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Phytopathogenic Fastidious Prokaryotes: Cultivation, Nutrition, and Metabolism

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ABSTRACT

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Fastidious prokaryotes that cause diseases of plants can be divided into two groups: prokaryotes with cell walls and prokaryotes without cell walls. They are considered as fastidious because they share at least one common feature that is distinct from most of other phytopathogenic bacteria. The unique feature is this they either require specific and enriched medium to grow or are yet to be cultured. Among the walled prokaryotes, three groups have been recognized: *Xylella fastidiosa*, often referred to as xylem-limited bacteria or rickettsia-like bacteria; *Clavibacter xyli* subsp. *xyli* and *C. xyli* subsp. *cynodontis*, and phloem-limited bacteria (PLB). Of the wall-free prokaryotes, spiroplasmas and phytoplasmas (newly proposed name for mycoplasma-like organisms) are two major groups. Media are accessible for the isolation and cultivation of most species of *Xylella, Clavibacter,* and *Spiroplasma,* yet no medium is available for the *in vitro* growth of PLB and phytoplasmas. Almost all media for growth of spiroplasmas contain three common ingredients-PPLO broth base, yeast extract, and animal serum-all of which are found in conventional mycoplasma (mollicute) culture media. Other supplements include tryptone, peptone, insect tissue culture medium (Schneider's *Drosophlia* medium), animal tissue culture medium (TC 199, CMRL-1066), and yeastolate. Major components in media for growth of *Xylella* and *Clavibacter* include peptone, soytone, citrate and succinate, amino acids, hemin chloride or ferric pyrophosphate, and bovine serum albumin, activated charcoal, or starch. A few defined media are ready for growth of several *Spiroplasma* spp. Whereas only one for *Xylella* and none for *Clavibacter*. Defined media provide opportunity for precise study of the nutritional requirements of the respective prokaryotes. *Spiroplasmas* require inorganic salts, carbohydrates, amino acids, nitrogenous bases or nucleosides, vitamins, and lipids which include fatty acids, sterols, and phospholipids. *X. fastidiosa* needs inorganic salts, tricarboxylic acids, and amino acids. The spiroplasmas are fermentative, possessing enzyme activities that convert glucose 6-phosphate to pyruvate and lactate by the Embden-Meyerhof-Parnas pathway. Substrate phosphorylation is found in spiroplasmas. Spiroplasmas can synthesize purine mononucleotides by using pyrophosphate (PP) as the orthophosphate donor. All spiroplasmas except *S. floricola* use adenosine tri-phosphate (ATP) to phosphorylate deoxyguanosine; no other nucleoside can be phosphorylated with ATP by any spiroplasmas. As in all other mollicutes, a tricarboxylic acid cycle is apparently absent in *Spiroplasma* spp. On the contrary, *X. fastidiosa* derives energy through the tricarboxylic acid cycle and does not possess lactate dehydrogenase for the completion of glycolysis.

Key words: fastidious prokaryotes, cultivation, nutrition, metabolism.

INTRODUCTION

Ever since Doi *et al.* (18) reported the association of mycoplasmalike organisms (MLOs) with several "yellows-type" diseases of plants in 1967, to date over three hundreds plant diseases have been reportedly attributed to the MLOs (28). Despite all the efforts trying to cultivate the MLOs for the last twenty-five years or so, none has been cultivated in any cell-free medium. Although not much about the culture of MLOs can be examined, two significant research breakthroughs related to the cultivation of other

fastidious prokaryotes occurred during that period of time. One was the *in vitro* growth of the MLO associated with citrus stubborn disease in SMC medium in 1971 (30) and the other the *in vitro* growth of rickettsia-like bacterium (RLB) from grape tissues with Pierce's disease symptoms in JD1 medium in 1978 (15), The MLO associated with citrus stubborn disease and the RLB associated with Pierce's disease of grape were later given binomial names as *Spiroplasma citri* in 1973 (31) and *Xylella fastidiosa* in 1987 (33), respectively. Numerous other undefined media were formulated for the isolation and cultivation of many other *Spiroplasma* spp. and other strains of *X. fastidiosa*. Two reviews (11,35) detailed the available undefined media formulations for the cultivation of spiroplasmas, whereas one (22) for the strains of *X. fastidiosa*.

In 1982, Chang and Chen (4) formulated the first chemically defined medium (CC-494) for the cultivation of three spiroplasmas: *S. melliferum* (AS 576), *S. floricola* (23-6), and *S. apis* (SR 3). A year later, Lee and Davis (24) developed a defined medium (LD 82) which could support the growth of *S. citri* as well as four epiphytic and insect-pathogenic spiroplasmas. Hackett *et al.* (21) in 1987 reported a successful cultivation of *S. mirum*, the suckling mouse cataract agent, in a defined medium (H-1). The accessibility of various defined media enabled the study of nutritional requirements of several spiroplasmas as detailed in a review by Chang (3). The first chemically defined medium for the growth of Pierce's disease strains of *X. fastidiosa* will be published in 1993 (6).

FASTIDIOUS PROKARYOTES

Fastidious prokaryotes that cause diseases of plants can be divided into two groups: one is prokaryotes with cell walls and the other without cell walls. They are described as fastidious because they share at least one common feature that distinguishes themselves from most of other phytopathogenic bacteria. The unique feature is this, they require specific and enriched medium to grow. In other words, conventionally available bacterial growth media, such as nutrient broth, nutrient agar, 523, and YDC are not able to support their in vitro growth. There are, however, exceptions that will be discussed in the "Cultivation of spiroplasmas and *X. fastidiosa*" section.

Three groups of microorganisms are recognized among the prokaryotes that possess cell walls: the first group is named *Xylella fastidiosa* (33), often referred to as xylem-limited bacteria or rickettsia-like bacteria; the second group includes *Clavibacter xyli* subsp. *xyli* (14), and *C. xyli subsp. cynodontis* (14), the causal agents of ratoon stunting disease of sugarcanes and stunting disease of Burmudagrass, respectively. Both are also xylem-inhabiting bacteria; and the third group is so-called phloem limited bacteria, such as citrus greening disease, rugose leaf curl of clover, clover club leaf, potato leaflet stunt, and little-leaf disease of *Sida cordifolia*. (We should add another group in here: those that are not tissue-limited, such as infectious necrosis of grapevines and crinkle disease of hop.) Most of the bacteria in the first two groups, *Xylella* spp. and *Clavibacter* spp., are cultivable in cell-free media, whereas none of the phloem-limited bacteria has been cultured. Of the cell wall-free prokaryotes, two groups of phloem-inhabiting microorganisms have been recognized: spiroplasmas and mycoplasma-like organisms (MLOs). Most of the spiroplasmas have been cultured, while none of the MLOs has been cultivated. In this paper, we review entirely the cultivation, nutrition, and metabolism of *X. fastidiosa* and spiroplasmas.

What is *X. fastidiosa*? It was first referred to as a rickettsia-like bacterium beacause it did not grow on conventional medium and it possessed rippled cell wall, both are the characteristics of rickettsia. It was later referred to as xylem-limited bacterium because it resided only in the xylem tissues of plants and no relationship was found between rickettsia-like bacterium and true rickettsia based on serological and DNA hybridization studies. *X. fastidiosa* is a Gram-negative bacterium with cell size rangeing from 0.2–0.4 μm × 1.0–4.0 μm which is generally smaller than that of other phytopathogenic bacteria in diameter. It requires special enriched medium to grow, such as CS20 (9,10), PD2 (16), PW (13), or BCYE (34). *X. fastidiosa* is associated with the following plant diseases: Pierce's disease of grape, almond leaf scorch, alfalfa dwarf, phony disease of peach, plum leaf scald, elm, sycamore and mulberry leaf scorch, periwinkle wilt, oak leaf scorch, ragweed stunt, pear leaf scorch, citrus blight (22), and citrus variegated chlorosis (CVC) (C. J. Chang, unpublished). Most of the diseases are found in North America, except pear leaf scorch found in Taiwan and CVC in Brazil. Koch's postulates have been fulfilled for all the diseases except CVC and citrus blight.

The disease symptoms caused by *X. fastidiosa* often consist of marginal necrosis of leaves, plant stunting, and general decline and reduced yields. Such symptoms are probably caused by plugging of the xylem by bacterial cells and by a matrix material partly of bacterial and partly of plant origin. No marginal necrosis occurs, however, in peach with phony disease, in periwinkle with wilt symptoms, and in citrus with variegated chlorosis.

The marginal necrosis of leaves develops on grapes with Pierce's disease. Usually scorching of leaves begins at outer leaf margins and progresses inward in

late summer. One unique symptom for Pierce's disease is the petioles to remain attached to the cane after leaves fall. The bacterium kills susceptible vines in 2–5 years in Georgia.

No marginal necrosis of leaves is found on peach trees with phony disease symptoms. The bacterium causes the shortening of the terminal growth every year which makes a severely infected peach tree look like an umbrella canopy. Other symptom on phony tree is that the infected leaves are darker green than healthy leaves.

The symptoms on citrus with variegated chlorosis. however, are different. The disease is characterized by a bright interveinal chlorosis resembling zinc deficiency on the upper side of the leaf and corresponding to the chlorotic areas there are slightly raised lesions with gum deposits on the top lower surface of the leaf. Fruits are usually smaller from a CVC-affected tree as compared with fruits from a healthy tree. Also the small fruits are hardened like rocks which could damage the machine designed for processing fresh orange juice.

What are spiroplasmas? Spiroplasmas are members in the Class Mollicutes. They are organisms without an organized and bounded nucleus; lack a true cell wall and are therefore bounded only by a single triple-layered "unit" membrane. They are small helical-shaped, with a genome size of 1000 MDa and sometimes seen as ultramicroscopic cells containing cytoplasm, randomly distributed ribosomes and strands of nuclear material. They measure from 175 to 250 nm in diameter during reproduction but grow into various sizes and shapes later on. They seem to be capable of reproduction by binary fission; have no flagella, produce no spores, and are Gram-negative. They can be grown on more or less complex artificial nutrient media in which they produce minute satellite-type colonies and require cholesterol for growth. They are completely resistant to penicillin, but sensitive to tetracycline, chloramphenicol, and certain other antibiotics. To date, there are fifteen recognized species of which *S. citri, S. kunkelii*, and *S. phoeniceum* are phytopathogenic.

CULTIVATION OF SPIROPLASMAS AND *X. FASTIDIOSA*

More than 200 spiroplasma isolates have been cultured since the citrus stubborn spiroplasma was first cultivated in 1971 in SMC medium (30). The development of enriched complex media and the improvement of environmental factors have contributed significantly to spiroplasma cultivation. In addition to SMC, media in common use include C-3, C-3G, M-1, SP-4, SM-1, LD8, and LD8A (11). Other media without designation were also used for spiroplasma isolations (11). Two reviews on the cultivation of spiroplasmas in undefined media have been published (11,35).

Almost all the mentioned media contain three common undefined ingredients-PPLO broth base which consists of beef heart infusion and peptone, yeast extract, and animal serum-all of which are found in conventional mycoplasma (=mollicute) culture media. Other supplements include DNA, tryptone, peptone, carbohydrate, inorganic salts and organic acids, amino acids, HEPES buffer, insect tissue culture medium (Schneider's *Drosophila* medium), animal tissue culture medium (TC 199, CMRL-1066), and yeastolate (11).

Of all the above mentioned media, C-3G seems to be the simplest one that contains only three ingredients: PPLO broth base (1.5%), orse serum (20%), and sucrose? (12%). This medium is easy to prepare and has been used to isolate and culture *S. citri, S. kunkelii*, the honeybee spiroplasma (*S. melliferum*), and various flower and insect spiroplasmas. Further modification of C-3G medium led to formulation of R_2 medium, which contains PPLO broth base (1.5%), fetal bovine serum or horse serum (15%), and sucrose (8–10%), and R_8 medium which contains PPLO broth base (2.1%), fetal bovine serum or horse serum (20%), and glucose (0.5%). The R_2 medium, has been used to make primary isolations and to grow a wide variety of cultivable spiroplasmas; of those tested, only *S. mirum* (SMCA) failed to grow. *Spiroplasma mirum* has been grown, however, in R_8 medium (12).

There are four commonly used media, PD2 (16), PW (13), BCYE (34), and CS20 (9,10) for the isolation and cultivation of *X. fastidiosa* strains. CS20 was formulated for the isolation and cultivation of *X. fastidiosa* associated with phony peach, plum leaf scald, and oak leaf scorch diseases, PW and BCYE for *X. fastidiosa* associated with phony peach and plum leaf scald, and PD2 for *X. fastidiosa* associated with Pierce's disease of grapes. PW was later used to isolate and cultivate *X. fastidiosa* associated with periwinkle wilt disease. In general, media used for *xylella* contain peptone, tryptone, soytone or yeast extract from various sources, hemin chloride or ferric pyrophosphate for iron sources, amino acids, inorganic salts, citrate and succinate, and starch, bovine serum albumin, or activated charcoal. All ingredients for CS20 medium were autoclavable, whereas glutamine and bovine serum albumin fraction V in PW medium, bovine serum albumin fraction V in PD2 medium and L-cysteine hydrochloride and ferric pyrophosphate in BCYE medium were sterilized through a 0.2-μm membrane filter. This clearly indicates that CS20 is easier to prepare than the other three media especially when large quantity of medium are needed. CS20 was shown to be able to grow all the cultivated *X. fastidiosa* strains (C. J. Chang, unpublished).

Can nutrient agar support the primary isolation and growth of any strains of *X. fastidiosa*? The answer is likely a "no" because it was reported in the paper

(15) describing the first success in the cultivation of PD bacterium that no any conventionally available bacterial medium could support the growth of Perce's disease bacterium. Becauses of this statement nutrient agar has been used as a control medium. If a bacterium grew in your enriched medium, not in nutrient agar, you know you may have isolated a fastidious prokaryote. This worked fine when we isolated *X. fastidiosa* from peach, plum and oak, but not from grapes with Pierce's disease and mulberry with leaf scorch disease (23). Kostka *et al.* (23) reported that mulberry strain of *X. fastidiosa* could grow in nutrient agar after having been isolated in an enriched medium. Whether nutrient agar can support the primary isolation of mulberry strain is unknown. Fry *et al.* (19) reported the success of using nutrient agar to isolate and grow *X. fastidiosa* from grape tissues with Pierce's disease symptoms. In the same year, Chang *et al.* (8) also described the primary isolation of *X. fastidiosa* in nutrient agar from grapes showing Pierce's disease symptoms. Pierce's disease strain and mulberry strain seem to be the least fastidious ones among all the cultivated strains.

NUTRITIONAL REQUIREMENTS OF SPIROPLASMAS AND *X. FASTIDIOSA*

A precise nutritional requirement for growth of any fastidious prokaryotes can only be investigated with a chemically defined medium. The above mentioned undefined media are useful for isolation and cultivation of various strains of fastidious prokaryotes, but not appropriate for the nutritional requirement study. However, as I mentioned above, there are two relatively simple media for the growth of fastidious prokaryotes: R2 for spiroplasmas and nutrient agar for Pierce's disease strains of *X. fastidiosa* which led to the success of formulating chemically defined media.

Chang and Chen (4) took two steps, firstly by formulating a solution with chemically known ingredients to replace PPLO broth base in R_2 medium and secondly by formulating a chemically known lipid solution to replace horse serum, to formulate the first defined medium (CC-494) for the growth of spiroplasmas. CC-494 medium (4) contains the following 71 chemically known ingredients: HEPES buffer, 8 inorganic salts, 2 keto acids, 10 nucleosides and nucleotides, 4 carbohydrates, 20 amino acids, 13 vitamins and cofactors, 5 lipids, bovine serum albumin, and 7 others. Three spiroplasmas (*S. melliferum, S. floricola,* and *S. apis*) grew in the CC-494 formulation. By using the CC-494 medium (4) and CC-494 M (7), the nutritional requirements of spiroplasmas including carbohydrate and lipid utilization, arginine metabolism, and nitrogenous base, ribonucleoside, amino acid, lipid, and vitamin requirements were thoroughly investigated.

A year after the development of CC-494 was reproted, Lee and Davis (24) formulated LD82 medium, which supported the growth of *S. citri* as well as other honeybee and flower spiroplasmas. Hackett *et al.* (21) have formulated H-1 medium for cultivation of *S. mirum*. A new medium, designated as CB3, was formulated for the investigation of the nutritional requirements of *S. citri* (C. J. Chang, unpublished). Recent successes in the development of chemically defined media for various spiroplasmas have not only revealed the nutritional requirements of spiroplasmas but also facilitated investigations of the metabolic pathways of spiroplasmas and have made sutdies of their pathogenicity to host plants more precise.

Twenty standard amino acids were included in the CC-494 (4), LD82 (24), CC-494M (7), CC-494N (1), and H-1 (21) defined media. Two other amino acids, β-alanine and hydroxy-L-proline, were also included in both LD82 and H-1 media. Whether they were essential for growth of *S. mirum* is unknown. It is, however, clear that *S. melliferum* (AS 576), *S. floricola* (23-6), *S. apis* (SR 3), and *S. citri* (R8A2) do not require β-alanine and hydroxy-L-proline for growth (C. J. Chang, unpublished). After the CC-494 medium was developed, Chang and Chen (5) grouped 20 amino acids into 10 combinations according to their structural differences, roles in the biosynthesis of sugars, and essentiality for higher animals and compared the growth of *S. melliferum, S. floricola,* and *S. apis* in the 10 combinations. The 10 combinations were (i) 20 amino acids; (ii) 14 glucogenic amino acids (Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Met, Pro, Ser, Thr, and Val); (iii) 3 ketogenic amino acids (Leu, Lys, and Trp); (iv) 3 glucogenic and ketogenic amino acids (Ile, Phe, and Tyr); (v) 10 essential amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val); (vi) 10 nonessential amino acids (Ala, Asn, Asp, Cys, Gly, Gln, Glu, Pro, Ser, and Tyr); (vii) 8 amino acids with nonpolar R groups (Ala, Ile, Leu, Met, Phe, Pro, Trp, and Val); (viii) 7 amino acids with uncharged polar R groups (Asn, Cys, Gly, Gln, Thr, Tyr, and Ser); (ix) 2 amino acids with negatively charged polar groups (Asp and Glu); and (x) 3 amino acids with positively charged polar groups (Arg, His, and Lys). It was interesting to note that *S. melliferum*, an insect pathogen, could grow in only 4 of the 10 amino acid combinations, whereas *S. floricola* and *S. apis* could grow in any of them. The medium supplemented with 20 amino acids supported the best growth for all three spiroplasmas. Generally, as more amino acids were deleted from the medium, lower yields resulted. For example, *S. melliferum* reached 2.46×10^9 cells/ml with 20 amino acids, compared with 1.03×10^8 cells/ml when only 7 uncharged polar R group amino acids were supplied. The four combinations that supported growth of *S. melliferum* were (i) 20 amino acids; (ii) 14 glucogenic

amino acids; (iii) 10 nonessential amino acids; and (iv) 7 uncharged polar R group amino acids. *S. citri* required 18 amino acids (with the omission of Asp and Glu or the omission of Asn and Gln) to grow in CB3 medium (C. J. Chang, unpublished).

Almost all cultivable spiroplasmas ferment glucose. Carbohydrates appear to be the major energy sources for spiroplasmas, although some glucose-utilizing spiroplasmas are also able to hydrolyze arginine. Media for primary isolation of spiroplasmas generally contain one, two, or three fermentable carbohydrates. SMC medium, the medium formulated by Fudl-Allak *et al.* (20) for *S. citri*, and M-1 (36) for *S. kunklii* contained glucose, fructose, and sucrose; LD8 and LD8A (25) were supplemented with fructose and sucrose; and C-3G (26) and SP-4 (32) contained sucrose and glucose, respectively. The successful growth of spiroplasmas in these media was first observed by the color change of the media, usually from red to yellow, indicating that acid was being produced, probably through glycolysis. Using CC-494 medium, Chang and Chen (5) compared the growth of *S. melliferum*, *S. floricola*, and *S. apis* in 12 carbohydrate-supplemented media. All three spiroplasmas produced acid in the presence of glucose, fructose, and mannose. Acid was also produced by *S. melliferum* when grown in the presence of trehalose, by *S. floricola* in the presence of sucrose, trehalose, and raffinose, and by *S. apis* in the presence of sucrose. Other carbohydrates included in the test were galactose, mannitol, sorbitol, lactose, maltose, and starch. These results differ substantially from those obtained with *S. melliferum* in undefined medium, in which glucose, fructose, maltose, starch, and trehalose were apparently utilized (27), but mannose was not (17). Since we know that horse serum and serum fraction are able to convert di-, oligo-, and poly-saccharides to glucose, and from the result obtained with defined media, *S. melliferum* appears definitely able to use glucose, fructose, mannose, and trehalose for energy. In contrast, positive results with maltose and starch could be an artifact. *S. citri* was able to use glucose, fructose, and trehalose, but not mannose, sucrose, and raffinose (C. J. Chang, unpublished).

Spiroplasmas, like *Mycoplasma* and *Ureaplasma* species, are sterol-requiring mollicutes. Spiroplasmas growing *in vitro* obtain sterols and other lipids mainly from supplemented animal sera in complex undefined media (11). Being considered one of the smallest living organisms, spiroplasmas do not possess enzymatic pathways to make lipids for their membrane via acetyl coenzyme A (2). Therefore, for the lipid components of their membranes, they depend totally on the supplementation of lipids in the medium. The strict requirement of cholesterol for spiroplasmal growth was proved even before defined media were formulated. One of the criteria in identifying a new spiroplasma species is the requirement of cholesterol for cell growth measured either by cell numbers or by cell proteins. In CC-494 medium, a lipid supplement containing (μg/ml) palmitic acid (12.3), oleic acid (9.2), and cholesterol (18.5) supported growth of *S. melliferum*, *S. floricola*, and *S. apis*. Chang and Chen (2) further reported that all three spiroplasmas could not grow when either oleic acid or cholesterol was deleted from CC-494 medium. In contrast, limited growth was obtained when palmitic acid was eliminated. It was concluded that not only cholesterol but also oleic acid was required by spiroplasmas, whereas palmitic acid, shown to be nonessential, promoted significant growth. Chang (2) substituted palmitic acid (16:0) with three other saturated fatty acids, oleic acid with three other unsaturated fatty acids, and cholesterol with five other sterols. It was concluded that lauric acid (12:0), myristic acid (14:0), or stearic acid (18:0) was able to replace palmitic acid; palmitoleic acid (16:1), linoleic acid (18:2), or linolenic acid (18:3) was able to replace oleic acid; and campesterol, ergosterol, β-sitosterol, or stigmasterol was able to replace cholesterol for the normal growth of *S. melliferum*, *S. floricola*, and *S. apis* in CC-494 medium. The CC-494 medium not only provided a useful tool for studying precise nutritional requirements (5) but also led to successes in the development of other defined media. Lee and Davis (24) formulated LD82 medium, which can support the growth of a phytopathogenic spiroplasma, *i.e.*, *S. citri*, and other spiroplasmas. The major difference between CC-494 and LD82 is in lipid formulation. Besides palmitic acid and cholesterol, linoleic acid, phosphatidic acid (egg yolk), phosphatidylcholine (egg yolk), lysophosphatidylcholine (egg yolk), and lysophosphatidylcholine (soybean) were included in LD82 medium. The essentiality of these lipids was, however, not determined. Because CC-494 medium cannot support *S. citri*, whereas LD82 medium can, it is apparent that in addition to palmitic acid and cholesterol, the combination of linoleic acid, phosphatidic acid, phosphatidylcholine, and lysophosphatidylcholine is essential to the growth of *S. citri*. Whether every component of the combination is essential is yet to be investigated. Hackett *et al.* (21) successfully developed H-1 medium for *S. mirum*. Sphingomyelin was supplemented in H-1 medium in addition to cholesterol, palmitic acid, and oleic acid. Sphingomyelin is the only lipid ingredient that is not included in CC-494 medium. The fact that CC-494 medium is unable to support *S. mirum* clearly indicates the significant role that sphingomyelin plays in H-1 medium for *S. mirum*. The requirement of sphingomyelin, the most abundant sphingolipid in the tissues of higher animals, for growth of *S. mirum*, a spiroplasma that causes experimental cataracts in suckling mice, may prove to be significant when pathogenesis is investigated.

Spiroplasmas, recognized as one of the smallest organisms capable of growth in cell-free medium nevertheless have typical prokaryotic organization, in which deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) serve the usual functions. Spiroplasmas must biosynthesize nucleotides and use these as the monomeric units of nucleic acids, just as they use amino acids as the building blocks of polypeptides. Chang and Chen (5) demonstrated that three spiroplasmas (*S. melliferum, S. floricola,* and *S. apis*), like other mollicutes, lack the orotic acid pathway for pyrimidine synthesis and the enzymatic pathway for *de novo* synthesis of purine base, because no growth occurred when nitrogenous bases or ribonucleosides were omitted from the formulation. Either nitrogenous bases or ribonucleosides are required by spiroplasmas for the synthesis of nucleic acids. In the same study, Chang and Chen showed that the four deoxyribonucleosides are required by spiroplasmas for the synthesis of nucleic acids. In the same study, Chang and Chen showed that the four deoxyribonucleosides, the two pentoses, and the nucleotide in CC-494 medium were nonessential to all three spiroplasmas. In other words, all three spiroplasmas were presumably able to synthesize ribose and deoxyribose from other carbohydrates and ultimately to synthesize deoxyribonucleosides from either nitrogenous bases or ribonucleosides.

The vitamins biotin, calcium pantothenate, folic acid, nicotinic acid, nicotinamide, para-aminobenzoic acid, pyridoxal hydrochloride, pyridoxine hydrochloride, riboflavin, and thiamin hydrochloride are found in CC-494, LD82, and H-1 defined media. In addition to these vitamins, CC-494 and H-1 contain ascorbic acid, whereas LD82 contains pyridoxal phosphate, riboflavin 5-phosphate, and thiamin pyrophosphate. It is known that spiroplasmas require some vitamins for growth. However, whether they are all required for spiroplasmas grown in LD82 and H-1 medium has not been determined. Chang (1) grouped the 13 vitamins and cofactors supplemented in CC-494M medium into the following six combinations for testing: (i) vitamin B group (calcium pantothenate, folic acid, nicotinic acid, nicotinamide, para-aminobenzoic acid, pyridoxal hydrochloride, pyridoxine hydrochloride, riboflavin, and thiamin hydrochloride); (ii) ascorbic acid; (iii) biotin; (iv) choline chloride; (v) *myo-inositol*; and (vi) a combination of all 13 vitamins and growth factors as control. Medium supplemented with the vitamen B group supported the growth of *S. melliferum, S. floricola,* and *S. apis.* In contrast, deletion of ascorbic acid, biotin, choline chloride, and *myo-inositol* did not prevent growth; these vitamins are therefore nonessential for the tested spiroplasmas. Further single-vitamin deletions from the vitamin B groups were conducted to find the essential vitamin for the growth of three spiroplasmas. Chang (1) concluded that although either nicotinic acid (or nicotinamide) or riboflavin was essential for spiroplasma growth, both vitamins were required for maximal growth of all three spiroplasmas.

Chang and Donaldson (6) formulated the first defined medium, designated as XF-26, for the primary isolation and growth of *X. fastidiosa* from grapevine tissues showing Pierce's disease symptoms. The XF-26 medium contains 3 inorganic salts [K_2HPO_4, $(NH_4)_2HPO_4$, and $MgSO_4 \cdot 7H_2O$], 2 tricarboxylic acids (trisodium citrate and disodium succinate), 17 amino acids (Ala, Arg, Asn, Cys, Gly, Gln, His, Ile, Leu, Lys, Mey, Phe, Pro, Ser, Thr, Trp, and Val), and 3 others (phenol red, potato starch, and Difco agar). Growth of cultivated Pierce's disease strains of *X. fastidiosa* in XF-26 was comparable to that in the undefined media CS20 or PD2. Medium XF-26 supported the primary growth of the bacterium isolated from grapevine tissues showing Pierce's disease symptoms as well as CS20 or PD2 did. XF-26 also supported the growth of two other cultivated, one originally isolated from periwinkle showing wilt symptoms and the other from oak with leaf scorch symptoms. Both XF-26 and PD2 contain trisodium citrate and disodium succinate which are likely the energy source for *X. fastidiosa* because no growth was observed when both acids were omitted in XF-26 (C. J. Chang, unpublished). Requirement of tricarboxylic acid for their growth may suggest that *X. fastidiosa* possess the Krebs cycle for energy. Of the four available undefined media, hemin chloride is included in CS20 (9,10), PW (13), and PD2 (16), whereas ferric pyrophosphate is the ingredient in BCYE (34). Davis *et al.* (15) reported that hemin chloride was not essential for growth of Pierce's disease bacterium, but hemin chloride enhanced growth of the bacterium. It is, however, obvious that *X. fastidiosa* associated with Pierce's desease requires no hemin chloride or ferric pyrophosphate for *in vitro* growth because the bacteria grew as well in a medium with or without hemin chloride. Whether hemin chloride or ferric pyrophosphate is required for the primary isolation of other strains of *X. fastidiosa*, such as those associated with phony peach disease, plum leaf scald, oak leaf scorch, and periwinkle wilt is unknown. Incorporation of starch, bovine serum albumin, or acid washed activated charcoal into the undefined media contributed to the successful isolation or culture for most strains of *X. fastidiosa.* The primary function of this group of components is probably to absorb and remove inhibitors in host tissues that contaminated the medium during primary isolation and in medium components. The accessibility of XF-26 would enable us to elucidate the role that starch plays in the primary isolation and culture of *X. fastidiosa.*

METABOLISM OF SPIROPLASMAS AND *X. FASTIDIOSA*

In order to determine the presence or absence and extent of a number of major interrelated metabolic pathways in members of the *Spiroplasmataceae*, Pollack *et al.* (29) examined cytoplasmic extracts from 10 strains of *Spiroplasma* species, which represented eight of the nine named species and seven serogroups (including four subgroups of group I) and were isolated from ticks, bees, mosquitoes, and different plants. Extracts were assayed for 67 enzyme activities that are components of the Embden-Meyerhof-Parnas (EMP) pathway, pentose phosphate (PP) shunt, tricarboxylic acid (TCA) cycle, and purine and pyrimidine pathways. The spiroplasmas were fermentative, possessing enzyme activities that converted glucose 6-phosphate to pyruvate and lactate by the EMP pathway. Substrate phosphorylation was found in all strains. A modified pentose phosphate shunt was present, which was characterized by a lack of detectable glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities. Spiroplasmas could synthesize purine mononucleotides by using pyrophosphate (PPi) as the orthophosphate donor. As in all other mollicutes, a tricarboxylic acid cycle is apparently absent in *Spiroplasma* spp. Reduced nicotinamide adenine dinucleotide oxidase activity was localized in the cytoplasmic fraction of all *Spiroplasma* species tested.

Cell-free extracts of four Pierce's disease strains of *X. fastidiosa* were assayed for 11 enzyme activities: phosphohexose isomerase, glucose 6-phosphate dehydrogenase, lactate dehydrogenase, and 8 enzymes associated with TCA cycle (aconitase, isocitrate and α-ketoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, fumarase, malate dehydrogenase, and citrate synthase). All enzymes assayed except lactate dehydrogenase were detected in all four Pierce's disease strains. We assume Pierce's disease strains possess pentose phosphate pathway and TCA cycle, but lack of glycolysis (C. J. Chang, unpublished).

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摘　　要

張宗仁．1993．營養苛求性植物病原原核菌之培養、養份及代謝．植病會刊2:218-226．（美國喬治亞大學植物病理系）

引起植物性病害之養份苛求性原核菌可分成具有細胞壁及不具細胞壁之二群。此些細菌因其生長常需含有特別成份或組成豐富之培養基，甚或迄今仍無法被培養，故被視爲養份苛求性細菌。至今已有三群具細胞壁之此類原核菌被發現，包括屬導管侷限性細菌或被稱爲似立克次

體之 *Xylella fastidiosa, Clavibacter xyli* subsp. *xyli* 及 *C. xyli* subsp. *cynodontis*，以及篩管侷限性細菌 (PLB)。而不具細胞壁者則主要有螺旋菌質體 (spiroplasmas) 及植物菌質體 (phytoplasmas) 二群。對 *Xylella, Clavibacter* 及 *Spiroplasma* 之大多成員 (種) 而言均能被分離及成功地加以培養，然而對 PLB 及植物菌質體則迄今仍無法成功地加以培養。在培養螺旋菌質體之絕大多數培養基中，幾乎均含有 PPLO 培養基礎原，酵母抽出液，及動物血清等三種常見於一般菌質體培養基之成份，此外常見的成份仍包括 tryptone, peptone，昆蟲組織培養基 (Schneider's *Drosophilia* 培養基)，動物組織培養基 (TC 199, CMRL-1006)，及 yeastolate。而培養 *Xylella* 及 *Clavibacter* 之培養基的主要成份則包括 peptone， soytone，檸檬酸，琥珀酸，胺基酸， hemin chloride 或焦磷酸鐵，牛血清，活性炭，或澱粉。有少數的組合式培養基適用於幾種螺旋菌質體。而適用於 *Xylella* 之組合式培養基只有一種，對 *Clavibacter* 則尚付闕如。此些組合式培養基可應用於探討此等微生物之營養需求。*Spiroplasma* 之生長需要無機鹽類、醣類、胺基酸、鹼基或核苷、維生素，及包括脂肪酸、固醇類、磷脂類等脂肪質。*X. fastidiosa* 則需要無機鹽類、三羧酸，及胺基酸。螺旋菌質體具發酵之能力，其可利用 Embden-Meyerhof-Parnas 代謝途徑將 6- 磷酸葡萄糖轉化成丙銅酸及乳酸。在螺旋菌質體中也可發現基質之磷酸化作用。螺旋菌質體亦可以焦磷酸 (PP) 當爲 orthophosphate 之提供者而合成嘌呤單核苷酸，除 *S. floricloa* 外之其他螺旋菌質體均可利用腺核苷三磷酸 (ATP) 去磷酸化去氧鳥糞核苷。除此以外，並無其他核苷可被任何螺旋菌質體利用 ATP 將其磷酸化。螺旋菌質體與其他菌質體一樣均缺乏三羧酸循環 (TCA cycle)。相反的， *X. fastidiosa* 則以三羧酸循環獲取能量，但其並不具有完成醣解作用所需之乳酸去氫酶。

關鍵詞：養份苛求性原核菌、培養、養份、代謝。