Xylella fastidiosa: Cultivation in Chemically Defined Medium

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This study was supported in part by State and Hatch Funds.
Accepted for publication 20 October 1992.

ABSTRACT

Chang, C. J., and Donaldson, R. C. 1993. *Xylella fastidiosa:* Cultivation in chemically defined medium. Phytopathology 83:192-194.

A chemically defined medium, XF-26, supported the in vitro cultivation of two strains of *Xylella fastidiosa* originally isolated from grapevines with symptoms of Pierce's disease and one strain from almond exhibiting leaf scorch symptoms. Growth 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 strains, one originally isolated from periwinkle showing wilt symptoms and the other from oak with leaf scorch symptoms.

Additional keywords: xylem-limited bacterium.

Bacterial strains of *Xylella fastidiosa,* which have been associated with diseases that cause tremendous losses in many economically important plants, including grape, alfalfa, peach, plum, almond, elm, sycamore, oak, maple, and possibly citrus (1,7), have been grown in media that are supplemented with one or more undefined constituents such as soy peptone, Bacto tryptone, phytone, and yeast extract (1,3–5,9). These constituents are complex and their exact role is difficult to define. No defined medium has been reported for the culture of *X. fastidiosa* strains, even though a formulation, namely amino acid-STABA medium, was described by Chen et al (2). This amino acid-STABA medium supported the growth of two strains of *X. fastidiosa*, namely the plum leaf scald and ragweed stunt bacteria (2). No details were given or published elsewhere, however, as to what extent the medium would support the growth of these bacteria or if the medium could support the growth of both bacteria isolated from plant tissue.

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Knowing the chemical nature of the cultural medium is necessary for determining the nutritional requirements, metabolic pathways, and biosynthetic capabilities of *X. fastidiosa* and for characterizing *X. fastidiosa* strains. We report here the successful culturing of strains of *X. fastidiosa* in a chemically defined medium.

MATERIALS AND METHODS

Three strains of *X. fastidiosa* were studied: ALS-BC (ATCC 35870), originally isolated from almond with leaf scorch symptoms, PCE-FG (ATCC 35881), isolated from grape with Pierce's disease symptoms, and R112V2 isolated from grape with Pierce's disease symptoms in a Georgia vineyard. All strains were maintained and subcultured weekly in CS20 or PD2 agar medium (1,4,5).

A defined medium, designated XF-26 (Table 1), was prepared by dissolving an adequate amount of K_2HPO_4, (NH_4) HPO_4, trisodium citrate, disodium succinate, potato starch (J. T. Baker

Chemical Co., Phillipsburg, NJ), and agar (Difco, Detroit, MI) in 800 ml of distilled water followed by adding the following stock solutions: 6 ml each of alanine, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, and valine; 12 ml each of arginine, asparagine, cysteine, glutamine, and histidine; 20 ml of $MgSO_4 \cdot 7H_2O$, and 10 ml of phenol red. Concentration of each amino acid stock solution was 3.3% except those of glutamine and tryptophan, which were 16.7 and 0.67%, respectively. Concentration of $MgSO_4 \cdot 7H_2O$ and phenol red was 10 and 0.2%, respectively. All stock solutions were sterilized by autoclaving for 15 min. The final volume of this medium was brought to 1 L with distilled water. The pH of the medium was adjusted to 6.6–6.7 by adding appropriate amounts of 5 N NaOH before the medium was autoclaved for 15 min. The medium was poured in plastic petri plates (100 × 15 mm) when cooled to 45–50 C. The XF-26 broth medium was prepared the same way without agar.

The XF-26 agar medium was first compared to two undefined media, CS20 and PD2, for growth of the three strains of *X. fastidiosa*. The amino acid-STABA medium described by Chen et al (2) was not compared with XF-26 throughout this study because no formal publication has described its suitability to support the growth of both plum leaf scald and ragweed stunt bacteria isolated from tissue and in maintaining the subcultures of both bacteria.

Both CS20 and PD2 were prepared as reported previously (1,4,5). Cells of each of the three strains were collected in CS20 broth individually from a 5-day-old CS20 culture. The cell suspension of each strain was adjusted to a Klett-Summerson photoelectric colorimeter reading of 50. A series of 10-fold dilutions were made to 10^{-6} cells with XF-26 broth to minimize the carryover of the undefined components from CS20 broth. A 0.1-ml aliquot from each of three dilutions, 10^{-4}, 10^{-5}, and 10^{-6} cells, was pipetted onto duplicate plates of XF-26, CS20, or PD2. After a 14-day incubation period at 30 C, number of colonies per plate was recorded, and diameters of 20 randomly selected colonies were measured.

The XF-26 was compared to CS20 and PD2 for primary isolation of *X. fastidiosa* from tissues of grapevines with Pierce's disease symptoms. Symptomatic leaves from two grapevines (R111V1 and R116V11) and asymptomatic leaves from two other grapevines (R57V16 and R117V3) were collected on 12 August 1991. Samples were placed in plastic bags and kept in an ice cooler when shipped from field to laboratory. The cooler containing collected samples was kept in cold room (5 C) for 2 days before isolation of the bacterium was attempted. Seven to 10 petioles from each grapevine sample were surface-sterilized in 1.06% sodium hypochloride for 15 min, rinsed three times in sterile distilled water, and air-dried under a Laminair hood (Environmental Air Control, Inc., Hagerstown, MD). Sterilized petioles were cut as finely as possible into 3 ml of XF-26 broth medium. The suspension was collected in a sterile test tube, mixed briefly and streaked onto duplicate plates of XF-26, CS20, and PD2 media and incubated at 30 C. Plates were observed for colony development at weekly intervals using a binocular microscope.

TABLE 1. Composition of XF-26 medium

Substance	(g/L)
Inorganic salts	
K_2HPO_4	1.5
$(NH_4)_2 HPO_4$	0.5
$MgSO_4 \cdot 7H_2O$	2.0
Tricarboxylic acids	
Trisodium citrate	1.5
Disodium succinate	1.5
Amino acids	
L-Alanine	0.2
L-Arginine	0.4
L-Asparagine	0.4
L-Cysteine	0.4
Glycine	0.2
L-Gutamine	2.0
L-Histidine	0.4
L-Isoleucine	0.2
L-Leucine	0.2
L-Lysine	0.2
L-Methionine	0.2
L-Phenylalanine	0.2
L-Proline	0.2
L-Serine	0.2
L-Threonine	0.2
L-Tryptophan	0.04
L-Valine	0.2
Others	
Phenol red	0.02
Potato starch	0.2
Difco agar	15.0

RESULTS AND DISCUSSION

The number of colonies per plate from the 10^{-4} dilution was too numerous to count, whereas those from 10^{-6} dilution were often too sparse to count. Therefore, the number of colonies and the diameter of 20 colonies were recorded and measured from plates of the 10^{-5} dilution. There were differences in growth among the three strains in the three media. For example, PCE-FG seemed to grow best in PD2, ALS-BC in CS20, and R112V2 in XF-26 (Table 2). However, there was no significant difference among the three media when the average number of colonies per plate or mean diameter of single colony from all three strains was compared (Table 2). All three strains have been subcultured for more than 20 passages in XF-26 with no change in growth rate.

Colonies of *X. fastidiosa* were visible in all three media when suspensions from symptomatic petioles of R111V1 or R116V11 were plated, whereas no colonies developed in any of media when suspensions from asymptomatic petioles of R57V16 or R117V3 were plated. Colonies were opalescent white and were 0.1–0.35, 0.04–0.43, and 0.08–0.40 mm in diameter in XF-26, CS20, and PD2, respectively, after 7 days of incubation. XF-26 medium has been successfully used to isolate *X. fastidiosa* from more than 25 different grapevine tissues with Pierce's disease symptoms.

All three media contain the same two inorganic salts, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$, whereas XF-26 and CS20 contain in addition to these salts, $(NH_4)_2HPO_4$, and PD2 includes K_2HPO_4. 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 tricarboxylic acids were omitted in XF-26 (C. J. Chang, *unpublished data*). Requirement of tricarboxylic acid for their growth may suggest that *X. fastidiosa* possess the Krebs cycle for energy.

Of the 17 amino acids in XF-26, glutamine and histidine, when incorporated into CS20, uniquely promoted growth of strains of *X. fastidiosa*, such as those that cause phony peach disease, plum leaf scald, and oak leaf scorch disease (C. J. Chang, *unpublished data*). Whether all 17 amino acids are required by *X. fastidiosa* for growth warrants further investigation. Chen et

TABLE 2. Average number of colonies per plate and mean diameter of a colony of three *Xylella fastidiosa* strains in XF-26, CS20, and PD2 media measured 14 days after inoculation

	Average number of colonies per plate[a]			Mean diameter[b] (mm) of single colony		
Strains	XF-26	CS20	PD2	XF-26	CS20	PD2
PCE-FG	213	95	259	0.66	0.72	1.09
ALS-BC	278	330	226	0.22	0.60	0.42
R112V2	325	275	145	0.64	0.95	0.78
Mean[c]	272 a	233 a	210 a	0.51 a	0.76 a	0.76 a

[a] Duplicate plates were inoculated for each medium.
[b] Twenty randomly selected colonies were measured (10 from each of the duplicate plates).
[c] Means followed by a common letter are not significantly different ($P = 0.05$) by Duncan's multiple range test.

al (2) stated that the Pierce's disease bacterium and the elm leaf scorch bacterium have basic growth requirements for as few as nine amino acids, a supplementary source of soluble iron, trace concentrations of minerals (available as contaminants in agar), and growth factors. Deletion of one amino acid or the iron supplement would reduce growth by 75% or more in the first passage on defined medium, and would prevent growth in subsequent passages. With the availability of XF-26 medium, the role of essential amino acids for growth of *X. fastidiosa* can lead to studies on the interconversion between tricarboxylic acids and amino acids. The metabolic pathway(s) of this newly named bacterium (8) can be defined in greater detail.

Of the four available undefined media (1,3–5,9), hemin chloride is included in CS20, PW, and PD2, whereas ferric pyrophosphate is the ingredient in BCYE. Davis et al (5) reported that hemin chloride is 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 disease 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 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. Two strains of *X. fastidiosa*, PWT-22 (ATCC 35878, originally isolated from periwinkle showing wilt symptoms) and oak (ATCC 35874, originally isolated from red oak showing leaf scorch symptoms), have grown in XF-26 for more than 15 passages to date (C. J. Chang, *unpublished data*), indicating that both strains are able to grow in a medium without the incorporation of hemin chloride or ferric pyrophosphate.

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* (1–5,9). The primary function of this group of components is probably to absorb and remove inhibitors in host tissues that contaminate the medium during primary isolation and in medium components (2). Feeley et al (6) successfully isolated *Legionella pneumophila*, a fastidious Gram-negative rod infectious to man, by adding charcoal additives to control the inhibitors in host tissue extracts. The accessibility of XF-26 would direct us to demonstrate the role that starch plays in the primary isolation and culture of *X. fastidiosa*.

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