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PCR Protocol- and Inulin Catabolism-Based Differentiation of Inulinolytic Soil Bacteria

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ABSTRACT

Bacteria collected from rotting dahlia tubers, instead of degrading inulin to D-fructose, preferentially formed the known DFA III (β-2.1′: α-2′,3 difructofuranose anhydride), inulobiose, higher inulo-oligosaccharides, and exoheteropolysaccharides. Owing to the morphological and Gram staining variability, the bacterial isolates designated YLW and CRM were examined to differentiate them from a reference strain *Arthrobacter ureafaciens*. The comparative analyses were whole DNA random amplification by *Taq* polymerase (RAPD-PCR protocol), culture media DFA III content in culture media, chromatographic profile of oligosaccharides formed, and exopolysaccharide fractionation/fragmentation.

A comparative study in liquid shake cultures showed that the isolate YLW was faster than the reference strain in the production of DFA III when the inulin/yeast extract ratio was maintained at 10 in the medium, although a similar maximum yield was displayed with both bacteria (13–14 mg of DFA/mL cell free media from the initial 30 mg/mL of inulin load). Doubling the yeast extract input, an even faster onset of DFA III production occurred with YLW but with no further improvement in the maximum yield. Both strains further degraded the resulting DFA during the stationary growth phase.

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The main ability of CRM when grown on inulin was the production of exopolysaccharides, although culture condition variation also allowed DFA III production, which was accompanied by somewhat lower amounts of its reducing analog, inulobiose.

Index Entries: DFA III; difructose anhydrides; inulobiose; exopolysaccharides; inulinolytic bacteria.

INTRODUCTION

Inulin processing has been undergoing increased interest since its main product, D-fructose, occupies a relevant position in the sweetener market (*1*). The reasons favoring its consumption are:

1. Its natural occurrence both as the free sugar in the majority of ripened fruits and as a plant reserve in its polymeric form, inulin (Jerusalem artichoke, chicory, and dahlia tubers).
2. Its physiological metabolic fate on entering the glycolytic pathway.
3. Its higher sweetening value when compared to invert sugar, sucrose, or glucose, having the advantage of no marked insulinogenic effects.

Two points deserve evaluation if one considers the occurrence of structurally related fructose dimers, such as inulobiose (a β-2,1′ fructobiose, the building block unit representative for inulin backbone) and DFA III (β-2,1′:α-2′,3 difructofuranose anhydride, the nonreducing derivative of inulobiose formed by elimination of water between positions 2′ and 3). First, the degree they retain the sweetness of the monomer, and second, the competitive to inhibitory effect of DFA III in the course of fructose metabolism itself, as indicated by our previous study using perfusion of isolated rat liver (*2*). This finding may mean some kind of beneficial dietetic effect.

The family of difructose anhydrides and diheterolevulosans appears as by-products during the acid hydrolysis of inulin but their amounts relative to fructose is low. One of these isomers, DFA III, accounts for ~5% of the free sugar (fructose) released in hydrochloric or sulfuric mild hydrolyzates of inulin (*3*), and it is also produced although in smaller amounts paralleling those of hydroxymethylfurfural during the mild phosphoric acid hydrolysis of inulin (*2*). On the other hand, DFA III may be almost quantitatively prepared from inulin by a microbial procedure using selected strains of the soil Gram(+) bacterium *Arthrobacter* (*4,5*). In addition, DFA III hydrolysis may lead to formation of both inulobiose or fructose, depending on substrate hydration with 1 or 2 mol of water.

DFA III was reported as having some sweetening properties (*4*). The possibility of its use in conjunction or as a replacement of fructose was

preliminarily evaluated in our laboratory using isolated rat liver perfusion and a marked negative effect on fructolysis, namely, a drop in both pyruvate and lactate production, was observed (2).

Most of the reported data on bacterial generation of DFA III is centered on *Arthrobacter ureafaciens* owing to the pioneering work carried out in Japan (4,5). On our part we decided to proceed with the biochemical, physiological, and genetic characterization of our previous bacterial isolates looking for advantages or alternatives in the bioprocessing of inulin.

MATERIALS AND METHODS

Substrates and Preparation of Standards

Inulin was obtained from cortex-free dahlia tuber slices by hot water extraction (70–80°C), the extract being then kept at 4°C overnight to render inulin insoluble, and the filtered polysaccharide finally washed ethanol and acetone. The resulting pale yellow powder was further purified by filtration through a bed of DEAE-cellulose to remove acidic contaminants, and the aqueous eluate was reprecipitated with 3 vol of ethanol and the residual water removed by lyophilization. The DFA III standard was kindly provided K. Tanaka (Osaka Kyoiku University, Japan). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Merck (Darmstadt, Germany).

Bacterial Sources, Maintenance, and Growth Conditions

The reference bacterium *Arthrobacter ureafaciens* (strain 7562; called Au) was obtained from ATCC (Rockville, MO). Two bacteria isolated from rotting dahlia tubers (2), YLW and CRM as well as the reference Au, were stored as lyophilized cells in skimmed milk. Two different culture media were used: 3 g% inulin, 0.3 g% yeast extract, and 10 m*M* pH 5.8 dihydrogen potassium phosphate; and with the same carbon source and concentration but with yeast extract raised to 0.6 g%, and potassium phosphate replaced by 0.1 g% dihydrogen ammonium phosphate, final pH 6.2. Both media also contained 0.08 g% of magnesium sulfate heptahydrate. All three lyophilized strains were submitted to two sequential cultures until the stationary phase in a 3 g% fructose-based media before transfer to the final inulin-based media. Cell transfer after centrifugation and resuspension in fresh medium ensured a light inoculum (initial absorbance $\geq$ 0.05–0.06 U at 650 nm). Volumes of air:culture medium were kept in the ratio 5:1 and scaling up was from 25 mL and 100 mL (fructose) to 200 mL (inulin). Growth in Erlenmeyer flasks at 100 rpm was carried out using a New Brunswick rotary shaker at 28°C. Three-mL samples were

collected at intervals from 12–36 h. The duplicate simultaneous experiments with the three strains was extended to 252 h, and pH measurements being made using a portable potentiometer from Cole-Parmer (Chicago, IL).

The CRM isolate was more extensively cultivated with yeast extract being reinforced by casein hydrolyzate or Vogel's salts (*6*). The effect of magnesium sulfate was also investigated in the 0.02–0.12 g% range.

Gram staining was routinely applied to bacterial smears from all stages of maintenance and growth (*7*).

The bacterial strains were tentatively characterized using the API 20E System kit (Div. Sherwood Medical, New York), along with the recommended pathogenic strains for the biochemical tests control (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*).

Metabolite Analyses

Three volumes of ethanol were added to the cell-free medium samples and the resulting supernatants analyzed by chromatography for the presence of DFA III, inulobiose, and higher oligosaccharides by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas-liquid chromatography (GLC), as indicated in the text or in the legends of Figs. 4 and 5. The respective precipitates, once dried and subjected to gravimetry, were examined for reduced viscosity in a Cannon-Feske viscometer (*8*), for residual inulin content and for exopolysaccharide fragmentation analyses. TLC plates were developed using orcinol (*9*) in order to ensure a clear color-based differentiation between the mono- and disaccharides. Preparative paper chromatography was applied specifically for the separation and recovery of DFA III and inulobiose, which were further used as standards. Crude exo(hetero)polysaccharides (EPS) were fractionated by anion exchange on a DEAE-cellulose column (acetate form), sequential elution being performed with water (neutral fraction), and 0.1 and 0.1*M* lithium chloride (acidic fractions). EPS fractionation was also carried by complexing with Cetavlon (cetyltrimethylammonium bromide) at a pH range from 7.0–12.0 (*10*). Two trifluoroacetic acid (TFA)-based procedures were adopted for polysaccharide hydrolysis, namely a mild option (selective for inulin and their fragments, except for DFA III), at pH 2 for 5 min, the resistant residual exopolysaccharide recovered by addition of an excess of ethanol (5 vol) to the partial hydrolysate, and stronger conditions with 1.3*M* TFA for 12 h as a conventional hydrolytic procedure for EPS. Both procedures were carried out at 100°C. DFA III purity was checked by ^{13}C-NMR spectroscopy with a 150-MHz Varian spectrometer using D_2O as solvents and TMS as external standard ($\delta = 0$). Chemical shifts were expressed as δ in ppm (parts per million). Total carbohydrate was assayed by the phenol-sulfuric acid method (*11*).

The random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) protocol was applied to whole bacterial DNA (*12,13*). In

summary, DNA was extracted and purified from freshly grown bacteria following lysis with lysozyme and Triton X-100. Short primers (10 bp) were used for the *Taq* (*Thermus aquaticus*) polymerase-mediated random DNA amplification step. The incubation system contained 37 μL of water, 5 μL of buffer (200 m*M* Tris-HCl pH 8.5 buffer, 500 m*M* KCl, 60 m*M* $MgCl_2$, and 0.1% BSA), 1 μL of dNTP mix (each deoxynucleotide at 10 m*M*), 2 μL of primer (10 μg/μL), and 5 μL of DNA (1 μg/μL). The temperature program was: first cycle at 98°C for 2 min, 50°C (addition of 4 U of enzyme), 35°C for 1 min, 72°C for 2 min, and additional cycles at 92°C (1 min), 35°C (1 min), and 72°C (5 min). This protocol was continued until 34 complete cycles for each primer was reached. The resulting products (five or more amplification bands, on average, owing to the selected primers) were examined comparatively on a 5% polyacrylamide gel using Tris-EDTA-borate buffer (*14*) developed with the silver stain. The amplification profiles were also confirmed using agarose gels revealed with ethydium bromide.

RESULTS AND DISCUSSION

The inulinolytic bacterial strains Au (ATCC 7562 reference strain), YLW, and CRM (morphologically different isolates from rotting dahlia tubers) were studied in order to evaluate some of their genetic and physiological characteristics. The intention is the production of fructose dimers, namely DFA III M and inulobiose, and to elucidate ability of CRM diverting inulin catabolism to viscous heteropolysaccharides.

The pigmentation of the gummy colonies (CRM: cream, Au: yellow, and YLW: deep yellow), cell morphologies (from wide to small coccoid rods), and Gram staining, consistently negative only for CRM, were not significant parameters for strain differentiation. The marked variability of shape and staining observed with Au and YLW was in full agreement with behavior previously reported for *Arthrobacter* spp. (*15–17*). The use, with reservations, of a biochemical kit for enteric bacteria allowed, at least, a partial differentiation of AU and YLW from CRM since the latter gave clear positive reactions in the *o*-nitrophenyl-galactoside (ONPG), lysine decarboxylase (LDC), citrate (CIT), Voges-Proskauer (VP); acetoin from sugars, and gelatin liquefation (GEL) tests.

Modern methodologies are being developed for the purpose of rapid and trustworthy differentiation and identification of several genotypes, and those based on PCR are applicable to polymorphism detection in plants, animals, and bacterial spp. (*18*). Such technical advances include the arbitrarily primed PCR (AP-PCR), which requires minute amounts of DNA (10–25 ng range) and random-amplified polymorphic DNA (RAPD), which is its subset employing reduced-size primers, and both were used in our

comparative study. The electrophoretic results for DNA amplification are shown in Figs. 1A,B. The reactions guided by the primers 8, 10, 11, 13, 63, and 64 pointed to three different strains. Primers 9 and 62, possibly as a result of weaker binding to YLW and Au DNA(s), gave poorer amplifications in contrast to the more marked amplification displayed by CRM with the same primers. Two well-matched bands of higher mol wt were clearly visible comparing Au and YLW amplification patterns using primers 9 and 62. Taking the latter distinct amplification patterns as a clear indication of some genomic similarity, they were in agreement with the Gram staining of the bacteria and with the data arising from inulin catabolism. Au and YLW isolates, as discussed below, really showed some similarities of biochemical behavior in terms of inulin bioconversion, which, in turn, were not detected in the CRM isolate, namely the generation of tri- to pentaose inulosaccharides.

The ^{13}C-NMR spectrum for the main product, DFA III, is shown in Fig. 2. As compared to those of inulin or inulobiose, the profile of a dimer with 12 signals is in accordance with the inverted anomericity of the two fructofuranose units. Inulin spectrum for instance, owing to the homogeneous β-2,1′-linked backbone, presented only six relevant signals. The quantitation of DFA III was rapidly carried out by HPLC using a carbohydrate column analysis (CAC) column from Waters, Millipore (Bedford, MA) eluted with acetonitrile:methanol:water (7:2:1, v/v/v) at 45°C and under a pressure of ~700 psi. Under these conditions, the increasing retention times, in minutes, were: 2.03 (DFA III), 2.28 (fructose), inulobiose (3.65), inulotriose (6.72), and inulotetraose (13.28). The identity between the authentic DFA III sample (ex-Japan) and our preparation was established by capillary GLC analysis on a HP-5 column (0.25 mm ϕ × 25 m, Hewlett Packard, isothermically at 280°C, nitrogen carrier at 20 psig, make up gage) using persilylated derivatives. The observed retention times were: derivatives of mixed isomers of fructose = 1.69; β-phenyl-D-galactoside internal standard = 2.68; DFA III = 3.34; inulobiose (α- and β-anomers) = 4.17 and 4.53. Sucrose could not be distinguished from inulobiose since its peak was superimposed on the second one eluted from inulobiose.

Data resulting from the simultaneous culture of the three bacteria in inulin are shown in Figs. 3A and 3B. Inocula for these inulin-based media were obtained by repeated culture in free fructose, which allowed a net induction and/or expression of the genomic capacity for inulin bioconversion(s). Distinctive pH curves of culture media were seen only for CRM isolate in both low (medium A) and high (medium B) yeast extract loads. In contrast, Au and YLW behaved very similarly. No great differences were observed for the growth curves ($A_{660\ nm}$), except for proportionally higher cell masses obtained for Au and YLW strains in the richer medium B. The more dramatic feature was the generation of DFA III, which was a biochemical ability of the two latter bacteria, and recalled the fructose (inoculum) to inulin (final culture) shift. The performance of the

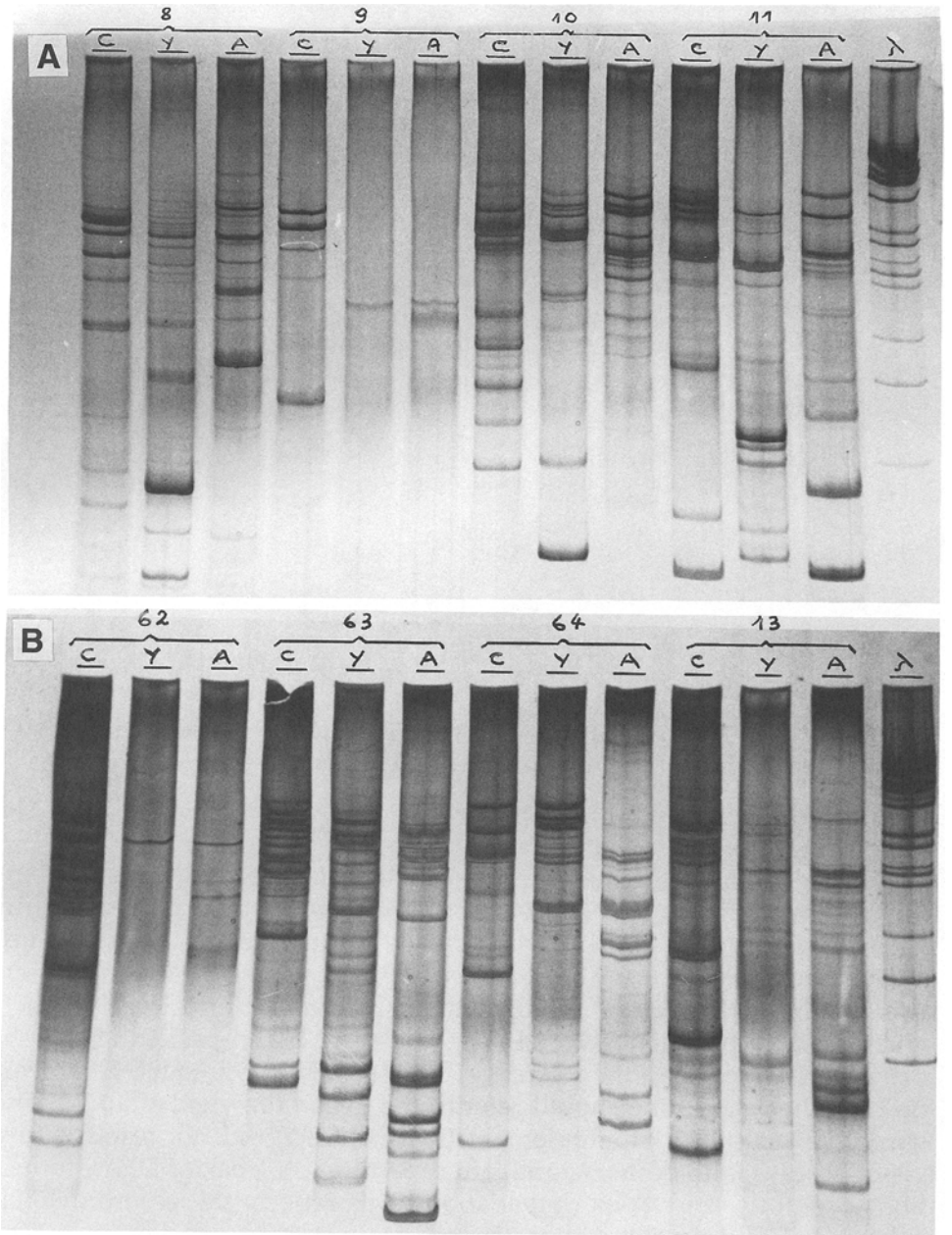

Fig. 1. Silver-stained PAGE eletrophoretogram of the RAPD-PCR on the whole DNA from CRM (C), YLW (Y), and Au (A) bacterial strains. Numbers refer to the 5′ → 3′ primers used in the PCR: 8 = TCACGTCCAC; 9 = CTGACGTCAC; 10 = AGGGCCGTCT; 11 = TGCCCGTCGT; 62 = GGGTAACGCC; 63 = CAGCACCAG; 64 = TGCCGAGCTG: and 13 = CTCTCCGCCA; last lane = phage λ digest with *Eco*R I plus *Hind* III restriction enzymes, as mol-wt marker; 0.56 kb as the shortest band and 3.48 kb as the largest clearly resolved band.

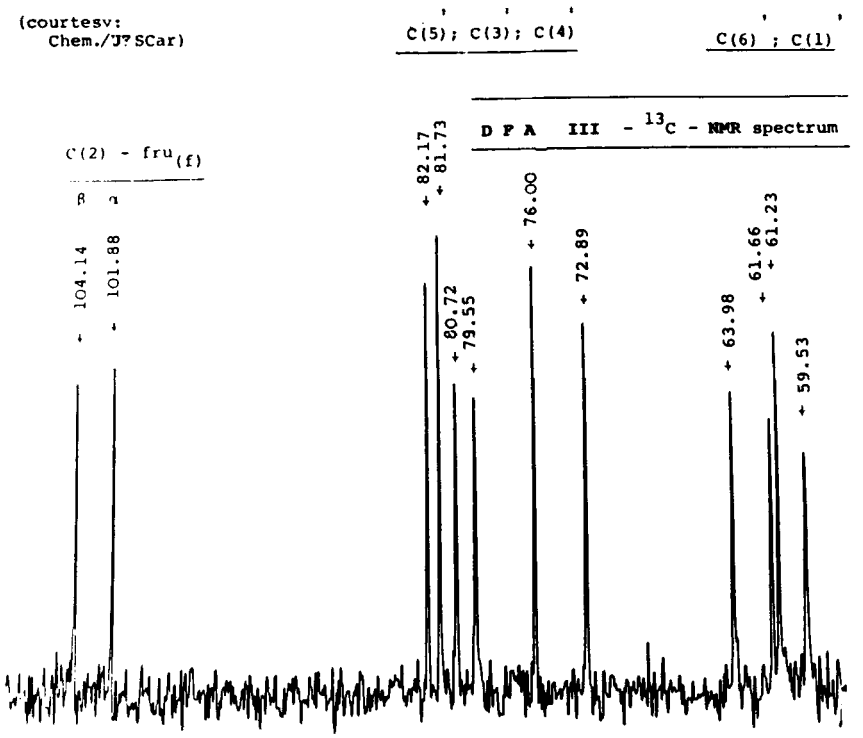

Fig. 2. ^{13}C-NMR spectrum of DFA III. The numbers heading the spectral signals are the chemical shifts expressed as δ in ppm).

Au reference strain (Fig. 3A) was identical to that of YLW in terms of bioconversion efficiency (ca. 13–14 mg/mL from 30 mg/mL inulin; medium A, low yeast extract), but the latter strain was superior since DFA production peaked earlier (about 90 h of advantage). The performance of our strain was confirmed using the richer medium B (Fig. 3B; high yeast extract) when DFA III production by YLW started ~12 h and peaked at ~31 h, being more rapid than the reference strain (~58 h advantage). However, on the other hand, one should refer to the maximum yield attained. The surplus expenditure of supplement (yeast extract) did not result in any yield increase, and both bacteria, after reaching the peak of DFA production, promptly started its degradation. This reflects the environmental behavior of this class of soil bacteria.

An overall view of the biochemical transformation of inulin up to 252 h in shake cultures of each of three isolates can be seen in Figs. 4A (Au), B (YLW), and C (CRM). The additional information concerned the release of inulooligosaccharides of up to 4 U, besides DFA III (the fasted spot in all chromatographic runs; R_f 0.52). It may be pointed out that free fructose was almost never detected (standard spot with R_f 0.36). A quite similar family of oligosaccharides was visible for the duplicate cultures with low and high loads of yeast extract of isolate YLW (Fig. 4B) but the high sup-

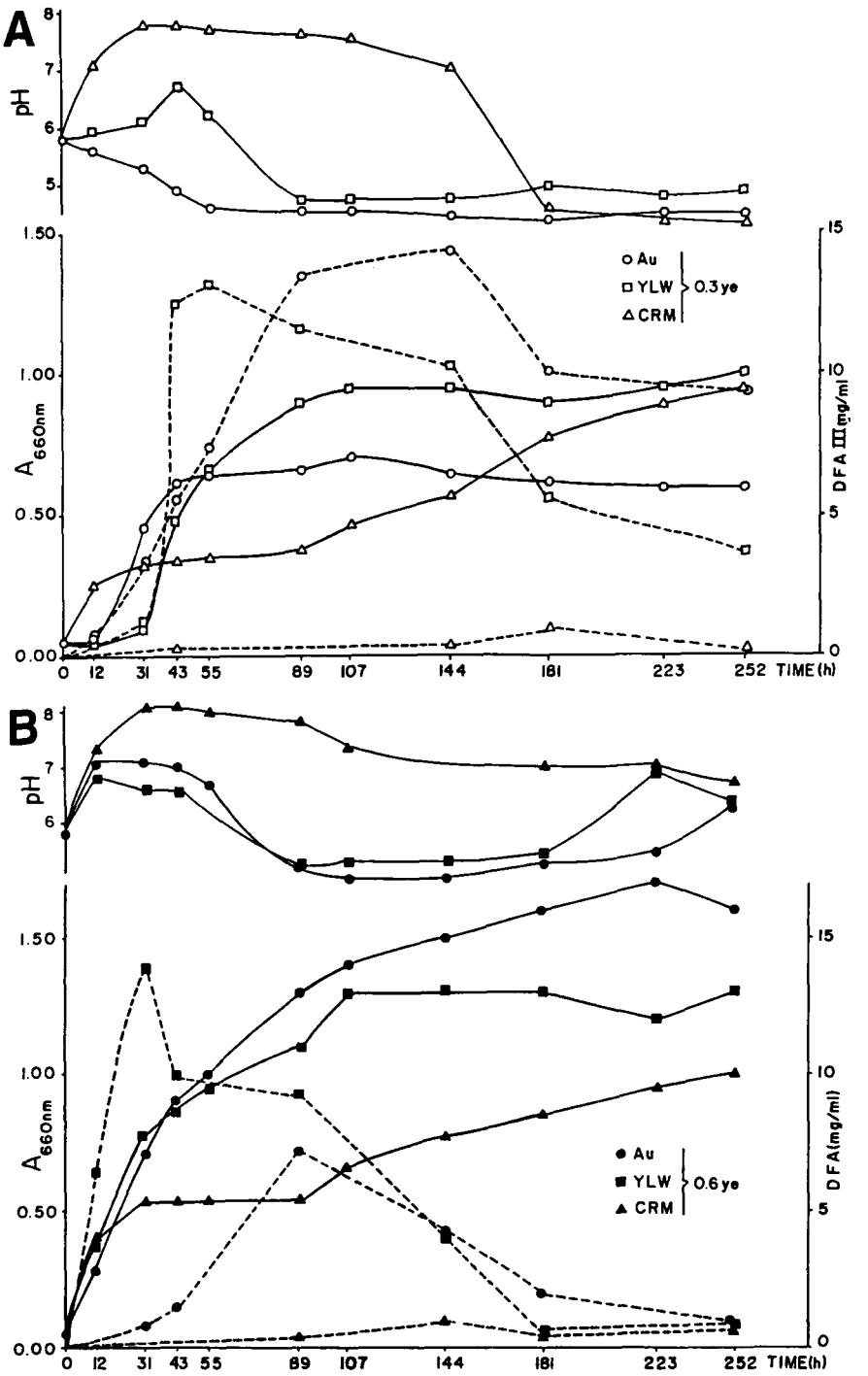

Fig. 3. Inulin bioconversion to DFA III: dependence on yeast extract load and on culture age. (-----) = growth (absorbance at 660 nm); (-----) = pH variation; (- - -) = DFA III (mg/mL) in the cell-free media. Note that 0.3 and 0.6 ye refers to the yeast extract concentration in g% as supplement to 3.0 g% inulin; initial buffering with phosphate salts as in Materials and Methods; symbols for the bacterial strains identification as in the figure inserts.

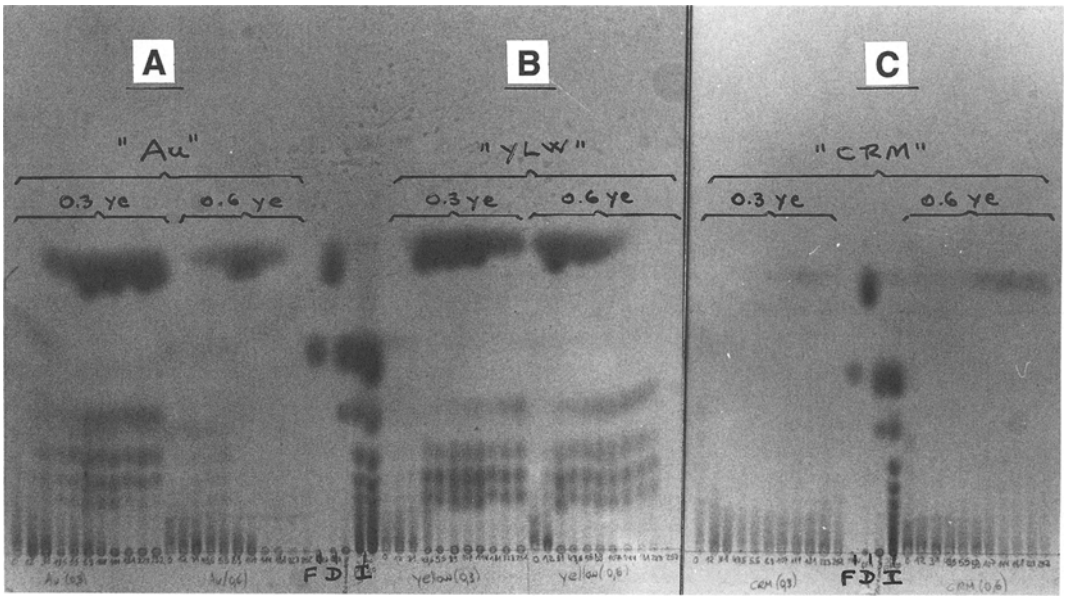

Fig. 4. TLC monitoring of the low-mol-wt carbohydrates in the cell-free media of the comparative cultures of inulin bioconversion depicted in Figs. 3A and 3B. Stationary phase = Merck's silica gel 60 chromatoplate; solvent = isopropyl alcohol:nitromethane:ethyl acetate:ethyl-methylketone:water = 50:45:50:25:30; revelation spray = 0.5 g% orcinol in methanol containing 5% sulfuric acid by heating at 100–120°C. The fastest spot in all cases is DFA III. Standards: F = fructose; D = DFA III; I = inulooligosaccharides obtained by mild TFA hydrolysis. The double-spot lane left-marginal do standard I is a mix of fructose > inulobiose. In order to render DFA III detectable in the time course of CRM culture (Fig. 4C), its cell-free medium was overloaded as compared to Au and YLW (Figs. 4A and B).

plement load did not favor this profile for the Au (Fig. 4A). The faster moving spot of this family (R_f 0.24) was inulobiose, as further checked by partial linearity of the classical plot of log [(1/R_f) - 1] vs DP (degree of polymerization). Figure 4C (sugar spots visible only owing to the overload of the samples in the TLC) demonstrated that the exopolysaccharide-producing strain, CRM, was the poorest DFA III and oligosaccharide producer under these experimental conditions, i.e., under the "shift effect" of going from fructose to inulin as carbon sources. However, CRM in repetitive liquid culture with inulin significantly changed the profile in inulin catabolism. Figure 5 shows that, not taking in account the inulin fraction diverted to exopolysaccharide production, only two by-products were released in significant amounts, namely DFA III and inulobiose, although this only occurred at the late phase of growth (>168 h until 360 h; right half; Fig. 5). The earlier stages of CRM adaptation (until 96 h; left half; Fig. 5) did not

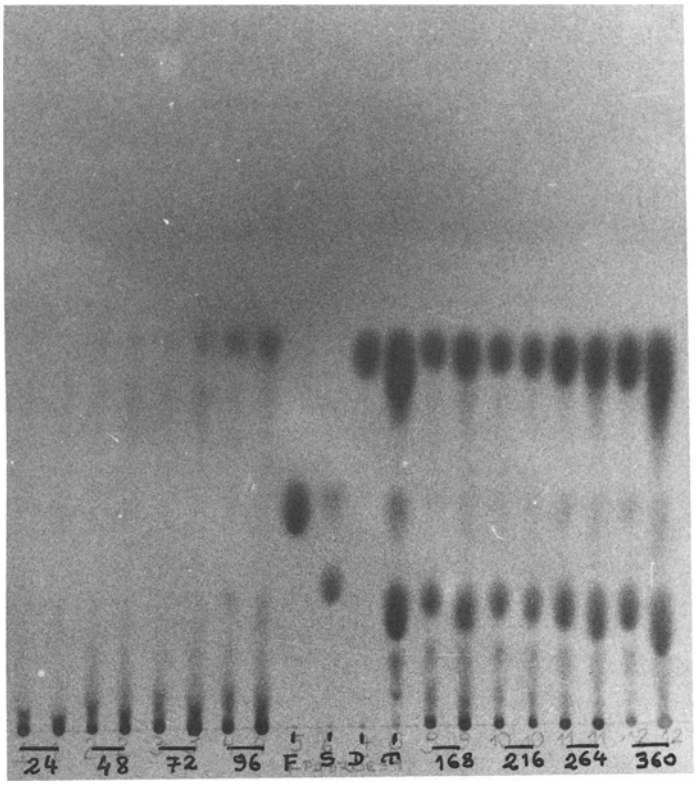

Fig. 5. TLC monitoring of DFA III and inulobiose production by the CRM strain following its full adaptation to inulin as carbon source. TLC conditions as in Fig. 4; spots with R_f 0.47 and 0.15 are DFA III and inulobiose; Standard: F = fructose, S = sucrose, D = (crystalline) DFA III, and T = Total free sugars from a CRM stationary phase culture using magnesium-free medium; numbers refer to the time of culture, in hours.

lead to DFA accumulation. TLC may also point to the onset of inulobiose production after that of DFA, since the rehydration of the latter effectively produces inulobiose.

Exopolysaccharide (EPS) production from CRM growing on inulin was further investigated and its framentation analysis partially carried out. The inclusion of 0.02–0.08 g% magnesium sulfate in the culture media was advantageous. Crude EPS, following cell removal by centrifugation, almost always contaminated with some residual inulin. The elimination or separation of the latter could be accomplished by one of three ways: mild TFA hydrolysis, DEAE-cellulose chromatography using warming for resin bed, sample, and eluant, or Cetavlon complexation. The first procedure was rapid and effective, since no labile sugar other than fructose was lost, such as rhamnose units of the EPS, but did not solve the

problem of EPS subfractions separation, which was easily achieved by complexation with Cetavlon at pH 7.0 (acidic fraction) and at pH 12.0 in the presence of sodium borate (neutral fraction) (average ratio of fractions 3:1). Reduced viscosity measurements of all stages of polysaccharide processing indicated the following values expressed as %/g: whole ethanolic precipitate from cell-free medium, 7.67; the same crude material after inulin removal by mild TFA, 5.80; neutral exopolysaccharide fraction, 0.0; and, acidic fraction, 10.07. The respective approximate flow times in the viscometer were 120, 105, 56, and 140, and water flow was 56 s. Thus, any viscosity could be attributed to the acidic fraction whose complete hydrolysis (strong TFA), followed by HPLC, revealed rhamnose (R_T = 6.95) and galactose (R_T = 11.71) as the only neutral monosaccharides (ratio ca. = 1:1), detected using a Waters FAM column eluted with 70% acetonitrile containing 3 m*M* phosphoric acid at 65°C. Some aldobiouronic acid was present in the TFA-hydrolyzed EPS sample, and it was isolated by preparative anion-exchange chromatography in Dowex-1 (acetate form) resin, from which it was eluted with 10% aqueous acetic acid or with the water acidified with formic acid to pH 2. Its complete hydrolysis was followed by TLC, and paper electrophoresis (*19*) indicated that its components were rhamnose and glucuronic acid.

The compositional profile of EPS from CRM was not markedly affected by the culture media variation (crude EPS recovery, before residual inulin removal, of about 1 g for the 3 g inulin input or 100 mL of cell-free medium). Despite the mixed composition and some dynamic interaction with residual inulin as indicated by the preliminary measurement of reduced viscosity, EPS production by CRM deserves further investigation since the complete consumption of the carbon source inulin was not achieved yet. From the comparative investigation on inulin, some EPS could be also isolated from Au and YLW (19–62 mg/100 mL of cell-free media). These latter polysaccharides, naturally free of any residual inulin, furnished on complete hydrolysis a different monosaccharide composition of mannose >> glucose. Thus, EPS production was also a valuable parameter for strain differentiation, confirming a close relation between the reference strain and our YLW isolate. The best EPS producer, CRM, resulted again different owing either to the polysaccharide nature (acidic rhamnogalactan) or to the higher EPS yield.

CONCLUSIONS

A satisfactory comparison was carried out on soil inulinolytic bacteria. The differential parameters were biochemical and physiological properties, such as the production of DFA III (difructofuranose anhydride), inulobiose, and EPS (exopolysaccharide) from inulin along with, from a genetic standpoint, the DNA amplification profile obtained using the PCR-guided protocol.

One of our bacterial isolates from rotting dahlia tubers, YLW, proved to produce DFA more rapidly than the *Arthrobacter ureafaciens* reference strain. Inulobiose and exopolysaccharide could be other interesting co-products of inulin to be obtained using the other isolate, CRM.

Since a previous experiment concerned with cloning of protoplasts of *Arthrobacter* indicated good genetic stability for the resulting recombinant strains (*20*), this approach could be useful in the reach for a recombinant, which combines the improved ability of YLW for DFA III production with the capability of CRM in the generation of its reducing counterpart, inulobiose.

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