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Genomics-based design of defined growth media for the plant pathogen *Xylella fastidiosa*

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Abstract

Based on the genetic analysis of the phytopathogen *Xylella fastidiosa* genome, five media with defined composition were developed and the growth abilities of this fastidious prokaryote were evaluated in liquid media and on solid plates. All media had a common salt composition and included the same amounts of glucose and vitamins but differed in their amino acid content. XDM_1 medium contained amino acids threonine, serine, glycine, alanine, aspartic acid and glutamic acid, for which complete degradation pathways occur in *X. fastidiosa*; XDM_2 included serine and methionine, amino acids for which biosynthetic enzymes are absent, plus asparagine and glutamine, which are abundant in the xylem sap; XDM_3 had the same composition as XDM_2 but with asparagine replaced by aspartic acid due to the presence of complete degradation pathway for aspartic acid; XDM_4 was a minimal medium with glutamine as a sole nitrogen source; XDM_5 had the same composition as XDM_4, plus methionine. The liquid and solidified XDM_2 and XDM_3 media were the most effective for the growth of *X. fastidiosa*. This work opens the opportunity for the in silico design of bacterial defined media once their genome is sequenced.

Keywords: *Xylella fastidiosa*; Genomics; Media design; Defined growth media

1. Introduction

The adjective 'fastidious' is applied to those microorganisms which require complex media for their adequate in vitro growth and which grow slowly even in such media. The bacterium *Xylella fastidiosa*, as its name indicates, is an important fastidious plant pathogen [1]. This bacterium is transmitted by xylem-feeding leafhoppers, and colonizes the xylem of plants causing diseases on several economic important crops such as citrus variegated chlorosis (CVC) in various citrus species, Pierce's disease (PD) of grapevine and coffee leaf scorch (CLS).

Sequencing and annotation of *X. fastidiosa* strain 9a5c which causes CVC [2] has shown that this bacterium should be efficient in energy production using a variety of different carbon sources. *X. fastidiosa* also possesses full catabolic pathways for amino acids such as threonine, serine, glycine, alanine, aspartate and glutamate that would supply the tricarboxylic acid cycle intermediary precursors needed for the synthesis of many other biomolecules. *X. fastidiosa* harbors complete anabolic pathways related to the synthesis of most purines, pyrimidines, nucleotides, enzyme cofactors and prosthetic groups, including vitamins such as biotin, folic acid, thiamine, pantothenate, riboflavin and coenzyme A, ubiquinone, glutathione, thioredoxin, glutaredoxin, FMN, FAD, porphyrin, pyridoxal-5′-phosphate and lipoate. The major amino acid biosynthetic pathways also are apparently complete, although some enzymes required for the synthesis of serine, cysteine and methionine apparently are missing. Missing enzymes in otherwise complete pathways is a relatively common feature among the recently sequenced bacterial genomes. It is not always clear whether those enzymes needed for a functional pathway are, in fact, missing or rather are a result of inaccuracies of the annotation process [3].

The five most widely used culture media for the isolation and cultivation of strains of *X. fastidiosa* are PD_2 [4],

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PW [5], BCYE [6], CS20 [7] and SPW [8]. All of these are complex media which include peptone, tryptone, soytone and yeast extract, hemin chloride or ferric pyrophosphate as iron sources, amino acids, inorganic salts, citrate, succinate, starch, bovine serum albumin or activated charcoal. Such formulations were empirically developed based on the reasoning that all bacteria needed sources of carbon, nitrogen, inorganic salts, iron and phosphate. Not only are such media costly and inconvenient to use, but they additionally complicate studies of the physiological characteristics of *X. fastidiosa*. A defined medium instead would be a great benefit in such studies.

The sequencing and annotation of *X. fastidiosa* genome [http://aeg.lbi.ic.unicamp.br/xf] has revealed that this bacterium has genes encoding all of the essential metabolic pathways for its growth, using many simple substrates such as carbohydrates, amino acids and inorganic salts. Interestingly, genes encoding individual enzymes were apparently sometimes missing though those for the rest of the metabolic pathways were present. A clear functionality of such pathways can be evaluated through the growth of such bacterium on defined media containing the substrate under investigation. The true absence of such genes could be ascertained by evaluating whether such pathways were, in fact, operational. Such studies would be most easily conducted by evaluating growth. Thus the objective of the present work was to design defined growth media for *X. fastidiosa*, based on genome analysis. The growth of *X. fastidiosa* on such media would verify complete sets of expressed genes required for the putative metabolic pathways and provide information on the growth processes of this bacterium in plant.

2. Materials and methods

2.1. Analysis of the annotated genome

To develop strategies for components to include in culture media the open reading frames (XF) from the *X. fastidiosa*–CVC genome project were analyzed focusing on biosynthetic catabolic pathways and ABC transporters for organic and inorganic components. Open reading frames were identified using the BLAST tool [9] by comparison with data in GenBank.

2.2. X. fastidiosa defined culture media

All of the minimal media developed contained the following components: K_2HPO_4 2.1 g l^{-1}; KH_2PO_4 0.8 g l^{-1}; $MgSO_4 \cdot 7H_2O$ 0.4 g l^{-1}; ferric pyrophosphate 0.125 g l^{-1}, glucose 10 g l^{-1} and 10 ml l^{-1} of a vitamin stock solution containing 0.2 mg D-biotin; 10 mg thiamine; 10 mg pyridoxine hydrochloride; 5.0 mg nicotinic acid; 0.050 mg vitamin B12 and 350 mg myo-inositol (all from Sigma Chemical Co.) dissolved in 100 ml in distilled water. The vitamin stock was filter sterilized through a 0.22-μm Millipore membrane and kept at 10°C until use. Different amino acids were also added in different combinations (Table 1). All media included 0.1% phenol red and were adjusted to pH 6.8 using 1 N KOH or HCl. The complete minimal media were filter-sterilized using 0.45 μm Millipore membranes. Solid media were prepared by adding an equal volume of agar (24 g l^{-1}) to a 2× concentrated minimal medium autoclaved at 120°C for 20 min.

2.3. Inoculum preparation

The inoculum of *X. fastidiosa* was always prepared in two steps. First a flask containing 30 ml of XDM_2 broth was inoculated with 1.0 ml of a freshly prepared *X. fastidiosa* 9a5c culture. After 5 days of growth at 28°C in a rotary shaker at 140 rpm, these cultures were used to inoculate (10% v/v) each medium used in the growth assay. Culture volumes did not exceed 10% of the capacity of the flask to ensure adequate aeration of the culture. Cultures were established in duplicate. A total of 14 flasks of each medium were prepared so as to allow destructive sampling of two flasks every other 48 h, during 14 days.

2.4. Growth curves

Two samples of each medium (20 ml) were collected every 48 h by centrifugation at 10 400×*g* for 20 min at 4°C and the pelleted cells were preserved by freezing. Growth was monitored by measuring the turbidity of resuspended cells and by protein abundance. Samples were resuspended in 1.0 ml of sonication buffer (10 mM Tris–HCl and 5 mM $MgCl_2$, pH 7.0) by vortexing. Before sonication, a 50 μl aliquot, was removed and diluted in 950 μl of water for measurement of turbidity. The remaining cells were used for protein determination. Cells extracts were prepared by sonication (Sonifier Branson, model 250 at 85W) in an ice-water bath for 5 min, and the sonicate was clarified by centrifugation at 10 600×*g* for 10 min at 4°C. The amount of extracted protein was estimated by the Hartree assay [10]. The purity of *X. fastidiosa* cultures in all experiments were carried out with PCR using specific primers [11,12] and by phase microscopy.

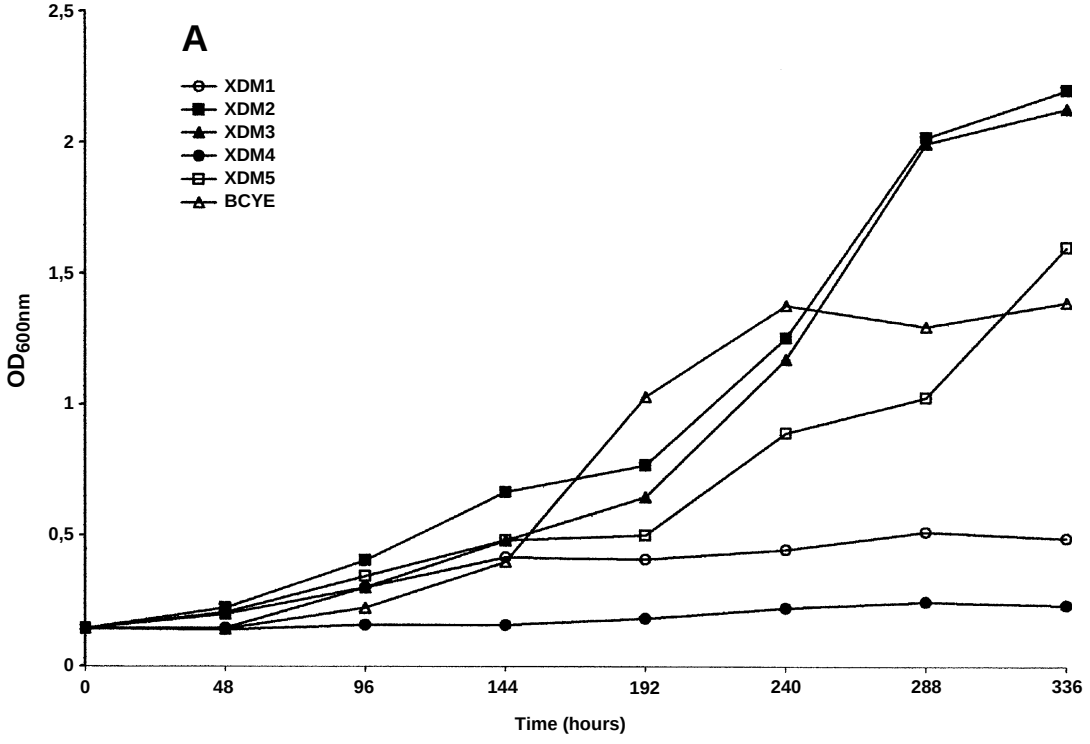
Table 1
Amino acids in a defined medium for *X. fastidiosa*

Amino acids (μg ml^{-1})	XDM_1	XDM_2	XDM_3	XDM_4	XDM_5
L-threonine	0.4				
L-serine	0.4	0.4	0.4		
L-glycine	0.4				
L-alanine	0.4				
L-aspartic acid	0.5		1.0		
L-methionine		0.4	0.4		0.4
L-glutamine	2.0	4.0	4.0	4.0	4.0
L-asparagine		1.0			

3. Results and discussion

3.1. Carbon and nitrogen sources

The growth of *X. fastidiosa* 9a5c was compared in XDM_1, XDM_2, XDM_3, XDM_4 and XDM_5 defined media as well as in BCYE by measuring cell turbidity and protein content for 14 days, at 28°C with shaking conditions (Fig. 1A,B). Since *X. fastidiosa* harbors all the genes for glycolytic pathway, tricarboxylic acid cycle and oxidative elec-



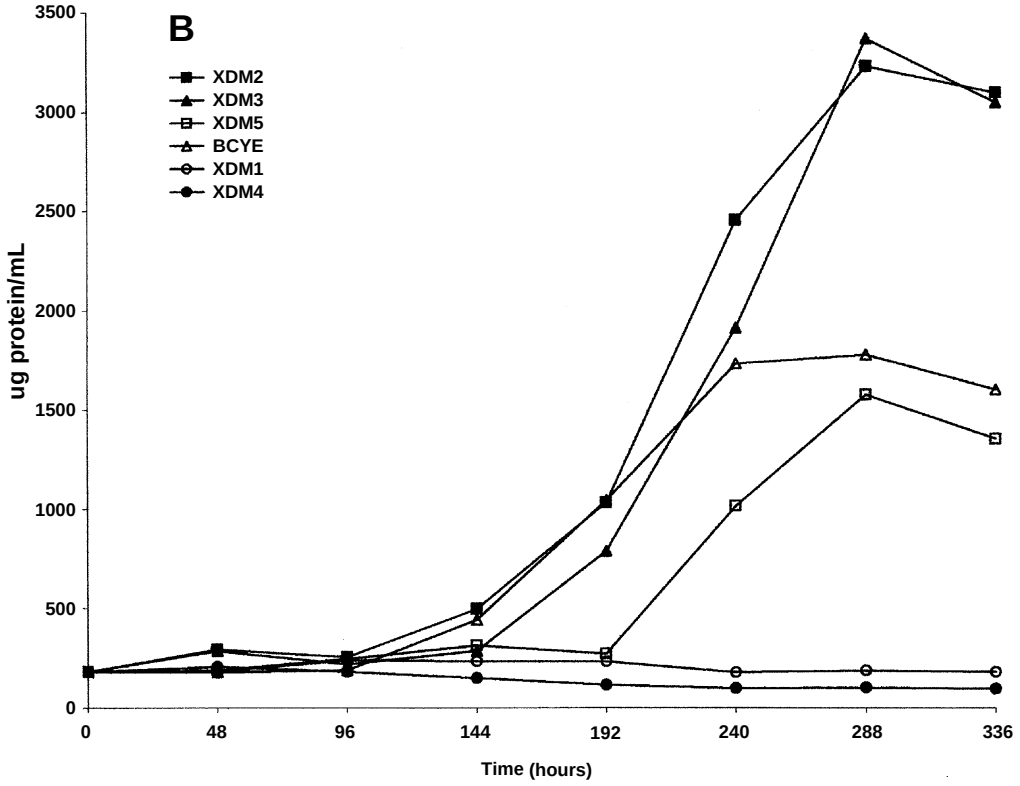


Fig. 1. Growth curves of *X. fastidiosa* 9a5c in different defined media. A: Protein content determination; B: OD_{600} during 14 days of growth at 28°C at 140 rpm.

tron transport chain, glucose was included in all media as a carbon source to meet the carbon and energy requirements for growth; there was no growth without glucose (data not shown). Evaluation of cell growth and growth rates of *X. fastidiosa* by culture turbidity or protein content gave very similar results (Fig. 1). Growth of *X. fastidiosa* in the XDM_2 medium which includes serine, methionine and the xylem components, asparagine and glutamine, was equivalent to the growth in the XDM_3 which has the same composition as XDM_2, except the replacement of asparagine by aspartic acid. Although *X. fastidiosa* could grow in XDM_1 and XDM_4 (which has only glutamine as a nitrogen source), the growth rate was much lower than on the other three defined media. In XDM_5 (XDM_4 plus methionine), however, there has been a noticeable growth after a 192-h lag phase. The growth rate of *X. fastidiosa* on the complex media such as BCYE was substantially lower than in XDM_2 or XDM_3 media. This strain grew equally well in liquid and on solidified XDM media of any given composition.

During cultivation of *X. fastidiosa* in liquid XDM media, cells adhered to the walls of the flasks, producing a biofilm (Fig. 2), which made quantification of growth difficult. The most abundant biofilm was observed in culture in XDM_2 and XDM_3 media (Fig. 2B,C). On solidified XDM_2 and XDM_3 media, strain 9a5c as well as different

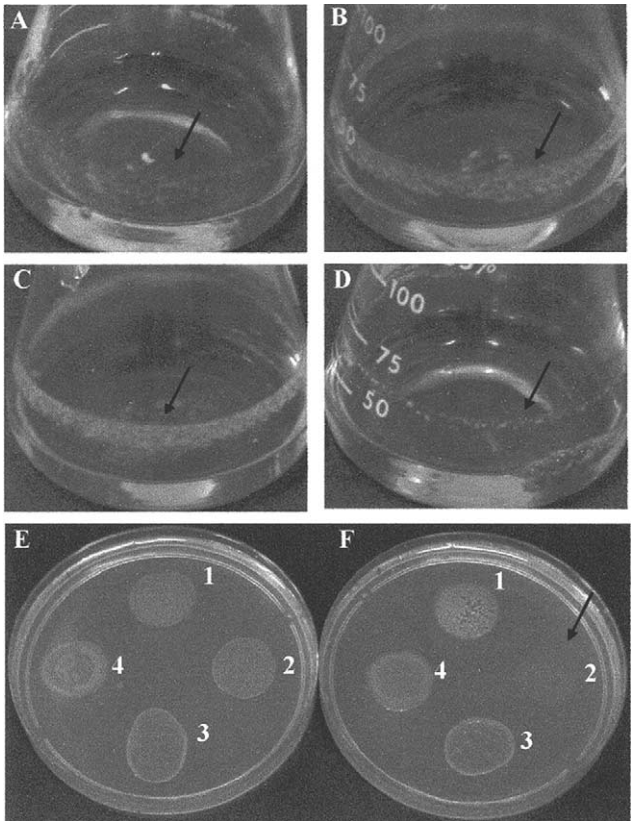


Fig. 2. Growth of *X. fastidiosa* 9a5c on liquid media XDM_1 (A), XDM_2 (B), XDM_3 (C), XDM_4 (D). *X. fastidiosa* growth on solid versions of XDM_2 (E) and XDM_3 (F): 9a5c-CVC (1), JAB1-CLS (2), 6747-Plum (3) and Temecula-PD (4).

X. fastidiosa isolates produced a sticky layer of gum (Fig. 2E,F). While CVC strain 9a5c, plum strain 6747 and the grape strain Temecula exhibited good growth on XDM_2 and XDM_3 media, strain JAB1-CLS obtained from coffee plants did not grow well on XDM_3. The growth of strains 9a5c and Temecula on such media was not surprising since the recent sequencing of the Temecula strain [Van Sluys et al., *X. fastidiosa* Genome – Pierce's Disease Strain Preliminary Public Website (http://onsona.lbi.ic.unicamp.br/world/xf-grape/; accessed on 30 March 2002) has revealed metabolic characteristics identical to those of CVC strain 9a5c [2].

Complete pathways for degradation of six amino acids – threonine, serine, glycine, alanine, aspartic acid and glutamine – are encoded in the *X. fastidiosa* genome, as well as a number of membrane transporters for their molecules. Considering these findings, the compounds above were chosen as carbon and nitrogen sources in the XDM_1 medium, but the growth on this medium was lower than on other defined media (XDM2, XDM3 and XDM5). Serine seemed to be particularly essential for *X. fastidiosa* growth since the key enzyme needed for its biosynthesis was not found during *X. fastidiosa* 9a5c annotation process. The main serine biosynthesis pathway is shared by most organisms, and for *X. fastidiosa* it was observed that, in the first step, the hydroxyl group of 3-phosphoglycerate is oxidized by D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95; XF2206) producing 3-phosphohydroxypyruvate. Transamination from glutamate by phosphoserine aminotransferase (EC 2.6.1.52; XF2326) normally yields 3-phosphoserine which should be hydrolyzed to serine by phosphoserine phosphatase (EC 3.1.33). However, since the ORF for the latter enzyme is missing within the *X. fastidiosa* genome (as well as for *Bacillus subtilis* [13]), we have expected *X. fastidiosa* to be a serine auxotroph. Since growth occurred in XDM_5 medium lacking serine, an alternative enzyme may be carrying out this enzymatic step in *X. fastidiosa*. In the xylem, the natural habitat for *X. fastidiosa*, there should be enough serine to supply its needs assuming that it can be transported into the cell (*X. fastidiosa* genome encodes several predicted amino acid transporters, but not the Na^+/serine symporter SstT).

Compositional analysis of citrus sap has shown that amino acids serine, aspartic acid, asparagine, glutamate/glutamine, lysine and proline are the primary carbon and nitrogen compounds there, but are found in low concentrations [14]. These compounds are available for the plant pathogen throughout its xylem colonization phase. Serine, asparagine/aspartic acid and glutamine were added to XDM_2 and XDM_3 media. However, the amino acids serine and glycine could also be obtained from degradation of threonine, since genes for serine hydroxymethyltransferase (EC 2.1.2.1; XF0946) and for the transfer of the 5,10-methylene-THF group (glycine cleavage system T protein, EC 2.1.2.10; XF0183) were found in *X. fastidiosa* genome. We then decided to add serine to three defined media

(XDM_1, XDM_2 and XDM_3), since this amino acid could enable the synthesis of cysteine, although the enzyme which performs the first step of this pathway, serine acetyltransferase (EC 2.3.1.30), was not found in *X. fastidiosa*.

3.2. Sulfur sources

Various sulfur compounds, especially cysteine, methionine and *S*-adenosylmethionine, are essential for growth and activities of all bacteria. Cysteine biosynthetic pathway plays an important role in the incorporation of inorganic sulfur into organic compounds like methionine, thiamine, biotin, lipoic acid and coenzyme A [15]. Besides the apparent absence of serine acetyltransferase, there are other enzymes missing in *X. fastidiosa* which are important for sulfur amino acids including cystathionine β and γ-lyases, homoserine-*O*-succinyltransferase and 2,4,5-methyltetrahydrofolate-homocysteine methyltransferase [2]. However, growth of *X. fastidiosa* was more vigorous when methionine rather then cysteine was added to the medium (data not shown). When the sole sulfate source in XDM_4 medium was $MgSO_4$, the cells grew very little in 14 days, showing that *X. fastidiosa* depends on sulfur-containing amino acids in order to support its in vitro growth. Indeed, *X. fastidiosa* grew much better in XDM_5 media

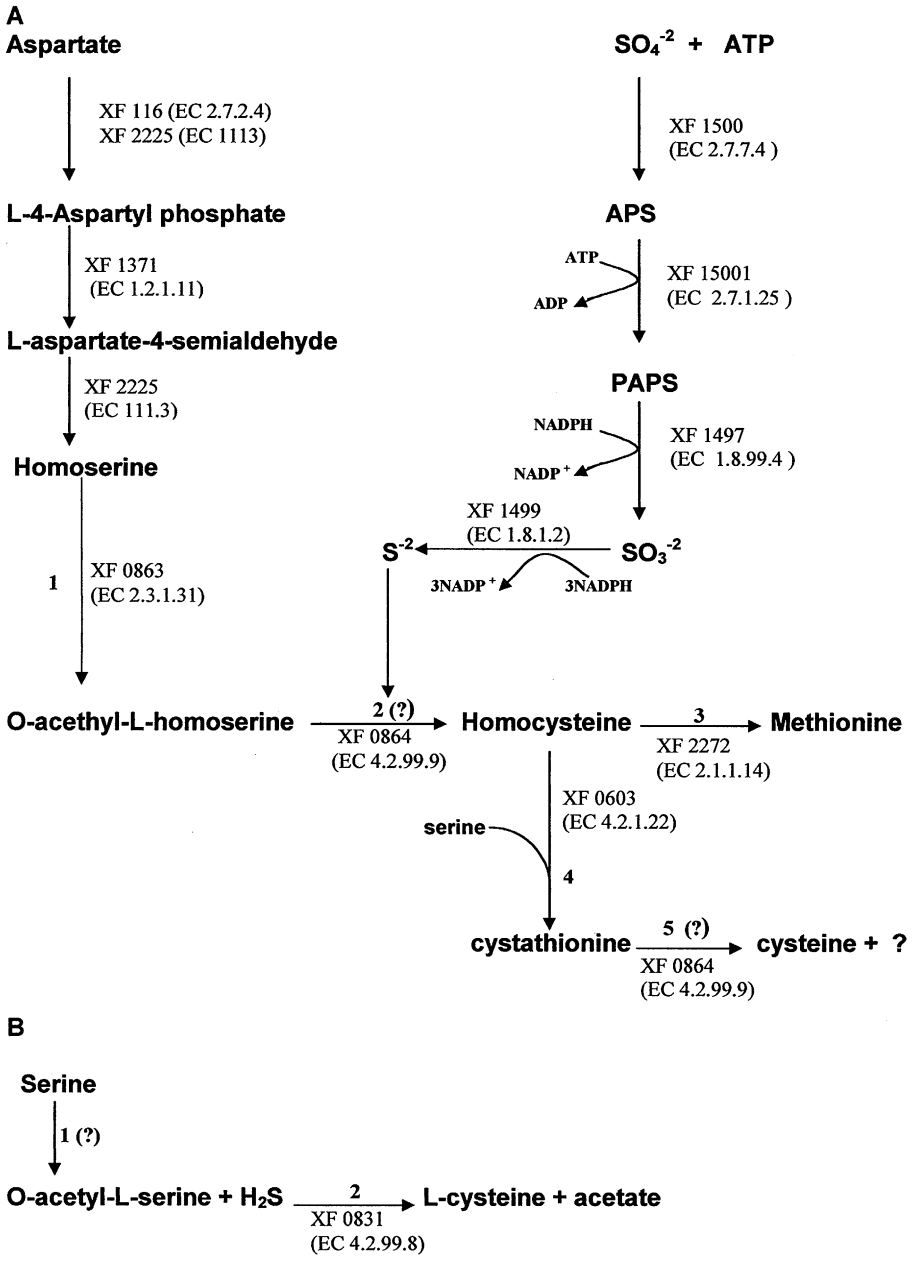


Fig. 3. Proposed pathways for the biosynthesis of methionine and cysteine in *X. fastidiosa*. Enzyme steps: (A) 1. homoserine O-acetyltransferase; 2. cystathionine-γ-synthase 3. methionine synthase; 4. cystathionine-β-synthase; 5. cystathionine-γ-synthase; (B) 1. serine acetyltransferase; 2. O-acetylserine sulfhydrylase. The question marks indicate a step that is incompletely characterized (A) or a missing enzyme (B).

that additionally contained methionine (Fig. 1). This observation becomes clearer when we consider the great amount of energy that is needed for sulfur reduction in the biosynthesis of cysteine and methionine. Sulfate is activated in two steps to produce 3-phosphoadenosine 5′-phosphosulfate, which then undergoes an eight-electron reduction to sulfide. The in vivo biosynthesis of these amino acids could be carried out by way of the aspartate to *O*-acetyl L-homoserine and reduced sulfur pathways, as all the necessary enzymes were found in *X. fastidiosa* genome (Fig. 3).

The transsulfuration of *O*-acetyl L-homoserine to L-homocysteine by cystathionine γ synthase (EC 4.2.99.9; XF 0864) could take place through two probable routes in *X. fastidiosa* (Fig. 3). The first one is related to methionine synthesis by way of methionine synthase (EC 2.1.1.14; XF2272), whose amino acid sequence has 56% identity ($E < 10^{-130}$) with the same protein from *Escherichia coli*, which transfers the methyl group from 5 methyltetrahydrofolate to homocysteine, producing methionine. The second route would involve cystathionine β-synthase (EC 4.2.1.22; XF0603), which acts on the reaction between serine and L-homocysteine, yielding cystathionine. At this point, another important question rises: which enzyme converts this substrate to cysteine, since the enzyme cystathionine β-lyase (EC 4.4.1.8) was not found among the genes described for *X. fastidiosa* as well as for *B. subtilis* [13]. In the proposed methionine biosynthesis pathway of *X. fastidiosa* found in the KEGG database ([16]; http://www.genome.ad.jp/dbget-bin/show_pathway?xfa00271) cystathionine γ-synthase (EC 4.2.99.9; XF0864) is considered a multifunctional enzyme, catalyzing different reactions of the cysteine and methionine metabolism.

The synthesis of cysteine by the condensation of *O*-acetylserine with sulfide, catalyzed by acetylserine sulfhydrylase (EC 4.2.99.8; XF0831) may occur in *X. fastidiosa*, although the first step of the reaction (which is serine acetylation by serine acetyltransferase) needs to be clarified since this enzyme was not found in the genome.

Transsulfuration is the main reaction in microorganisms [17] and plants [18] that incorporates sulfur into L-homocysteine, which in turn, is produced from L-cystathionine through a reaction catalyzed by cystathionine β-lyase (EC 4.4.1.8). These organisms first synthesize L-cysteine with *O*-acetyl-serine and sulfide and subsequently synthesize L-cystathionine with L-cysteine and L-homoserine by the catalytic action of cystathionine γ-synthase (EC 4.2.99.9). This enzyme reacts with acetyl-L-homoserine (fungi and bacteria), *O*-succinyl-L-homoserine (enteric and other bacteria), or *O*-phosphoryl-L-homoserine (plants). In another plant pathogen, *Pseudomonas syringae*, methionine biosynthesis is achieved by an acylhomoserine intermediate and sulfur assimilation is by transsulfuration [19]. Some archaea synthesize cysteine from methionine through reversed transsulfuration from homocysteine to cysteine [20] but an *O*-acetyl-L serine sulfhydrylase (EC 4.2.99.8) has been found in an archaeon, suggesting that the enzyme is a functional as a cysteine synthase. Based on these considerations, we have chosen to add methionine to the XDM_2 and XDM_3 media in order to allow a faster growth with a lower energy requirement.

3.3. Vitamin sources

Although genomic analysis suggested that vitamins thiamine, biotin, nicotinic acid and pyridoxine are synthesized de novo in *X. fastidiosa*, their addition to the defined media promoted faster growth in all XDM media. Myo-inositol was added to the media since we did find in the genome the gene encoding inositol monophosphatase (IMPase, EC 3.1.3.25; XF2476), an enzyme that catalyzes the dephosphorylation step in the de novo biosynthetic pathway of inositol and is crucial for all inositol-dependent processes [21]. Genes encoding IMPase homologs have been identified in the genomes of *Escherichia coli* [22], *Thermotoga maritima* [23] and *Methanococcus jannaschii* [24], and in the bacterium *Rhizobium leguminosarum* bv. *trifolii* [25] and bv. *viciae* [26]. The role played by this enzyme in prokaryotes is still unknown, but it probably has a pleiotropic role in metabolism [22]. Recently, the IMPase was implicated in exopolysaccharide synthesis in *R. leguminosarum* bv. *trifolii* [27].

Inositol is a component of plant galactinol, which may be a specific precursor of cell wall polysaccharides. Interestingly, phytone peptone, a papaine digest of soybean meal, is a major component of the traditional *X. fastidiosa* growth medium PW [5]. Large amounts of phytic acid are present in grains, usually as calcium or mixed Ca^{2+}–Mg^{2+} salts [28]. This is consistent with the improved growth of *X. fastidiosa* on media containing such material. It is hypothesized that *X. fastidiosa* degrades the pit membrane in xylem vessels using pectolytic enzymes as well as cellulases and polygalacturonases to move within the plant. Such a process may mobilize cell walls for nutritional purposes and sugars like inositol would be available to fulfill *X. fastidiosa* metabolic requirements.

4. Conclusions

Although some essential enzymes for important biochemical pathways were not apparent in the *X. fastidiosa* genome, these enzymes may in fact be present. Possible reasons for the apparent existence of such 'missing enzymes' in microbial genomes are: (a) incorrectly assigned gene identities in the genome databases; (b) enzymes encoded by novel, 'analogous' or low-similarity genes; (c) the presence of multifunctional enzymes; and (d) presence of unknown 'bypass' pathways [3].

From these simple experiments on bacterial growth in culture media of variable composition, it was clear that *X. fastidiosa* can grow using glucose as carbon source, a

few amino acids as nitrogen sources, and inorganic components such as iron, phosphate, potassium, magnesium and sulfate and the need of only a few vitamins for growth. This work demonstrated that analysis of growth requirements is a useful way to test the annotation of bacterial genomes.

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