Hydrolysis of Aesculin and Phosphatase Production by Members of the Order *Mycoplasmatales* Which Do Not Require Sterol

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Two bacteriological methods, one to demonstrate phosphatase production and the other to demonstrate aesculin hydrolysis, were tested on sterol-requiring *Mycoplasma* and sterol-nonrequiring *Acholeplasma* strains to determine their utility in differentiating species. The phosphatase test, which gave weak and variable reactions with most of the sterol-nonrequiring strains, was judged ineffectual for differentiating these strains. The aesculin test distinguished all *Mycoplasma* strains and *Acholeplasma granularum*, both of which gave negative reactions, from most *Acholeplasma laidlawii* strains and from *Acholeplasma axanthum*, which gave positive reactions.

The bacteriological characterization of *Mycoplasma* species by standardized methods was recently described by Aluotto et al. (1). Of the 22 species studied by these authors, half displayed distinctive patterns of biochemical activities. The remaining strains fell into groups containing several species with biochemically similar patterns. One such group included *Mycoplasma gallisepticum*, *Mycoplasma histotropicum* [now regarded as a strain of *Mycoplasma pulmonis* (3)], *Mycoplasma granularum*, and *Mycoplasma laidlawii*. Application of the test of Razin and Tully (6) for determining cholesterol requirement readily permits differentiation of the sterol-requiring species in this group from the two sterol-nonrequiring species, *M. granularum* and *M. laidlawii*. [For these and other sterol-nonrequiring species, a new generic name, *Acholeplasma*, was recently proposed (4) and will be employed hereafter in this report.] *Acholeplasma granularum* and *Acholeplasma laidlawii* can be differentiated from each other by serological methods and gel electrophoretic patterns (7, 8), but a simple biochemical test for distinguishing them has been lacking. These strains react somewhat differently from each other when tested for phosphatase production and hemolysis; however, neither test alone is capable of distinguishing the species. The phosphatase reaction was reported to be weakly positive for *A. laidlawii*

type A but negative for *A. laidlawii* type B and *A. granularum* (1). The type of hemolytic reaction for various *A. laidlawii* strains was examined (*unpublished data*) and found to vary from strain to strain. Now that a new and distinct species of *Acholeplasma* has been recognized (9), further biochemical tests for characterizing and differentiating them are all the more desirable.

A survey of biochemical reactions applicable to the problems of *Mycoplasma* and *Acholeplasma* characterization and differentiation pointed to aesculin hydrolysis as a possibility. A group of more than 22 types or representative strains of *Mycoplasma* and 32 *Acholeplasma* strains were, therefore, examined for ability to hydrolyze aesculin. In addition, the test for phosphatase activity was reexamined on all *Acholeplasma* strains to clarify and expand the findings of Aluotto et al. (1).

MATERIALS AND METHODS

Organisms and maintenance media. The organisms used by the authors were obtained from the American Type Culture Collection, from J. G. Tully, and from W. A. Clyde, Jr. Additional organisms from the collection of E. A. Freundt were tested by T. Sander at the University of Aarhus, Denmark. Strain designation and source of each organism are given in Table 1.

All cultures were grown in Heart Infusion Broth (Difco) enriched with 20% (v/v) sterile, heated (56 C for 30 min) horse serum (Microbiological Associates,

TABLE 1. *Hydrolysis of aesculin by Mycoplasma and Acholeplasma species*

Species	Strain	Source	Reaction
<i>M. agalactiae</i>	PG2	Freundt ^a	— ^b
<i>M. arthritidis</i>	PG6 (Preston)	ATCC 19611 ^c	—
<i>M. bovigenitalium</i>	PG11 (B2)	ATCC 19852	—
<i>M. canis</i>	PG14 (C55)	ATCC 19525	—
<i>M. edwardii</i>	PG24 (C21)	ATCC 23462	—
<i>M. felis</i>	CO	ATCC 23391	—
<i>M. fermentans</i>	PG18 (G)	ATCC 19989	—
<i>M. gallinarum</i>	PG16 (Fowl)	ATCC 19708	—
<i>M. gallisepticum</i>	PG31 (X95)	ATCC 19610	—
<i>M. gateae</i>	CS	ATCC 23392	—
<i>M. hominis</i>	PG21 (H50)	ATCC 23114	—
<i>M. hyorhinis</i>	BTS-7	ATCC 17981	—
<i>M. iners</i>	PG30 (M)	ATCC 19705	—
<i>M. maculosum</i>	PG15 (C27)	ATCC 19327	—
<i>M. mycoides</i> subsp. <i>capri</i>	PG3	Freundt	— ^b
<i>M. mycoides</i> subsp. <i>mycoides</i>	PG1	Freundt	— ^b
<i>M. neurolyticum</i>	Sabin A	ATCC 19988	—
<i>M. orale</i> 1	CH19299	ATCC 23714	—
<i>M. orale</i> 2	CH20247	ATCC 23636	—
<i>M. pneumoniae</i>	FH	ATCC 15531	—
<i>M. pulmonis</i>	PG34 (Ash)	ATCC 19612	—
<i>M. salivarium</i>	PG20 (H110)	ATCC 23064	—
<i>Mycoplasma</i> sp.	C30KS-1	ATCC 15718	—
<i>Mycoplasma</i> sp.	Navel	ATCC 15497	—
<i>Mycoplasma</i> sp.	67-166	ATCC 23243	—
<i>M. spumans</i>	PG13 (C48)	ATCC 19526	—
<i>A. axanthum</i>	S-743	ATCC 25176	+
<i>A. granularum</i>	BTS-39	ATCC 19168	—
<i>A. granularum</i>	BTS-39	Tully ^d	—
<i>A. granularum</i>	39D	Tully	—
<i>A. granularum</i>	31B-1	Tully	—
<i>A. granularum</i>	Friend	Tully	—
<i>A. laidlawii</i>	Laidlaw A	ATCC 14089	+
<i>A. laidlawii</i>	Laidlaw B	ATCC 14192	+
<i>A. laidlawii</i>	PG8 (Sewage A)	ATCC 23206	+
<i>A. laidlawii</i>	PG9 (Sewage B)	ATCC 23217	+
<i>A. laidlawii</i>	Laidlaw A (F1)	Freundt	+ ^b
<i>A. laidlawii</i>	Laidlaw B (F8)	Freundt	+ ^b
<i>A. laidlawii</i>	Algen	Tully	—
<i>A. laidlawii</i>	Mist	Tully	+
<i>A. laidlawii</i>	L	Tully	+
<i>A. laidlawii</i>	PG10 (B-15)	Tully	+
<i>A. laidlawii</i>	643-N	Tully	+
<i>A. laidlawii</i>	J-18S	Tully	+
<i>A. laidlawii</i>	Haig 179-L (clone 1)	Tully	—
<i>A. laidlawii</i>	Haig 179-L (clone 6)	Tully	—
<i>A. laidlawii</i>	PG47 (3M-152)	Tully	+
<i>A. laidlawii</i>	H3-10	Tully	+

TABLE 1. *Hydrolysis of aesculin by Mycoplasma and Acholeplasma species (Con't.)*

Species	Strain	Source	Reaction
<i>A. laidlawii</i>	STR	Tully	+
<i>A. laidlawii</i>	OR	Tully	+
<i>A. laidlawii</i>	Flamm 583	Tully	+
<i>A. laidlawii</i>	R-4	Tully	+
<i>A. laidlawii</i>	Granoff 8	Tully	—
<i>A. laidlawii</i>	TC7277	Tully	+
<i>A. laidlawii</i>	R-1	Tully	+
<i>A. laidlawii</i>	PG5	Tully	+
<i>A. laidlawii</i>	B4	Tully	+
<i>A. laidlawii</i>	KHS	Tully	+
<i>A. laidlawii</i>	S	Clyde ^e	+
<i>A. laidlawii</i> subsp. <i>inocuum</i>	A4H3-10	ATCC 25937	+

^a Cultures in the collection of E. A. Freundt, University of Aarhus, Aarhus, Denmark.

^b Test conducted by Torben Sander at University of Aarhus, Aarhus, Denmark.

^c Cultures received from the American Type Culture Collection, Rockville, Md.

^d Cultures received from Joseph G. Tully, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

^e Cultures received from Wallace A. Clyde, Jr., University of North Carolina School of Medicine, Chapel Hill, N.C.

Inc., Bethesda, Md.) and 10% (v/v) of a 25% (w/v) fresh yeast extract solution (Grand Island Biological Co., Grand Island, N.Y.). Incubation was at 37 C for 24 hr.

Test media and methods. The phosphatase test method was essentially that of Aluotto et al. (1). The medium was composed of Heart Infusion Agar (Difco), 20% (v/v) sterile, heated (56 C for 30 min) horse serum, 10% (v/v) of a 25% (w/v) fresh yeast extract solution, and 1% (v/v) of a sterile 1% (w/v) stock solution of the sodium salt of phenolphthalein diphosphate (Sigma Chemical Co., St. Louis, Mo.). Plates were inoculated in triplicate with 24-hr-old broth cultures and incubated at 37 C. Readings were made after 3, 7, and 14 days of growth. The presence of free phenolphthalein was tested for on each occasion by flooding the surface of one plate of the triplicate set with 5 N NaOH. A red color in the 3- or 7-day-old plate, with the coloration becoming more intense in the 7- or 14-day-old plate, respectively, was recorded as positive. A faint red color produced only in the 14-day-old plate was recorded as weakly positive.

Aesculin hydrolysis was determined by a modification of the method described by Cowan and Steel (2) using Heart Infusion Agar (Difco) supplemented with 20% (v/v) sterile, heated (56 C for 30 min) horse serum, 10% (v/v) of a 25% (w/v) fresh yeast extract solution, 0.1% (w/v) aesculin (BBL), and 0.05% (w/v) ferric citrate (Fisher Scientific Co., Fair Lawn, N.J.). Plates were inoculated with 24-hr-old broth cultures, incubated at 37 C, and read at 3, 7, and 14 days. The development of a black color, whether intense or faint, in the test medium was indicative of a positive reaction.

Controls. Test cultures were examined for freedom from contamination by inoculation into Thioglycolate fluid medium (BBL) to which 20% (v/v) sterile, heated horse serum had been added, onto an agar gel of the broth growth medium, and onto a blood-agar plate. Gram stained preparations of all cultures were examined for contaminants.

Organisms known to give positive or negative reactions were included as controls for both the phosphatase and the aesculin tests. To ensure that test organisms and not media constituents were responsible for positive reactions, test media were inoculated with the sterile infusion-serum-yeast broth medium.

For the aesculin test, cultures were also grown on test media from which only aesculin was excluded, from which only ferric citrate was excluded, and from which both substrates were excluded. In all cases, reactions were seen only on the complete test medium containing both substrates.

RESULTS AND DISCUSSION

Phosphatase production. A few *Acholeplasma* strains gave consistent phosphatase results. *A. granularum* strains 31B-1 and Friend were negative; *A. laidlawii* type A strains, Laidlaw A, PG8, Laidlaw A (F1), OR, Flamm 583, and R-4, and type B strains, Granoff 8 and TC7277, were consistently weakly positive. However, most *Acholeplasma* strains reacted with marked inconsistency; they exhibited weakly positive reactions some of the time and negative reactions at other times. The explanation for such variation is not clear. The positive

control organism, *Mycoplasma arthritidis* strain Preston, like the other *Mycoplasma* species that have exhibited a positive reaction, produced an intense red color by the seventh day of incubation. The negative control organism, *Mycoplasma gallisepticum* strain S6, was negative at each reading. Uninoculated control plates containing the test substrate were negative at 7 days but did occasionally exhibit a faint reddening after 14 days of incubation, indicating partial breakdown of the test substrate with liberation of some free phenolphthalein. Thus, the weakly positive reactions that appeared for a majority of the *Acholeplasma* strains might have been false positive reactions, although the appropriate control plates were negative at the time. Detection of phosphatase activity in *Acholeplasma* strains by the particular method here used cannot be considered reliable. Since only 2 of 32 of those strains gave frankly negative reactions, and since an explanation for the late and variable reactions of the others is required, production of phosphatase by these organisms needs further investigation.

Aesculin hydrolysis. The results of tests for hydrolysis of aesculin are given in Table 1. Twenty-six *Mycoplasma* strains representing 22 or more species were negative. Also negative were four *A. granularum* strains, including two different culture lines of BTS-39. Twenty-four strains of *A. laidlawii* and the new sterol-nonrequiring species *A. axanthum* (9) gave consistently positive reactions for aesculin hydrolysis. Most reactions developed by the third day, and only a few strains required as long as 7 days to produce the color change. Three remaining *A. laidlawii* strains, Algen, Haig 179-L (clones 1 and 6), and Granoff 8, were judged negative, although a very slight blackening of the test medium could occasionally be seen at the 14-day reading. Tully and Razin (7) noted that two of these strains showed atypical fermentative results, i.e., Haig 179-L attacked neither glucose nor mannose, and Granoff 8 attacked both carbohydrates in contrast to Algen and other *A. laidlawii* strains that attacked glucose but not mannose. By fluorescent-antibody techniques (7) and by gel electrophoretic analysis (8), Haig 179-L, Granoff 8, and Algen were identified as *A. laidlawii* strains. Whether their atypical behavior on the glucoside aesculin and perhaps on the above-mentioned carbohydrates is meaningful for taxonomic purposes is not yet clear.

Apart from the above exceptions, hydrolysis of aesculin appears to be characteristic for two *Acholeplasma* species: *A. laidlawii* and *A. axanthum*. Henrikson and Smith (5) examined

strains of three *Mycoplasma* species, one fermentative, sterol-nonrequiring (*A. laidlawii*), one fermentative, sterol-requiring (*M. gallisepticum*), and one nonfermentative, sterol-requiring (*M. hominis*), for glucosidase activity by using several glucosides other than aesculin. They found that *M. hominis* hydrolyzed none of the glucosides tested, whereas *A. laidlawii* and *M. gallisepticum* hydrolyzed certain of the β -glucosides. In contrast, the strain of *M. gallisepticum* studied here showed no glucosidase activity for aesculin.

Tully and Razin (8) recently showed that *A. laidlawii* and *A. granularum* could be differentiated from *A. axanthum* by the ability of the two former species to produce pigmented carotenoids, an ability that the latter species lacked. Tully and Razin (8), therefore, suggested that species differentiation among sterol-nonrequiring strains might be based on (i) serological characteristics, (ii) unique properties of lipid metabolism such as lack of pigmented carotenoids, and (iii) additional nutritional and biochemical distinctions. Based on carotenoid production and aesculin hydrolysis alone, each of the three species presently comprising the genus *Acholeplasma* appears distinct. *A. laidlawii* is aesculin positive, carotenoid positive; *A. granularum* is aesculin negative, carotenoid positive; and *A. axanthum* is aesculin positive, carotenoid negative. Thus, the ability to hydrolyze aesculin provides a simple and useful differential biochemical test for the species of the genus *Acholeplasma* inasmuch as all *Mycoplasma* species examined to date are negative in this test.

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