain	Fraction	Iron ^b	Acid-labile sulfide ^c	Molar Fe/S ratio
<i>Acholeplasma</i> sp.				
<i>A. axanthum</i> S743	Whole cells	8.2 ± 2.2 (5)	1.07 ± 0.41 (3)	10.5
	Membranes	3.3 ± 0.4 (4)	0.66 ± 0.17 (4)	5.0
	Supernatant	2.8 ± 0.7 (4)	0.21 ± 0.09 (4)	13.3
<i>A. equifetale</i> N93	Whole cells	6.5 ± 3.2 (4)	0.82 ± 0.72 (4)	15.2
	Membranes	4.3 ± 1.2 (3)	0.92 ± 0.25 (3)	4.7
	Supernatant	8.2 ± 2.1 (3)	0.36 ± 0.11 (3)	22.7
<i>A. granularum</i> BTS39	Whole cells	7.4 ± 1.8 (4)	0.50 ± 0.12 (3)	14.8
	Membranes	3.0 ± 0.9 (4)	0.32 ± 0.10 (4)	9.4
	Supernatant	4.9 ± 1.6 (4)	0.30 ± 0.16 (3)	16.3
<i>A. laidlawii</i> B, PG9	Whole cells	7.7 ± 2.1 (4)	0.42 ± 0.04 (3)	18.3
	Membranes	4.9 ± 1.4 (5)	0.81 ± 0.40 (8)	6.0
	Supernatant	4.1 ± 1.0 (3)	0.60 ± 0.11 (3)	6.8
<i>A. laidlawii</i> B, PG9-PS	Whole cells	ND ^d	0.85 (1)	— ^e
	Membranes	6.7 (2)	0.81 ± 0.23 (4)	—
	Supernatant	5.3 (2)	0.61 (2)	—
<i>A. modicum</i> PG49	Whole cells	15.3 (2)	0.88 ± 0.64 (3)	—
	Membranes	7.4 ± 3.1 (4)	0.82 ± 0.79 (3)	9.0
	Supernatant	2.7 (2)	0.20 (1)	—
<i>A. oculi</i> 19L	Whole cells	ND	0.21 ± 0.81 (3)	—
	Membranes	6.8 (2)	0.31 (2)	—
	Supernatant	4.2 (2)	0.10 (2)	—
<i>Mycoplasma</i> sp.				
<i>M. arthritis</i> 07	Whole cells	5.9 ± 3.4 (6)	0.99 ± 0.14 (3)	5.9
	Membranes	10.7 ± 3.8 (6)	0.75 ± 0.38 (6)	14.2
<i>M. pneumoniae</i> FH	Whole cells	7.9 ± 1.2 (4)	0.61 ± 0.07 (3)	12.9
	Membranes	5.7 ± 2.0 (6)	0.35 ± 0.05 (5)	16.3
<i>M. pulmonis</i> JB	Whole cells	5.7 ± 1.4 (3)	0.36 ± 0.10 (3)	15.8
	Membranes	8.9 ± 3.2 (3)	0.28 ± 0.02 (3)	31.8
Beef heart mitochondria ^f		7.56 ± 2.2 (5)	6.98 ± 0.81 (5)	1.08
Ferredoxin V (<i>C. pasteurianum</i>) ^g		846.1 ± 121.1 (2)	810.21 ± 44.22 (2)	1.04

^a Mean nanomoles of iron or acid-labile sulfide per milligram Lowry protein in different batches of lyophilized *Mollicutes* fractions ± standard deviation; in parentheses is the number of different batches assayed. Each batch was assayed two to five times.

^b For the iron assay, the samples of lyophilized *Mycoplasma* sp. grown with 3% serum ranged from 2.04 to 4.93 mg (dry weight); the samples of *Acholeplasma* sp. grown with 0.5% serum ranged from 3.10 to 5.21 mg (dry weight).

^c For the acid-labile sulfide assay, as in footnote *b*, the samples of lyophilized *Mycoplasma* sp. ranged from 2.81 to 10.69 mg (dry weight); the *Acholeplasma* sp. ranged from 1.96 to 11.07 mg (dry weight).

^d ND, Not done.

^e —, None.

^f Iron and acid-labile sulfide values for fresh beef heart mitochondria are reported as nanomoles per milligram of biuret protein ± standard deviation; in parentheses is the number of different batches assayed. Each batch was assayed three to six times. The amount of fresh beef heart mitochondria used in all assays was 1 to 7 mg (as biuret protein).

^g Each lot was examined in quintuplicate. Molecular weight, 6,000 (7).

pigments when grown statically in our modified Edward or SSR2 medium (unpublished data). *A. laidlawii* B-PG9, grown in either medium, while continuously sparged with air with or without 0.01% (wt/vol) hemin, did not produce detectable cytochrome pigments (unpublished data). The resultant cell mass was considerably less than that obtained from statically grown cultures with or without hemin. The diminution in growth was expected since Tourtellotte and Jacobs indicated that *A. laidlawii* B grows optimally between 1 and 8% oxygen (31).

Using 10 mg (as protein) of whole cells, we were also unable to detect reduced pyridine hemochrome in *M. arthritidis* 07, *M. pneumoniae*, and *A. laidlawii* B-PG9 grown with sparging by the technique of Rieske (24) (unpublished data).

The direct spectra of the oxidized membranes of three *Mycoplasma* species are quite different from those of *A. laidlawii* (Fig. 1). The differences may have taxonomic value. The negative spectral scan of *M. pneumoniae* may be attributable to low levels of membranes analyzed (Fig. 1); scans of more concentrated preparations (1.8 mg of protein per ml) were similarly blank.

It has been reported that considerable quantities of iron were incorporated into the membrane fraction of *Mycoplasma capricolum* (1). We were unable to localize iron in the three *Mycoplasma* species examined because we could not concentrate the mycoplasmal supernatant fractions by lyophilization; however, the data suggest that there may be some localization of iron in the membrane fractions. In the *Acholeplasma* species, we could not interpret the data to indicate any localization pattern for iron. Jinks and Matz found 5.0 nmol of iron per mg of membrane protein in *A. laidlawii* (ATCC 14192) (11). Our results are in close agreement as we found 4.9 ± 1.4 nmol of iron per mg of membrane protein in *A. laidlawii* B-PG9. In our controls, the iron content was (in nanomoles of iron per milligram of protein): 952 ± 172 nmol for type V *C. pasteurianum* ferredoxin and 7.56 ± 2.2 nmol for beef heart mitochondria. These values were close to the reported values of 830 (15) and 6.0 nmol (6), respectively.

The function of iron in *Mollicutes* has not been established. Tarshis et al. (30) found that phenanthroline, which forms a complex with ferrous iron, reduced respiration and the active transport of 3-O-methyl-D-glucose in *A. laidlawii* Köller. These workers suggested that electrons are transported from flavoproteins to oxygen by means of some intermediate carrier of nonhemic nature which is sensitive to phenanthroline. The data support the view that in

aerotolerant *Mollicutes* the iron, perhaps with sulfur, is involved with flavin (flavin adenine dinucleotide or flavin mononucleotide or both) in the transfer of electrons via NADH to oxygen with the association of NADH:ferricyanide oxidoreductase or NADH oxidase activities or both (20, 30). In the obligately anaerobic members of the genus *Anaeroplasma*, also presumably lacking cytochromes, the transfer of electrons proceeds by some other route. In these hypotheses, iron may act as a reversible electron carrier cycling between Fe(III) and Fe(II). Bauminger et al. (1) have found Fe(III) and Fe(II) in *M. capricolum* and noted the possibility that iron exists in an iron-containing electron storage protein to supply low potential redox equivalents.

In general, the absolute amount of acid-labile sulfide we detected in *Mollicutes* fractions was low, close to our limit of detection. The low recoveries were probably due to minimal or even inadequate sample sizes, ≤ 16 mg of dry weight, but it is also possible that the sulfide was extruded from the protein during preparation (17). It is less likely that these organisms contain an iron-sulfur protein like rubredoxin which lacks acid-labile sulfide (3). In our controls, the acid-labile sulfide content was (in nanomoles of acid-labile sulfide per milligram of protein): 810 ± 44 nmol for type V *C. pasteurianum* ferredoxin and 6.98 ± 0.81 nmol for beef heart mitochondria. These values were near the reported values of 730 nmol (15) and 7.1 to 7.3 nmol (8), respectively.

If we assume that iron-sulfur proteins are present and have a molecular weight of 10,000, contain four acid-labile sulfur atoms and four iron atoms per molecule and our sulfide assays are appropriate, we calculate that the concentration of iron-sulfur proteins would be less than 10 μ g per mg of whole cell dry weight. Further, the *Mollicutes* would contain additional iron of unknown function not associated with sulfide.

We have been unable to detect cytochrome pigments in four *Mycoplasma* and six *Acholeplasma* spp. Our data, as well as an earlier report of Holländer (9), are in disagreement with the finding that *M. arthritidis* 07 has cytochrome pigments (33). Further, we could not find cytochrome pigments in *M. arthritidis* ATCC 14124 and ATCC 14152 by using a Cary 14 spectrophotometer with 0.02-nm full-scale slide wire (J. D. Pollack, unpublished data). Our data suggest that *Mollicutes* do not synthesize ATP at positions analogous to the electron transport chain found in many other procaryotes. Therefore, barring the possibility that ATP is synthesized by *Mollicutes* at a locus-like site I of mitochondria, it appears that these organisms synthesize

most, if not all, of their ATP at the substrate level. Hence, some specific inhibitors of electron transport or phosphorylation at the cytochrome level might be useful in isolating or distinguishing *Mollicutes*.

Many *Mollicutes* were originally categorized by Paul VanDemark as having a "flavin-terminated respiratory chain" (32). Excluding the wall-less obligately acidophilic and thermophilic *Thermoplasma*, the evidence supports the adoption and expansion of VanDemark's concept. We now suspect that members of the Class *Mollicutes* may lack quinones and cytochromes and are, in this regard, metabolically homogeneous and similar to the cytochromeless facultative anaerobic clostridia and lactic acid bacteria.

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