Reduction of Benzyl Viologen Distinguishes Genera of the Class *Mollicutes*

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We tested the ability of 62 growing strains belonging to the class *Mollicutes* to reduce the redox indicator and free-radical generator 1,1'-dibenzyl-4,4'-bipyridinium dichloride (benzyl viologen [BV]) to a blue-violet-purple color. BV was reduced by 12 *Acholeplasma* species but not by *Acholeplasma multifforme* PN525^T (T = type strain). BV was also reduced by five of nine *Mesoplasma* species and by four of six *Entomoplasma* species. BV was not reduced by 19 *Mycoplasma* species, six *Spiroplasma* species, five unnamed *Spiroplasma* strains belonging to different serogroups, three *Ureaplasma* species, and one unnamed *Ureaplasma* strain. The BV-reducing ability was localized in the membrane of *Acholeplasma laidlawii* B-PG9 and was dependent on NADH. Reduction of BV could be expressed in mixed cultures, and this activity may be useful for recognizing the contaminating presence of an *Acholeplasma* species. The reductive BV response may have phylogenetic value. We believe that the test described in this paper readily distinguishes all *Acholeplasma* species and some *Mesoplasma* and *Entomoplasma* species from all *Mycoplasma*, *Spiroplasma*, and *Ureaplasma* species tested.

The use of redox indicators like tetrazolium salts to study the wall-less members of the class *Mollicutes* was first described by Somerson and Morton (19). There have been numerous applications of the observation of these authors (1, 4, 8, 17). Although most members of the *Mollicutes* have been found to reduce tetrazolium salts (8, 21), this characteristic is now thought to have little value in identification or in the development of a taxonomy for the *Mollicutes* (20). Nevertheless, the use of tetrazolium salts to monitor the growth kinetics and metabolism of these organisms is still a valuable experimental and clinical tool (8, 18). Besides tetrazolium salts there are other redox indicators that may be useful in distinguishing and characterizing *Mollicutes*. Among the indicators that we examined were the bipyridinium compounds 1,1'-dibenzyl-4,4'-bipyridinium dichloride (benzyl viologen [BV]) and methyl viologen (paraquat). In this study of 62 strains belonging to the *Mollicutes*, we found that BV added to broth cultures of members of the non-cholesterol-requiring *Mollicutes* genus *Acholeplasma* underwent a rapid and profound color change. There are six genera of facultatively anaerobic *Mollicutes*, and the response which we describe below distinguished the members of the genus *Acholeplasma* from all *Mycoplasma*, *Spiroplasma*, and *Ureaplasma* species and most *Mesoplasma* and *Entomoplasma* species tested.

MATERIALS AND METHODS

Mollicutes, growth media, and culture conditions. The members of the *Mollicutes* that we studied are listed in Table 1. It was necessary to employ a number of different media to grow the members of the six facultatively anaerobic *Mollicutes* genera that were studied. Two of the media were variations of modified

Edward's medium. One of these media was called SNE (Table 1) and contained (per liter) 20 g of Mycoplasma Broth Base (BBL), 2 g of Bacto Yeast Extract (Difco Laboratories), 11 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Research Organics, Inc., Cleveland, Ohio), 10 g of Bacto Peptone, (Difco), and 0.1 g of herring sperm DNA (Sigma); the final pH was 7.6. After this basal broth had been autoclaved, we added (per liter) 14 ml of a separately autoclaved 50% (wt/vol) glucose solution and 6 ml of a separately autoclaved 41% (wt/vol) anhydrous K₂HPO₄ solution. Horse serum (Hyclone or Oxoid) was added to a concentration of 10% (vol/vol). The second variation of modified Edward's medium was not autoclaved. This medium was similar to SNE; however, PPLO Broth w/o CV (Difco) was used instead of Mycoplasma Broth Base, peptone was omitted, the level of yeast extract was 5 g/liter, the concentration of horse serum (GIBCO) was only 3.0% (wt/vol) and 1.8 g of "ultrapure" urea (Research Organics, Inc.) per liter was added. The ingredients of this medium were combined with distilled water, and the pH was adjusted to 7.4 with 2 N NaOH. The solution was clarified by sequential filtration through 1.2- and 0.45- μ m-pore-size filters and then sterilized by passage through a 0.2- μ m-pore-size filter. This medium was called MEM+U (Table 1) and was used exclusively for the growth of all *Ureaplasma* isolates.

To grow *Spiroplasma* species, we used the following three media (Table 1): BSR (3), M1D (23), and H-2 (6). The growth medium and incubation temperature used for each *Mollicutes* strain are shown in Table 1.

In the experimental assay to determine growth reduction of BV, a filter-sterilized aqueous solution (50 mM) of BV (Sigma) was added to tubes containing sterile medium to a final concentration of 1 mM. As the reduced BV was readily reoxidized by air and thereby decolorized, the tubes were filled with 5 to 10 ml of medium as the generally preferred configuration. In some experiments, to impede the diffusion of air and reduce reoxidation, 1.5 to 2 cm of sterile (autoclaved) mineral oil (USP) was added as an overlay after inoculation. The stock solution of BV and media containing BV were stored at 4°C for several months with no apparent loss of activity.

With the exception of the ureaplasmas, cultures were incubated for 10 to 14 days depending on the isolate, before they were characterized as BV negative. The animal *Ureaplasma* isolates were subcultured only three times. *Ureaplasma urealyticum* T960^T (T = type strain) was subcultured seven times. *Mollicutes* strains that were always capable of reducing BV within five to seven consecutive transfers were considered BV positive. *Mollicutes* cultures in which no reduction of BV occurred were subcultured with fresh BV-containing medium for 4 to 12 consecutive transfers. Only if all of the resulting cultures failed to reduce BV were the isolates considered BV negative.

Other assays. In order to identify the cellular localization of the BV-reducing ability, membrane and cytoplasmic fractions of *Acholeplasma laidlawii* B-PG9 were prepared by osmotic shock as previously described (12). The reduction of

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TABLE 1. BV reduction by members of the class *Mollicutes*

Organism	Response ^a	Growth medium containing 1 mM BV	Growth temp (°C)
<i>Acholeplasma axanthum</i> S-743 ^T	+	SNE	37
<i>Acholeplasma brassicae</i> 0502 ^T	+	SNE	37
<i>Acholeplasma cavigenitium</i> GP3 ^T	+	SNE	37
<i>Acholeplasma equifetale</i> C112 ^T	+	SNE	37
<i>Acholeplasma granularum</i> BTS-39 ^T	+	SNE	37
<i>Acholeplasma hippikon</i> C1 ^T	+	SNE	37
<i>Acholeplasma laidlawii</i> PG8 ^T	+	SNE	37
<i>Acholeplasma modicum</i> PG49 ^T	+	SNE	37
<i>Acholeplasma morum</i> 72-043 ^T	+	SNE	37
<i>Acholeplasma multilocale</i> PN525 ^T	—	SNE	37
<i>Acholeplasma oculi</i> 19L ^T	+	SNE	37
<i>Acholeplasma palmarum</i> J233 ^T	+	SNE	37
<i>Acholeplasma parvum</i> H23N ^T	+	SNE	37
<i>Mycoplasma alkalescens</i> PG51 ^T (= NCTC 10135 ^T)	—	SNE	37
<i>Mycoplasma arginini</i> G230 ^T (= NCTC 10129 ^T)	—	SNE	37
<i>Mycoplasma bovis</i> PG45 ^T (= NCTC 10131 ^T)	—	SNE	37
<i>Mycoplasma bovovulvi</i> M165/69 ^T (= NCTC 10141 ^T)	—	SNE	37
<i>Mycoplasma buccale</i> CH20247 ^T (= NCTC 10136 ^T)	—	SNE	37
<i>Mycoplasma californicum</i> ST-6 ^T (= NCTC 10189 ^T)	—	SNE	37
<i>Mycoplasma capricolum</i> subsp. <i>capricolum</i> California Kid ^T (= NCTC 10154 ^T)	—	SNE	37
<i>Mycoplasma fermentans</i> PG18 ^T (= NCTC 10177 ^T)	—	SNE	37
<i>Mycoplasma felis</i> CO ^T (= NCTC 10160 ^T)	—	SNE	37
<i>Mycoplasma flocculare</i> Ms42 ^T (= NCTC 10143 ^T)	—	SNE	37
<i>Mycoplasma gallinarum</i> PG16 ^T (= NCTC 10120 ^T)	—	SNE	37
<i>Mycoplasma gallisepticum</i> PG31 ^T (= NCTC 10115 ^T)	—	SNE	37
<i>Mycoplasma hyopneumoniae</i> J ^T (= NCTC 10110 ^T)	—	SNE	37
<i>Mycoplasma imitans</i> 4229 ^T (= NCTC 11733 ^T)	—	SNE	37
<i>Mycoplasma penetrans</i> GTU-54-6A1 ^T (= ATCC 55252 ^T)	—	SNE	37
<i>Mycoplasma pirum</i> HRC70-159 ^T (= NCTC 11702 ^T)	—	SNE	37
<i>Mycoplasma primatum</i> HRC292 ^T (= NCTC 10163 ^T)	—	SNE	37
<i>Mycoplasma salivarium</i> PG20 ^T (= NCTC 10113 ^T)	—	SNE	37
<i>Mycoplasma verrecundum</i> 107 ^T (= NCTC 10145 ^T)	—	SNE	37
<i>Spiroplasma apis</i> B31 ^T	—	BSR	32
<i>Spiroplasma citri</i> R8A2 Maroc ^T	—	BSR	30
<i>Spiroplasma citri</i> R8A2 Maroc ^T	—	M1D	32
<i>Spiroplasma clarkii</i> CN-5 ^T	—	BSR	32
<i>Spiroplasma diabolicae</i> DU-1 ^T	—	BSR	32
<i>Spiroplasma ixodetis</i> Y32 ^T	—	M1D	26, 32
<i>Spiroplasma ixodetis</i> Y32 ^T	—	BSR	32
<i>Spiroplasma ixodetis</i> Y32 ^T	—	H-2	32
<i>Spiroplasma leptinotarsae</i> LD-1 ^T	—	H-2	32
<i>Spiroplasma mirum</i> SMCA ^T	—	M1D	32
<i>Spiroplasma monobiae</i> MQ-1 ^T	—	BSR	32
<i>Spiroplasma taiwanense</i> CT-1 ^T	—	BSR	32

Continued

TABLE 1—Continued

Organism	Response ^a	Growth medium containing 1 mM BV	Growth temp (°C)
<i>Spiroplasma</i> group II strain DW-1	—	BSR	32
<i>Spiroplasma</i> group XXIII strain TG-1	—	BSR	32
<i>Ureaplasma diversum</i> T44	—	MEM+U	37
<i>Ureaplasma gallorale</i> D6-1 ^T (= ATCC 43346 ^T)	—	MEM+U	37
<i>Ureaplasma urealyticum</i> T960 ^T	—	MEM+U	37
<i>Ureaplasma urealyticum</i> Pi	—	MEM+U	37
<i>Ureaplasma</i> sp. ovine strain 1692	—	MEM+U	37
<i>Mesoplasma chauliocola</i> CHPA-2 ^T	+	SNE	30
<i>Mesoplasma entomophilum</i> TAC ^T	—	SNE	30
<i>Mesoplasma florum</i> L1 ^T	Weak, variable ^b	SNE	30
<i>Mesoplasma florum</i> GF	Weak, variable ^b	SNE	30
<i>Mesoplasma grammopteriae</i> GRUA-1 ^T	+	SNE	30
<i>Mesoplasma lactucae</i> 831-C4 ^T	—	SNE	30
<i>Mesoplasma pleciae</i> PS-1 ^T	+	SNE	30
<i>Mesoplasma seiffertii</i> F7 ^T	—	SNE	30
<i>Mesoplasma syrphidae</i> YJS ^T	—	SNE	30
<i>Mesoplasma tabanidae</i> BARC 857 ^T	+	SNE	30
<i>Entomoplasma elychniae</i> ELCN-1 ^T	—	SNE	30
<i>Entomoplasma lucivorax</i> PIPN-2 ^T	—	SNE	30
<i>Entomoplasma luminosum</i> PIMN-1 ^T	—	SNE	30
<i>Entomoplasma melaleuca</i> M-1 ^T	Variable ^b	SNE	30
<i>Entomoplasma somniflux</i> PYAN-1 ^T	—	SNE	30

^a +, blue-violet-purple coloration of the medium appeared within 4 to 48 h at 30.0 or 37.0°C and in all five to seven subcultures; —, no coloration of the medium occurred during 3 to 12 consecutive subcultures.

^b Blue-violet-purple coloration of the medium was observed discontinuously during 9 or 10 consecutive subcultures.

BV by membranes was monitored spectrophotometrically at 555 nm. Each reaction mixture (final volume, 1 ml) contained 50 mM HEPES (pH 7.4), 155 mM NaCl, 1 mM 2-mercaptoethanol, and 20 to 300 µg of cell membrane protein. The reaction was started by adding NADH to a final concentration of 5.2 mM. Protein contents were determined by using the Bio-Rad standard microassay and bovine serum albumin as the protein standard.

RESULTS

Table 1 shows the ability of 62 members of the *Mollicutes* representing the six facultatively anaerobic genera in the class to reduce BV during growth. Twelve *Acholeplasma* species but not *Acholeplasma multilocale* PN525 were BV positive. Of the nine *Mesoplasma* species studied, four were always BV positive, and another (*Mesoplasma florum*) was weakly reductive (consecutive subcultures were not consistently BV positive). Four mesoplasmas were BV negative. Five *Entomoplasma* species were examined; one was variable like *Mesoplasma florum*, and four were BV negative. Nineteen *Mycoplasma* species, seven *Spiroplasma* species, five *Spiroplasma* strains representing four other different serogroups, three *Ureaplasma* species, and two other *Ureaplasma* strains were all BV negative.

With most *Acholeplasma* species a blue-violet-purple color appeared within 12 h, but with *A. laidlawii* B-PG9 the color appeared within 4 to 5 h. The color generally filled the growth tubes, making them almost completely opaque. After shaking, aeration, or prolonged incubation the color disappeared. Cultures were examined frequently during the first 24 h.

The $\lambda_{\max}$ of the blue-violet-purple color in BV-positive cultures was 553 to 556 nm, and the color was lost upon aeration.

We were also able to reproduce the color response in a mixture containing 1 mM BV and 0.1 mM sodium borohydride in distilled water. The λ_{max} of this solution was 555 nm, and the color was lost upon aeration. The visible color, the λ_{max} , and the sensitivity to aeration were compatible with the previously reported properties of reduced BV (2). We concluded that the color which we observed in BV-containing media inoculated with *Acholeplasma* species was due to reduced BV.

The BV activity was localized in the membrane fraction of *A. laidlawii* B-PG9. The specific activity of the reaction was estimated by determining the increase in A_{555} as the highly colored reduced reaction product was synthesized. The reaction was entirely dependent on NADH. The specific activity of the cytoplasmic fraction was $0.37 A_{555} \text{ unit min}^{-1} \text{ mg of protein}^{-1}$, while the specific activity of the membrane fraction was $111.1 A_{555} \text{ units min}^{-1} \text{ mg of protein}^{-1}$. The NADH oxidase activity of all *Acholeplasma* species has also been shown to be localized in the membrane (11).

In preliminary studies (data not shown) we found that broth cultures of the BV-negative isolates *Mycoplasma capricolum* subsp. *capricolum* 14, *Mycoplasma fermentans* PG18^T, and *U. urealyticum* T960^T grown in the presence of BV rapidly became BV positive when *A. laidlawii* B-PG9 was added as a contaminant. This suggests that the BV test may be useful for determining the presence of *Acholeplasma* species in mixed cultures containing *Mollicutes* belonging to some other genera.

In other experiments (data not shown), we attempted to use the property of BV reduction by *Acholeplasma* strains as an indicator of *Acholeplasma* contamination of tissue cultures. However, the experiments failed because at the concentrations of BV tested (0.063 to 4 mM), the growth of HeLa cells was inhibited.

The usefulness of the BV-reducing ability of *A. laidlawii* B-PG9 was also tested aerobically on SNE containing 0.9% Noble agar (Difco) and 1 mM BV. In this test the organism grew, although poorly. No color change was detected. These experiments failed, apparently because of rapid and complete reoxidation of the colored reduction product by air and the apparent toxicity of BV.

DISCUSSION

Upon one-electron reduction, the essentially colorless compound BV (or BV^{2+}) becomes highly colored (λ_{max} , 555 nm) and a singly charged stable free radical (reduced $\text{BV}^{\bullet}$ or $\text{BV}^{2\bullet}$) (2, 10). The color rapidly disappears upon aeration. We speculate that the NADH-dependent reduction of BV by *A. laidlawii* B-PG9 membranes is specifically related to the organism's NADH-dependent membrane-localized NADH oxidase. These properties may be affected by the same protein. The membrane-localized enzyme NADH oxidase is a flavin mononucleotide—copper-containing iron-sulfur protein (15). We hypothesize that the same enzyme or a similar enzyme (i.e., an enzyme with a comparable or lower redox potential) is present in all other *Acholeplasma* species, but not in all other genera of the *Mollicutes*.

The NADH oxidase of *Mycoplasma capricolum* subsp. *capricolum* is the only other *Mollicutes* NADH oxidase that has been isolated and characterized (9). This enzyme differs markedly from the *A. laidlawii* enzyme (9). The prosthetic group of the *Mycoplasma capricolum* subsp. *capricolum* NADH oxidase is flavin adenine dinucleotide, not flavin mononucleotide. Also, unlike the *A. laidlawii* enzyme, this enzyme has no associated metals, cannot donate electrons to cytochrome *c*, dichlorodolphenol, or ferricyanide, or produce superoxide radicals.

If our hypothesis is correct, the redox potentials of the acho-

leplasmal NADH oxidases are similar to the redox potential of the *A. laidlawii* B enzyme (9) and are more negative than the E'_0 of BV, since the flow of electrons is from more negative to more positive. The E'_0 of BV is -0.359 V (22). The E'_0 of the *Mycoplasma capricolum* subsp. *capricolum* NADH oxidase is more positive than -0.359 V , and hence this enzyme is not able to donate electrons to an acceptor like BV, which has a more negative potential.

In preliminary growth curve (CFU) experiments (data not shown), we found that the BV-positive organism *A. laidlawii* B-PG9 died after about 6 h of incubation in the presence of 1 mM BV. At this time the level of reduced BV was maximal. We assume that the organism was in a sense "starved" for NADH; that is, as NADH was synthesized by the cells, it was nonenzymatically oxidized by BV, which removed it from the metabolic pool. BV is quickly reoxidized in air and then is able to accept electrons from more NADH as it is synthesized by cells. The organisms become transiently depleted of NADH and die. When the reoxidation is impeded, the colored compound can be detected. Another explanation for the apparent toxicity of BV reduction is based on the results of animal studies and is related to the production of hydrogen peroxide (10). The highly reactive free radical reduced BV interacts with O_2 , producing a peroxide that inhibits essential metabolic processes. In the case of BV-negative *Mollicutes*, such as *Mycoplasma capricolum* subsp. *capricolum* and *Mycoplasma fermentans*, BV appears to be inert.

We also tested methyl viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride or paraquat) in the same way (data not shown). Paraquat has a redox potential ($E'_0 = -0.446 \text{ V}$) (2) that is even more negative than that of BV. Paraquat was also reduced by *A. laidlawii* B-PG9. However, the reduced colored compound (λ_{max} , 601 nm) (2) seemed considerably more labile in air than reduced BV, and therefore, this compound was not studied further.

The variable and frequently weaker but positive BV responses that we detected in some cultures of non-*Acholeplasma* species, especially mesoplasmas, may also have been due to the presence of an NADH oxidase with similar or intermediate properties.

BV reduction may be associated with the NADH oxidase of acholeplasmas, because the NADH oxidase of the BV-positive *Acholeplasma* species may act as a diaphorase. Diaphorases are enzymes that are capable of mediating the transfer of electrons from a donor such as NADH, to both unnatural electron acceptors, like BV, tetrazoliums, or ferricyanide, or a natural electron acceptor, such as (presumably) O_2 . *A. multilocale* PN525^T (7) did not reduce BV. We have shown that the NADH oxidase of this acholeplasma is localized in the cytoplasmic fraction, in contrast to all other acholeplasmas, whose NADH oxidase activities are membrane associated (11, 14). The absence of BV activity in *A. multilocale* PN525^T and other BV-negative *Mollicutes* may be related to a lack of a membrane-localized flavin mononucleotide—copper-containing iron—sulfur-containing NADH oxidase activity that can act as a diaphorase.

We have also reported that *A. multilocale* PN525^T, unlike all other acholeplasmas, lacks dUTPase, PP_i -dependent phosphofructokinase, and uracil DNA glycosylase activities (5, 12–14, 24). Furthermore, this isolate can use both ATP and PP_i in the deoxyguanosine kinase reaction (14). This characteristic also distinguishes *A. multilocale* PN525^T from all other acholeplasmas, which use either ATP or PP_i or are completely unable to phosphorylate nucleosides. These findings and the negative BV response are exceptions to the general metabolic pattern that we have found in 12 *Acholeplasma* species (13, 14). However, the data of Rose et al. indicate that *A. multilocale*

PN525^T, like other achleplasmas, has no growth requirement for cholesterol or fatty acids (16). We believe that the lack of a BV response by *A. multilocale* PN525^T, a member of the only BV-negative *Acholeplasma* species, coupled with our other findings, indicates that *A. multilocale* PN525^T belongs to a distinct unrecognized metabolic subgroup of the genus *Acholeplasma* or is not an *Acholeplasma* strain.

Our survey is incomplete. We have tested only about 40% of the 150 species belonging to the class *Mollicutes*, and therefore, we can only suggest that the simple growth assay used in this study may be useful for distinguishing the BV-negative *Mycoplasma*, *Spiroplasma*, and *Ureaplasma* species from the BV-positive *Acholeplasma* species and some other *Mollicutes* that we have tested. The BV response may have some phylogenetic relevance. We also suggest that the BV test may be useful for determining the presence of *Acholeplasma* species in broth cultures of other members of the *Mollicutes*.

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