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Substrate Utilization in Defined Media

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Substrate utilization in defined media for two flower spiroplasmas (*S. floricola* and FS SR-3) and honeybee spiroplasma (HBS AS-576) was investigated. Glucose, fructose, and mannose were utilized by all three spiroplasmas. In addition, HBS (AS-576) could ferment trehalose; FS (SR-3), sucrose; and *S. floricola,* trehalose, sucrose, and raffinose. The three spiroplasmas varied greatly in growth requirements for amino acids. Only *S. floricola* utilized arginine. HBS (AS-576) required at least one purine and one pyrimidine base for growth, while both flower spiroplasmas grew with only one base in the medium. Oleic acid, cholesterol, and BSA were essential to all three spiroplasmas. Palmitic acid, which was non-essential, promoted growth significantly.

INTRODUCTION

Although more than 60 isolates of spiroplasmas have been successfully cultivated, including some pathogenic to plants, insects, and vertebrate animals, and some not yet shown to be associated with any disease, the precise nutritional requirements for these isolates is still unclear [1,2,3].

Previous studies [4-12; Chang CJ, Chen TA: Phytopathology 69:1024, 1979; Lee IM: Ph.D. thesis, University of California, Riverside, 1977; Lee IM, Davis RE: Phytopathlogy News 12:215, 1978; Malloy KM, Chen TA: Phytopathology 70:465, 1980] using undefined media attempted to show the comparative capabilities of spiroplasmas to utilize various nutrients. The results were frequently conflicting. For example, Saglio et al. [11] showed that *Spiroplasma citri* was unable to metabolize arginine, whereas Townsend [12] reported that with a limited supply of a fermentable carbohydrate *S. citri* was able to metabolize arginine. Igwegbe et al. [8] showed that *S. citri* (Morocco isolate) could ferment mannose, whereas Davis' result [4] showed otherwise. Thus data on substrate utilization and nutrient requirement of spiroplasmas based on cultivation in a complex medium are very difficult to interpret.

We report herein the precise nutritional requirements for carbohydrates, amino acids, nitrogenous bases, ribonucleosides, fatty acids, and cholesterol of two flower spiroplasmas (*S. floricola* and FS SR-3) and honeybee spiroplasma (HBS AS-576) cultured in a chemically defined medium [13]. Substrate utilization in undefined and defined media is discussed.

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MATERIALS AND METHODS

Spiroplasmas

Spiroplasma floricola (ATCC 29989) [6], FS (SR-3) (ATCC 33095, and HBS (AS-576) (ATCC 29416) were used throughout this study. They were maintained in CC-494 medium at 31 ± 1°C and subcultured every two, four, and three days respectively [13].

Spiroplasma Growth

Cell growth and yields were determined as previously reported [13]. At least 10 passages in a given test medium were made before results were considered positive. Growth yields were measured at two, four, and three days after inoculation for *S. floricola,* FS (SR-3), and HBS (AS-576), respectively, unless stated otherwise.

Carbohydrate Utilization

Each of the following carbohydrates was tested as the sole carbohydrate source at a concentration of 0.8 percent in CC-494 [13]: D-glucose, D-fructose, D-galactose, D-mannose, D-mannitol, D-sorbitol, α-lactose, maltose, sucrose, D-trehalose, D-raffinose, and starch. All stock solutions were 20 percent, except for lactose and starch which were 10 percent and were filter-sterilized (millipore filter 0.45 μm). Ribose and deoxyribose, which were the original constituents of medium CC-494, were deleted in this study.

Amino Acid Requirements

The following ten combinations were grouped from 20 amino acids (all L-form except glycine) according to their structural differences, their roles in the biosynthesis of sugars, and essentiality for higher animals: glucogenic (14 amino acids); ketogenic (three amino acids); glucogenic and ketogenic (three amino acids); essential (ten amino acids); nonessential (ten amino acids); uncharged nonpolar R group (eight amino acids); uncharged polar R group (seven amino acids); positively charged polar R group (three amino acids); negatively charged polar R group (two amino acids); and one containing all 20 amino acids. The concentration of each amino acid was the same as that in medium CC-494 [13].

Arginine Metabolism

Arginine at a concentration of 0.42 percent [12] was used in medium CC-494 with the complete exclusion of glucose, ribose, and deoxyribose. Growth in this medium and shifting of medium pH upward are taken as an indication of the organism's use of the arginine dehydrolase pathway for energy production.

Nitrogenous Base Utilization

The free bases adenine, guanine, cytosine, thymine, and uracil were used to replace nucleosides, deoxyribonucleosides (including 5-methy-deoxycytidine), and UTP which were originally in medium CC-494. The following ten combinations were grouped and tested: adenine (0.0048 percent) + guanine (0.003 percent) + cytosine (0.003 percent) + uracil (0.003 percent) + thymine (0.003 percent); guanine (0.003 percent) + cystosine (0.003 percent); adenine (0.0048 percent) + thymine (0.003 percent) + uracil (0.003 percent); adenine (0.0048 percent) + guanine (0.003 percent); cytosine (0.003 percent) + thymine (0.003 percent) + uracil (0.003

percent); adenine (0.0096 percent); guanine (0.0048 percent); cytosine (0.0048 percent); thymine (0.0048 percent); and uracil (0.0048 percent).

Ribonucleoside Requirement

The same ten combinations as used in the free nitrogenous base study were replaced with corresponding ribonucleosides. The concentration of each nucleoside was the same as that used in the original medium CC-494 [13]. Deoxyribonucleosides (including 5-methy-deoxycytidine) and UTP, which were constituents of CC-494, were excluded.

Fatty Acids, Cholesterol, and BSA

Based on the lipid composition in medium CC-494 [13], the following four groups were tested: (*1*) without cholesterol, (*2*) without Tween 40 and palmitic acid, (*3*) without Tween 80 and oleic acid, and (*4*) without bovine serum albumin. The concentration of each ingredient remained the same as that in medium CC-494 [13].

RESULTS

Carbohydrate Utilization

The three spiroplasma strains showed differences in metabolizing various carbohydrates. All could ferment glucose, fructose, and mannose. In addition, HBS (AS-576) utilized trehalose, *S. floricola* utilized sucrose, trehalose, and raffinose, and FS (SR-3) utilized sucrose. None could utilize galactose, mannitol, sorbitol, lactose, maltose, or starch. Glucose was the best carbohydrate source for all the spiroplasmas. There were significant differences in yield among the spiroplasmas growing in media supplemented with other carbohydrates (Table 1).

Amino Acid Metabolism

Both *S. floricola* and FS (SR-3) grew in any of the ten amino acid combinations, whereas HBS (AS-576) grew only in four: (*1*) 20 amino acids, (*2*) glucogenic amino acids, (*3*) nonessential amino acids, and (*4*) uncharged polar R group amino acids.

The medium supplemented with 20 amino acids supported the highest growth for all three spiroplasmas. Generally, deletion of amino acids from the medium resulted in lower yield. For example, HBS (AS-576) reached 2.46×10^9 cells/ml with 20

TABLE 1
Growth Comparison of HBS (AS-576), *S. floricola*, and FS (SR-3) in Various Carbohydrates[a]

Carbohydrates	Growth (cells/ml)		
	HBS (AS-576)	*S. floricola*	FS (SR-3)
Glucose	2.38×10^9	2.71×10^9	2.23×10^9
Fructose	2.00×10^9	2.33×10^9	1.93×10^9
Mannose	3.62×10^{8}[b]	1.20×10^9	1.51×10^9
Sucrose	–[c]	1.60×10^9	1.21×10^9
Trehalose	1.51×10^9	7.84×10^8	–
Raffinose	–	7.99×10^7	–

[a]Other tested nonutilizable carbohydrates to all three spiroplasma are galactose, mannitol, sorbitol, lactose, maltose, and starch.

[b]Growth reached 1.53×10^9 cells/ml in the second transfer.

[c]No growth

amino acids compared with 1.03×10^8 cells/ml when only seven uncharged polar R group amino acids were supplied.

Arginine Metabolism

Based on cell growth and pH change of the culture medium it was determined that *S. floricola,* among the three strains tested, could obtain energy through an arginine dihydrolase pathway.

Growth of *S. floricola* in media containing either glucose or arginine reached its peak in two and five days, respectively. *S. floricola* grew much more slowly in the arginine medium; its yield was reduced almost fivefold.

Nitrogenous Base Requirement

The requirement for nitrogenous bases differed between the two flower spiroplasmas and honeybee spiroplasma. HBS (AS-576) required at least one free pyrimidine base and one free purine base. On the other hand, any pyrimidine or purine base could support the growth of *S. floricola* and FS (SR-3).

Growth of the three spiroplasmas varied significantly with different combinations of nitrogenous bases. The highest yield was obtained when all five nitrogenous bases were added. Growth in the medium containing guanine and cytosine was significantly greater than that containing adenine, thymine, and uracil.

Ribonucleoside Requirement

Similar results were obtained when five ribonucleosides were used to replace the corresponding five nitrogenous bases. HBS (AS-576) required at least one ribonucleoside from a pyrimidine base and one from a purine base, whereas *S. floricola* and FS (SR-3) grew in a medium supplemented with any single pyrimidine or purine ribonucleoside.

The medium supplemented with all five ribonucleosides supported the highest growth for the three spiroplasmas. The addition of guanosine and cytidine resulted in higher yields than that of adenosine, thymidine, and uridine. Other combinations resulted in significantly different and reduced yields for the two flower spiroplasmas.

Fatty Acids, Cholesterol, and BSA Requirement

All three spiroplasmas have the same requirements for fatty acids, cholesterol, and BSA. Oleic acid, cholesterol, and BSA were found to be essential. Although palmitic acid was not required, it promoted significant growth of the three spiroplasmas.

DISCUSSION

Carbohydrate Utilization

This study indicated that the spiroplasmas of distinct serogroups show differences in the utilization of carbohydrates. Of the twelve carbohydrates tested, *S. floricola* could utilize six whereas HBS (AS-576) and FS (SR-3) could each utilize four. A possible explanation is that *S. floricola* has more enzymes for carbohydrate utilization than the other two spiroplasmas.

Considering that trehalose is the major disaccharide found in the hemolymph of most of the insect habitats of HBS, it is not surprising that this spiroplasma could cleave trehalose into two glucose residues. Therefore, it is quite possible that *S.*

floricola, at some time in its life cycle, may also reside in the hemolymph of an insect.

Both *S. floricola* and FS (SR-3) were originally isolated from the surfaces of flowers. It has been suspected that the two spiroplasmas multiply in the nectaries of flowers, but no direct evidence of this has ever been reported. The ability of *S. floricola* and FS (SR-3) to ferment sucrose is consistent with this.

Our results contradict earlier reports on carbohydrates utilization studies. For example, HBS (AS-576) was reported to use glucose, fructose, maltose, trehalose, and starch [Malloy KM, Chen TA: Phytopathology 70:465, 1980] but not mannose [4]. Since in our defined medium the presence or absence of a particular sugar can be precisely controlled, different results of carbohydrate utilization obtained from undefined and chemically defined conditions should be expected. Recently, Malloy and Chen [Phytopathology 71:892, 1981] showed that di-, oligo-, and polysaccharides are converted to glucose when incubated with horse serum or serum fraction in the absence of spiroplasma. Therefore, the validity of carbohydrate utilization as a criterion for mycoplasma taxonomy when using undefined media is questionable.

Amino Acid and Arginine Metabolism

It is possible that free amino acids are present in the complex components of undefined culture media because the three spiroplasmas required all 20 amino acids for maximum growth in our defined medium. Cell yields decreased with reduced amino acids supplements. This is not surprising since the amino acids are important metabolic building blocks for spiroplasmas which carry a minimum genome size. However, it was unexpected that both *S. floricola* and FS (SR-3) grew in a medium supplemented with only two negatively charged polar R group amino acids (aspartic and glutamic acids) or with any of the ten amino acid combinations used in this study. This suggests that they are able to transaminate amino acids readily. On the other hand, HBS (AS-576) required at least seven uncharged polar R group amino acids in the medium, indicating a lesser transamination ability. Positive results have been reported for HBS (AS-576) and *S. floricola* [4] for arginine metabolism. In our defined medium, however, only *S. floricola* utilized arginine but growth was much less than that obtained with glucose as the energy source. Since ATP production from arginine catabolism is less than that through glycolysis, the arginine dihydrolase pathway may function as a minor energy-generating system for *S. floricola.*

Like carbohydrate utilization, studies on arginine metabolism obtained from defined and undefined conditions produced conflicting results. Previous studies [4,7,12] indicated that minute amounts of glucose were required for arginine metabolism of *S. citri.* In our defined medium, however, *S. floricola* required a period of adjustment before adapting to the arginine medium while HBS (AS-576) and FS (SR-3) did not survive through the first passage.

Nitrogenous Base and Ribonucleoside Requirement

Our studies indicated that spiroplasmas, like other mycoplasmas, lack the orotic acid pathway for pyrimidine synthesis and the enzymatic pathway for *de novo* synthesis of purine bases [14].

HBS (AS-576) could only interconvert bases within the same group (either purine and pyrimidine) whereas *S. floricola* and FS (SR-3) could convert not only within groups but also between groups. HBS (AS-576) required the same base precursors as

Mycoplasma mycoides subsp. *mycoides* [15] and *Acholeplasma laidlawii* strain B [16]. *S. floricola* and FS (SR-3) require fewer base precursors for nucleic acid synthesis than any of the above organisms. Since the biosynthesis of purine and pyrimidine bases are different, it is very difficult to explain the growth of flower spiroplasmas in a medium in which only single bases or ribonucleosides were supplemented.

Deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxycytidine, and 5-methyl-deoxycytidine), ribose, and deoxyribose are constituents of medium CC-494 [13]. In both nitrogenous base and ribonucleoside utilization studies they were therefore deleted from the medium. Thus our results have clearly shown that spiroplasmas are able to synthesize ribose and deoxyribose from other fermentable carbohydrates and make deoxynucleotides from either nitrogenous bases or ribonucleosides.

Fatty Acids, Cholesterol, and BSA

The requirement for sterols by spiroplasmas and mycoplasmas has been recognized universally. Cholesterol is supplied from the serum in the culture media. Beside sterols, a number of glycerides, free fatty acids, phospholipids, and proteins also play important roles in spiroplasma nutrition. Only in the development of chemically defined media [13] have we realized the precise importance of these active chemical components from the serum.

In our medium CC-494, horse serum was replaced by a combination of palmitic acid, oleic acid, cholesterol, and BSA. CC-494 supported excellent growth of *S. floricola,* FS (SR-3), and HBS (AS-576). We found that oleic acid, cholesterol, and BSA were required by the three spiroplasmas. Palmitic acid was not essential but promoted significantly greater growth. This confirms the previous suggestion that spiroplasmas are incapable of *de novo* biosynthesis of long-chain fatty acids and cholesterol for their cell membranes from acetyl-CoA [14].

Using defined medium, we are convinced that spiroplasmas do require cholesterol. Such requirement is a taxonomic criterion for the family spiroplasmataceae.

Apparently spiroplasmas need BSA in the medium to act as a carrier and detoxifier for the free fatty acids [17]. Whether BSA also functions directly as a nutrient, e.g., as a nitrogen source, requires further investigation.

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