Differential Inhibition of Mollicute Growth: an Approach to Development of Selective Media for Specific Mollicutes

S. A. Keçeli* and R. J. Miles†

Department of Microbiology, Division of Life Sciences, King’s College London, London W8 7AH, United Kingdom

Received 15 April 2002/Accepted 17 July 2002

The energy-generating pathways of Mycoplasma spp. are diverse. Thus, it was predicted that the ability of inhibitors of these pathways to block growth would vary among species. This prediction was tested with 14 Mycoplasma species and potential inhibitors. The greatest differentiation among test species was obtained using fluoride, iodoacetate (IAA), β-fluoropyruvate (FP), cibacron blue (CB), L-citrulline, and carbonyl cyanide m-chlorophenylhydrazone. A range of other potential inhibitors, including l-arginine analogues, had little inhibitory effect on growth, and d-arginine was shown to be a growth substrate for arginine-hydrolyzing species. Fluoride selectively inhibited the growth of mycoplasmas that were able only to ferment sugars to lactate and/or to hydrolyze arginine. In contrast, IAA was most effective against organic acid-oxidizing species, and L-citrulline inhibited arginine-hydrolyzing species. Mycoplasma verecundum, a species for which energy sources have not been identified, was relatively resistant to FP. Similarly, Acholeplasma laidlawii was distinguished by its CB resistance.

Mollicutes (“soft skin,” a reference to the lack of a cell wall) are prokaryotes that are widely distributed as pathogens and commensals of animals and plants. They include eight recognized genera: Acholeplasma, Anaeroplasma, Asteroplasma, Entomoplasma, Mesoplasma, Mycoplasma, Spiroplasma, and Ureaplasma. In humans and domestic animals the majority of mollicutes isolated belong to the genus Mycoplasma, although Ureaplasma and certain Acholeplasma spp. may also be present in clinical specimens.

Mollicutes are slow growing and nutritionally demanding, and it is likely that their significance in human and animal disease is presently underestimated because of low rates of isolation. Improved rates of isolation of recognized animal pathogens may also aid in disease control. A major consideration in designing isolation media for mollicutes is the inclusion of selective inhibitors to reduce the growth of cell-walled bacteria. The selective agents usually used are antibiotics which inhibit cell wall synthesis and thallium salts. However, the isolation of potentially pathogenic mycoplasmas may also be prevented by the more rapid growth of commensal Mycoplasma and Acholeplasma species. Selective media in which the growth of nontarget mollicutes is inhibited have not yet been described, although Abu-Amero et al. (1) showed that the bacteriocin nisin inhibited the growth of Acholeplasma, but not Mycoplasma, species.

There appear to be few variable cellular targets among Mycoplasma species which might be selectively inhibited. However, energy-generating mechanisms are diverse, and one possible approach to the development of selective media for specific Mycoplasma groups is the inhibition of particular routes to ATP synthesis. In Mycoplasma, the recognized mechanisms of energy generation are the fermentation of sugars to lactate, the oxidation of lactate or pyruvate to acetate plus carbon dioxide, and the metabolism of l-arginine to ornithine (11, 17). Individual species may be able to use one, any two, or all three of these mechanisms (12), although in certain species energy sources have not been identified and in Mycoplasma verecundum it appears that energy generation occurs by some other mechanism (13). Acholeplasma spp. possess pathways for the fermentation of sugars to lactate and the oxidation of lactate or pyruvate to acetate plus carbon dioxide (18). The diversity of energy generation among Mycoplasma spp. suggests that the susceptibility of species to inhibitors of specific energy-generating pathways will vary. Thus, such inhibitors may be useful in developing selective media for specific Mycoplasma species or groups of species. In addition, the identification of inhibitors active against only certain Mycoplasma spp. may enable the development of simple growth inhibition tests for screening unknown isolates.

In the present study, inhibitors tested for differential effects on mycoplasma growth were sodium fluoride (NaF), iodoacetate (IAA), β-fluoropyruvate (FP), cibacron blue (CB), d-arginine, l-lysine, L-citrulline, t-canavanine, t-ornithine, methylyamine, N⁵-methyl-L-arginine, carbonyl cyanide m-chlorophenylhydrazone (CCCP), dicyclohexylcarbodiimide (DCCD), and the ionophores monensin and nigericin.

NaF inhibits enolase, and IAA inhibits glyceraldehyde-3-phosphate dehydrogenase (9). Thus, both NaF and IAA would be predicted to be most effective against mycoplasmas dependent upon the glycolysis for energy generation. Both NaF and IAA have been reported to block glycolysis and growth in a range of fermentative cell-walled bacteria (6, 16, 20, 22).

FP is known to inhibit the oxidation of pyruvate and was included in this study as a potential selective inhibitor of lactate- and pyruvate-oxidizing mycoplasmas. The pyruvate dehydrogenase complex of Escherichia coli was rapidly inactivated.
by low FP concentrations (0.008 mM) (5), and in Mycoplasma gallinatum, FP (300 μM) blocked oxygen uptake by cells metabolizing pyruvate (300 μM) or lactate (3 mM) (24).

CB shares structural similarities with NADH and was considered for use in this study as a potential inhibitor of NADH oxidase. This enzyme is found in all Mycoplasma spp.; however, its role would appear to be more significant in organic acid-oxidizing species in which there is a net accumulation of NADH during energy-generating metabolism (12). In addition, the cellular location of NADH oxidase differs among mollicutes. In acholeplasmas, the enzyme is predominantly membrane bound (17); in contrast, in mycoplasmas, activity is usually reported as being predominantly intracellular, although cell surface-bound NADH oxidase activity has been reported in Mycoplasma bovoculi (2).

L-Citrulline, L-ornithine, d-arginine, L-lysine, L-canavanine, methylamine, and Nε-methyl-L-arginine were included in the study as potential inhibitors of L-arginine metabolism by the ATP-generating arginine dihydrolase pathway (19). They were considered potential analogues of L-arginine which might compete with it for uptake or subsequent metabolism. There are no previous reports of the effect of arginine analogues on mollicute growth.

NADH oxidase activity has been reported as being predominantly intracellular, although the cellular location of NADH oxidase differs among mollicutes. In acholeplasmas, the enzyme is predominantly membrane bound (17); in contrast, in mycoplasmas, activity is usually reported as being predominantly intracellular, although cell surface-bound NADH oxidase activity has been reported in Mycoplasma bovoculi (2).

MATERIALS AND METHODS

Organisms. The following microorganisms were obtained from the National Collection of Type Cultures (London, United Kingdom): Mycoplasma arginini NCTC 10129, Mycoplasma agalactiae NCTC 10123, M. bovoculi NCTC 10141, Mycoplasma felis NCTC 10160, Mycoplasma fermentans NCTC 10117, Mycoplasma gallinatum NCTC 10120, Mycoplasma gallisepticum NCTC 10115, Mycoplasma pinun NCTC 11702, Mycoplasma verecundum NCTC 10145, and Acholeplasma laidlawii NCTC 10116. Mycoplasma imitans and Mycoplasma penetrans were given by J. Bradbury (University of Liverpool Veterinary School, Liverpool, United Kingdom) and D. Taylor-Robinson (St. Mary's Hospital, London, United Kingdom), respectively. Mycoplasma bovis 159B96 was kindly provided by R. Nickerson (Veterinary Laboratories Agency, Weybridge, United Kingdom). Mycoplasma hominis NCTC 10112 were tested in the study as potential inhibitors of L-arginine metabolism by the ATP-generating arginine dihydrolase pathway (19). They were considered potential analogues of L-arginine which might compete with it for uptake or subsequent metabolism. There are no previous reports of the effect of arginine analogues on mollicute growth.

Substrate oxidation experiments. To obtain cell suspensions, the growth of broth cultures was monitored by changes in opacity at 550 nm (Gallenkamp Viss-spec; Fisons Scientific Equipment) and cells were harvested towards the end of the exponential growth phase by centrifugation at 13,000 × g for 4 min. The cell pellets obtained were washed twice and resuspended in RH solution, which was prepared by dissolving 18 g of HEPES liter⁻¹ and 160 U of catalase ml⁻¹ (C-10; Sigma) in one-quarter-strength Ringer solution (Oxoid; pH 7.6). The optical density of cell suspensions (550 nm) was adjusted to 0.80, which is equivalent to approximately 200 μg of cell protein ml⁻¹ and a viable count of 8 × 10⁹ CFU ml⁻¹. The amount of protein was determined by the method of Markwell et al. (6).

Rates of oxygen uptake by cell suspensions were determined, as described previously (15), from changes in dissolved oxygen tension measured with an oxygen electrode system (Rank Brothers, Bottisham, Cambridge, United Kingdom) linked to a chart recorder and calibrated with air-saturated water (dissolved oxygen tension, approximately 210 nmol ml⁻¹). One millilitre of cell suspension was added to the electrode vessel, which was magnetically stirred and maintained at 37°C. The air-saturated suspension was then isolated from the air, and test substrates and inhibitors were added with a microsyringe. Except where otherwise stated, the initial concentrations of test substrates (glucose, pyruvate [Na salt], L-lactate [Na salt], and NADH) were 10 μM. In experiments with lysed cells, cells were lysed by addition of 5 μl of Triton X-100 per ml of cell suspension 2 min before the addition of test substrate and inhibitor (CB). Rates of oxygen uptake were determined from the rate of decline in DOT (11).

Viable counts. Viable counts were determined as described previously (16). The 95% confidence limits of counts were determined as described by Meynell and Meynell (10) and were, in all cases, within ±15% of the values given. All experiments were repeated; the data shown are mean values from two or more replicate experiments.

RESULTS

Inhibition by fluoride. In initial experiments the MICs of NaF for a metabolically diverse range of mycoplasmas varied from ≤0.2 to 2 g liter⁻¹ (Table 1). The most sensitive organisms tested were, as expected, M. felis (glucose fermenting) and M. fermentans (glucose fermenting and arginine hydrolyzing).
M. verecundum was relatively sensitive to NaF; this organism apparently does not oxidize organic acids, although the energy source(s) required for growth is unknown (13). A. laidlawii is able to oxidize organic acids and was relatively resistant to NaF.

Inhibition by IAA. The MICs of IAA for a metabolically diverse range of Mycoplasma spp. varied from only 0.1 to 0.3 g liter$^{-1}$ (Table 1). Surprisingly, and in contrast to results obtained with fluoride, the species most sensitive to IAA were those solely dependent upon the oxidation of organic acids for energy (M. agalactiae and M. bovis). The most resistant species were all able to use arginine.

Inhibition by FP. The MICs of FP for the test Mycoplasma spp., determined in agar medium, varied from 0.5 to 2 g liter$^{-1}$ (Table 1), except for M. verecundum, which was resistant (MIC of <3 g liter$^{-1}$). However, among these other strains, there was no obvious correlation of degree of sensitivity with metabolic type.

The presence of 5 g of FP liter$^{-1}$ did not reduce the growth yield of M. verecundum in broth, although maximum cell populations were reached after 72 h of incubation, compared with 48 h in the absence of FP. In contrast, cells of other species were killed; using an inoculum size equivalent to approximately 10$^7$ CFU ml of broth medium$^{-1}$, survivors were not detected after incubation for 48 h (M. arginini, M. bovis, M. fermentans, and M. gallinarum) or 8 h (M. penetrans and M. pirum). In the absence of FP, population sizes increased by 10- to 100-fold within 48 h.

Inhibition by CB. The MICs of CB for a range of Mycoplasma spp. and A. laidlawii, determined on agar plates, varied by more than 10-fold (Table 1). There was no reduction in the viable counts of A. laidlawii on plates containing 10 g of CB liter$^{-1}$, whereas, even with an inoculum of 10$^7$ CFU per plate, colonies of M. verecundum were not detected with only 1 g of CB liter$^{-1}$. There appeared to be no general association of degree of susceptibility to CB with dependence upon organic acid oxidation, since M. fermentans and M. verecundum (neither of which oxidizes organic acids) were among the more sensitive of the test species. Also, there was a large difference in sensitivity between M. penetrans and M. pirum (both are able to oxidize organic acids, ferment glucose, and hydrolyze arginine).

Effect of inhibitors on substrate oxidation by washed cell suspensions. The effect of the inhibitors on the oxidation of glucose and organic acids by various test species was also determined. The effects of CB on substrate oxidation in A. laidlawii, M. bovoculi, and M. imitans were compared. In A. laidlawii, CB at concentrations of up to 2.5 g liter$^{-1}$ (the highest tested) had no effect on the rate of glucose (2.8 mM) oxidation (equivalent to approximately 30 nmol of O$_2$ consumed · min$^{-1}$ · mg of cell protein$^{-1}$), when added either 5 min prior to addition of glucose or during glucose oxidation, after a constant rate of metabolism had been achieved. However, consistent with the observed effects of CB on growth (Table 1), a marked inhibition of glucose oxidation was seen in the test Mycoplasma species (Table 2).

The effect of CB on NADH oxidation was also determined. Experiments were carried out using M. imitans (glucose and organic acid oxidizing and relatively sensitive to CB) and the metabolically similar M. bovoculi. Rates of NADH oxidation by washed cell suspensions and lysed cells of the test organisms are shown in Table 3. In both M. imitans and M. bovoculi, NADH oxidase activity was inhibited by CB, although inhibition was much greater in whole cells.

Growth inhibition of arginine-hydrolyzing mycoplasmas. The potential inhibitors were screened using M. arginini, M. hominis, and M. orale as representative arginine-hydrolyzing species. In broth medium, d-arginine, which was included in the study as an analogue of L-arginine, appeared to be an effective energy source for arginine-utilizing mycoplasmas (Table 4). On agar medium, without added arginine, no zones of growth inhibition were evident around disks containing 0.05 mg of N$^\text{6}$-methyl-L-arginine or 2 mg of d-arginine, L-canavanine, L-lysine, methylamine, or L-ornithine. A low concentra-

<table>
<thead>
<tr>
<th>Species</th>
<th>Added substrate</th>
<th>CFU ml$^{-1}$ (after 24 h)</th>
<th>Culture optical density at 550 nm after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>M. arginini</td>
<td>None</td>
<td>4.8 × 10$^7$</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>d-Arginine</td>
<td>2.7 × 10$^8$</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>L-Arginine</td>
<td>2.4 × 10$^8$</td>
<td>0.058</td>
</tr>
<tr>
<td>M. gallinarum</td>
<td>None</td>
<td>8.0 × 10$^8$</td>
<td>0.079</td>
</tr>
<tr>
<td></td>
<td>d-Arginine</td>
<td>6.0 × 10$^8$</td>
<td>0.149</td>
</tr>
<tr>
<td></td>
<td>L-Arginine</td>
<td>5.8 × 10$^8$</td>
<td>0.212</td>
</tr>
</tbody>
</table>

TABLE 2. Effect of inhibitors on the oxidation of glucose and organic acids by mycoplasma cell suspensions in RH buffer$^a$

<table>
<thead>
<tr>
<th>Test species</th>
<th>Inhibitor (concn, g liter$^{-1}$)</th>
<th>Rate of oxygen uptake (% of rate without inhibitor)$^b$ with:</th>
<th>Glucose</th>
<th>Pyruvate</th>
<th>l-Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. penetrans</td>
<td>NaF (2.5)</td>
<td>&lt;15</td>
<td>60–80</td>
<td>Not tested</td>
<td></td>
</tr>
<tr>
<td>M. penetrans</td>
<td>IAA (2.5)</td>
<td>Not detected</td>
<td>15</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>M. bovis 159B96</td>
<td>FP (0.25)</td>
<td>Not utilized</td>
<td>0–17</td>
<td>5–11$^c$</td>
<td></td>
</tr>
<tr>
<td>M. bovoculi</td>
<td>CB (0.15)</td>
<td>51</td>
<td>31</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>M. imitans</td>
<td>CB (1.20)</td>
<td>63</td>
<td>31</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Cells were suspended in RH buffer, and inhibitors were added 5 min prior to substrate addition. Substrates were added at saturating concentrations for the test species.

$^b$ In experiments conducted simultaneously.

$^c$ Increased with increase in substrate concentration from 50 to 250 μM.

TABLE 3. Inhibition of NADH oxidase activity by CB in whole and lysed cells of M. bovoculi and M. imitans

<table>
<thead>
<tr>
<th>Cell prepn</th>
<th>Initial rate of oxygen uptake (nmol · min$^{-1}$ · mg of cell protein$^{-1}$)</th>
<th>% Inhibition by CB (0.25 g liter$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. bovoculi</td>
<td>Whole cells</td>
<td>296</td>
</tr>
<tr>
<td></td>
<td>Lysed cells</td>
<td>566</td>
</tr>
<tr>
<td>M. imitans</td>
<td>Whole cells</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Lysed cells</td>
<td>230</td>
</tr>
</tbody>
</table>
tion of N⁶-methyl-L-arginine was used, as the cost of this arginine analogue would prevent its routine use at a higher concentration. However, L-citrulline was inhibitory, and with 2-mg disks, zones of growth inhibition were 10 mm (M. orale) to >25 mm (M. arginini and M. hominis). This effect appeared to be specific for mycoplasmas dependent solely upon arginine hydrolysis for energy generation, as no inhibition zones were seen with M. gallinarum, M. penetrans, and M. pirum, which additionally metabolize organic acids and/or glucose.

The effects of certain inhibitors were also determined in broth medium to which arginine had been added to enable growth quantification by culture opacity. In these experiments, 50 mM L-citrulline, L-lysine, and L-ornithine had little effect on the growth of M. arginini or M. gallinarum after 48 h of incubation.

Inhibition by ionophores and inhibitors of proton-translocating ATPase. On agar medium, zones of growth inhibition around disks containing 2 mg of CCCP were evident for all test species: 2 mm for M. verecundum and 6 to 15 mm for M. arginini, M. bovis, M. fermentans, M. gallisepticum, and M. penetrans. In contrast to results obtained with CCCP (0.2 mg per disk), the use of monensin (2 mg per disk), DCCD (2 mg per disk), and nigericin (20 μg per disk) caused no growth inhibition of M. arginini and M. gallinarum. Nigericin is generally used in metabolic studies at only micromolar concentrations (21).

**DISCUSSION**

Selective inhibitors are widely used in isolation media for specific cell-walled bacterial groups. The identification of inhibitors acting selectively among mycoplasmas would enable the similar development of media for specific groups of *Mycoplasma* spp. The availability of such media would be expected to lead to improvements in mycoplasma detection rates, particularly for slower-growing species, from clinical specimens subject to contamination by commensal mycoplasmas. In addition, tolerance to inhibitors is the basis of many biochemical identification techniques. The range of biochemical tests for mollicute identification is small, and definitive identification tests are usually serological or PCR based. However, as the number of recognized *Mycoplasma* spp. is increasing rapidly, further biochemical tests would be useful in restricting the number of possible species to which a new isolate could belong.

Few previous studies have attempted to differentiate mollicutes on the basis of their tolerance to inhibitors. However, among species from the human oral cavity, *M. salivarium* was distinguished from *M. orale* by its ability to grow on medium containing 0.2 mM MnCl₂ (27). Similarly, *M. gallisepticum* was distinguished from certain avian species by its tolerance to Cu²⁺ (4). The basis of mollicute resistance to metal ions is unknown. In contrast, in this study, the aim was to use inhibitors for which the principal cell targets were likely to be energy-generating pathways. Thus, it appeared to be possible that the more susceptible species could be predicted from their major type(s) of energy metabolism.

Growth on an agar medium was generally used to determine growth effects. The nature of the medium used and the type of test would be expected to influence the results obtained, and this was evident in some cases. For example, citrulline inhibited the growth of arginine-hydrolyzing species on agar but not in broth medium supplemented with arginine. Conversely, L-canavanine (30 mM) and N⁶-methyl-L-arginine inhibited the growth rates of arginine-hydrolyzing species in broth culture but did not appear to affect the number of colonies appearing on agar.

Overall the greatest differentiation of the test species was obtained with fluoride, IAA, FP, CB, citrulline, and CCCP. Fluoride and IAA were predicted to be most effective against mycoplasmas solely dependent upon the fermentation of sugars to lactate for energy generation. Fluoride sensitivity did differentiate the test species according to their metabolic type: species capable of oxidizing organic acids were resistant; species able only to ferment sugars to lactate and/or to hydrolyze arginine were sensitive. The fluoride sensitivity of species dependent upon arginine hydrolysis for energy generation was unexpected and may suggest that fluoride is an inhibitor of enzymes in this pathway. However, other explanations are also possible; for example, in certain nonfermentative mycoplasmas, enzymes of the glycolytic pathway may be required for the synthesis of sugars from pyruvate. In contrast, IAA was most effective against organic acid-oxidizing strains, and strains able to hydrolyze arginine were relatively resistant. Metabolic experiments using *M. penetrans* and *M. pirum* showed that the metabolism of glucose was more susceptible to inhibition than that of pyruvate. This is consistent with the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase being a target of IAA inhibition (9); however, the high resistance of these species to IAA suggests that there may be other, more significant targets.

The presumed target for FP was the pyruvate dehydrogenase complex (PDHC). PDHC has been reported in all *Acholeplasma* and *Mycoplasma* species studied, including *M. verecundum* (3, 7). Nevertheless, it appeared to be possible that the role of PDHC might be greater in organic acid-oxidizing species, especially those that appear to be dependent solely on the oxidation of pyruvate for ATP synthesis. The resistance of the test strains to FP varied widely. However, there was no obvious correlation with metabolic type except that *M. verecundum* was highly resistant; this organism, a rare isolate from cattle, appears to be unable to ferment sugars, hydrolyze arginine, or oxidize organic acids (12).

It appeared that, while all test *Mycoplasma* spp., particularly *M. verecundum*, were sensitive to CB, the test species *A. laidlawii* was resistant. These two genera are differentiated by the cellular location of NADH oxidase. In acholeplasmas, the enzyme is predominantly membrane bound; in mycoplasmas, it is generally stated to be cytoplasmic (17), although *M. bovoculi* appears to be exceptional in having surface-bound NADH oxidase activity (2). However, while CB significantly inhibited NADH oxidation by whole *M. bovoculi* cells, among the test *Mycoplasma* spp. used, *M. bovoculi* was not particularly sensitive to CB. Thus, the mechanism of CB inhibition of growth is unclear.

A range of potential inhibitors of arginine-hydrolyzing mycoplasmas were tested. L-Citrulline appeared to be the most useful and selectively inhibited those test species dependent solely upon arginine for energy generation. In addition, preliminary experiments (data not shown) indicate that combinations of other test inhibitors may also be effective. For exam-
ple, l-ornithine plus either monensin, nigericin, or DCCD inhibited the growth of M. arginini (arginine hydrolyzing) but not M. gallinarum (arginine hydrolyzing and organic acid oxidizing). Interestingly, d-arginine, which was included in the study as an analogue of l-arginine, appeared to be an effective energy source for arginine-utilizing mycoplasmas. The ability of d-arginine to stimulate mycoplasma growth has not previously been reported, although Smith et al. (23) showed that it was hydrolyzed by the M. arthritidis arginine deiminase but at a lower rate than l-arginine.

In conclusion, this study has identified a range of inhibitors which might be used in the development of identification tests or selective media for specific mycoplasmas. The inhibitors were chosen because they were likely to affect known enzyme activities associated with particular types of energy-generating pathway. For most of the inhibitors, patterns of mycoplasma growth inhibition suggested that additional, unknown metabolic activities were also inhibited. Nevertheless, the results suggest that certain metabolic types of mycoplasmas may be selected for or against by using single inhibitors or combinations of inhibitors. For example, organisms dependent upon arginine as an energy source might be selectively inhibited by using fluoride and/or citrulline and selected for by using IAA. Similarly, the organic-oxidizing species M. agalactiae and M. bovis might be selectively inhibited by using IAA and selected for using a medium containing added pyruvate (as an energy source) (25) with fluoride and citrulline. The results also suggest that selective media for the metabolically unusual M. ve- recundum might be based on FP.

ACKNOWLEDGMENT
This work was supported by a grant from the Turkish High Educational Council to S.A.K.

REFERENCES