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ORIGINAL ARTICLE

Enterobacterial repetitive intergenic consensus (ERIC) PCR based genetic diversity of Xanthomonas spp. and its relation to xanthan production

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ABSTRACT

Background and Objective: The genus *Xanthomonas* is composed of phytopathogenic bacterial species. In addition to causing crops diseases, most of the *Xanthomonas* species especially *Xanthomonas campestris* produce xanthan gum via an aerobic fermentation process. Xanthan gum is, an important exopolysaccharide from *Xanthomonas campestris*, mainly used in the food, petroleum and other industries. the purpose of this study was assessment of relationship between genetic diversity and xanthan production in *Xanthomonas spp*.
Materials and Methods: In this study 15 strains of Xanthomonas spp. which had previously been isolated from soils of vegetable farms, were discriminated from each other using Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR and 16S rDNA sequencing methods. Xanthan production of strains was measured in 250 ml flask. The results of ERIC PCR and xanthan production was compared.
Results: ERIC-PCR patterns not only could differentiate all *Xanthomonas campestis* from the control i.e. *Xanthomonas translucent* but also discriminate strains of *Xanthomonas* to three clusters with 40% similarity based on Jaccard's coefficient. This clustering of the strains was in agreement with other characteristics including xanthan production and biochemical features.
Discussion: The results showed that genomic fingerprinting conferred adequate genetic data for discriminating between strains of the species *Xanthomonas campestris*. The data indicated a partial relationship between ERIC-PCR patterns and xanthan production by the strains.
Conclusion: Further development of experiments may result in making good prediction about xanthan production capability of the X*anthomonas* strains on the basis of ERIC-PCR method.

Keywords: Genetic diversity, *Xanthomonas*, Xanthan, ERIC-PCR

INTRODUCTION

The genus *Xanthomonas* is composed of phytopathogenic bacterial species that cause diseases in diverse crops and results in declined yields. Strains of the genus also can produce xanthan gum through an aerobic fermentation (1). Xanthan gum is a high molecular weight anionic polysaccharide. This polymer displays appropriate properties: high viscosity at low concentrations, pseudoplasticity, and insensitivity to a wide range of temperature, pH and electrolyte variations. Because of its special rheological properties, xanthan is used in food, cosmetics, pharmaceuticals, paper, paint, textiles, adhesives and oil and gas industry (2). The flow characteristics of xanthan, coupled with its stability to salts and extremes of pH, gives it a technical advantage over most polymers used in drilling. The greatest potential for xanthan gum appears to lay in

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Table 1. The *Xanthomonas* isolates used in this study and their geographical origins and xanthan production capabilities

Numbers assigned to isolates	Isolates	Province	Field	Crops	Apparent viscosity of broth (cP)	Raw xanthan (g l^{-1})
1	*X. translucens* SAM 0402	Tehran	1	Cauliflower (Brassica oleracea)	~1 [a]	~0 [a]
2	*X. campestris* SAM 0302		1		960 [b]	9.69 [b]
3	*X. campestris* SAM 0401		1		870 [b]	9.34 [b]
4	*X. campestris* SAM 0301		1		904 [b]	9.68 [b]
5	*X. campestris* SAM 3301	Alborz	2	Red Cabbage (Brassica oleracea)	1426 [c]	11.92 [c]
6	*X. campestris* SAM 3302		2		1524 [c]	11.42 [c]
7	*X. campestris* SAM 3303		2		1526 [c]	11.25 [c]
8	*X. campestris* SAM 4101		3	Green Cabbage (Brassica oleracea)	1532 [c]	11.61 [c]
9	*X. campestris* SAM 4204		4		1242 [c]	10.73 [c]
10	*X. campestris* SAM 4205		4		412 [b]	7.12 [b]
11	*X. campestris* SAM 4210		4		1514 [c]	12.10 [c]
12	*X. campestris* SAM 4213		4		1554 [c]	11.56 [c]
13	*X.campestris* SAM 4215		4		1547 [c]	11.61 [c]
14	*X. campestris* SAM 4217		4		1525 [c]	11.96 [c]
15	*X. campestris* SAM 4220		4		1505 [c]	11.05 [c]

[a,b,c] These results were significantly different ($p<0.05$).

the enhanced oil recovery operations (3).

"Many reports showed high level of polymorphism among *Xanthomonas* species affecting their biological and fermentation properties". Genomic fingerprinting by PCR amplification, with primers specific to the highly conserved repetitive elements such as the 35–40 bp repetitive extragenic palindromic (REP) sequence, and the 124–127 bp enterobacterial repetitive intergenic consensus (ERIC) have been used successfully to characterize a large number of bacteria and distinguish closely related strains (4-6).

The screening of *Xanthomonas* strains with potential in xanthan production industry is a continuous need. Although the use of genomic fingerprinting techniques to study variability in microorganisms is fairly common, there are few reports on screening associated with gum production, especially in relation to xanthan production. The objectives of this study were to: (i) assess genetic diversity and evaluate the use of rep-PCR to differentiate among some Iranian *Xanthomonas* strains, and (ii) investigate the correlation between the genetic diversity and xanthan production capabilities of the strains.

MATERIALS AND METHODS

Bacterial strains. Fifteen isolates of the genus *Xanthomonas* were used in this study. The isolates

were isolated in our previous study from soil of four farms gone under cultivation of cauliflower and cabbage *(Brassica oleraceae)* crops in Tehran and Alborz Provinces, Iran (7). The disease is not widespread in Iran, and the strains were isolated during an intensive soil screening program. The isolates and their geographical origins are listed in Table 1. Pure cultures of the bacteria were maintained on Yeast Malt (YM) agar slants at 4 ºC and transferred to fresh medium every 14 days to prevent strains from losing their production capability (8).

Xanthan production. Actively growing cells from 24-h slant cultures of each isolate were inoculated to test tubes containing YM broth. The cultures were incubated at 28 ºC overnight and then transferred to 100-ml flasks containing 20 ml of YM broth. After incubation at 28 ºC on an orbital shaker at 140 rpm, the inocula were added to 250-ml flasks containing 50 ml of production medium. The composition of the medium was the same as synthetic medium introduced by Roseiro (9). Following incubation at 28ºC and 140 rpm for 72 h, the apparent viscosity of fermentation broth was measured at room temperature using a Brookfield system viscometer (Anton Paar, DV1) with spindle number 3 at 60 rpm. Raw xanthan was precipitated with 1.5 volumes of isopropyl alcohol and 0.5 g l^{-1} NaCl and dried in an oven. The experiments were carried out in four replicates.

Genomic fingerprinting. Bacteria were cultured in a liquid medium containing (g l^{-1}) Trypton 10, NaCl 5 and yeast extract 5; pH 7.2. Total genomic DNA was extracted from overnight cultures by the method of Boucher (10). ERIC-PCR. Primer sequences corresponding to ERIC (ERICIR [5'-ATGTAAGCTCCTGGGGATTCAC-3'] and ERIC2 [5´-AAGTAAGTGACTGGGG TGAGCG-3´] were used (11). The PCR was performed with 25-µl volumes containing the Taq DNA polymerase buffer 1X, 200 mM dNTP, 0.4 mM primer, and 1.0 U of Taq DNA polymerase. Fifty nanograms of bacterial DNA or approximately 10,000 bacterial cells were used as the template. PCR amplification was performed with a PEQLAB Thermocycler using the following cycles: 1 initial cycle at 95°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min with ERIC primers, and extension at 72°C for 1 min with a single final extension cycle at 72°C for 10 min and store at 4°C.

A 6 to 8 µl aliquot of amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels in 0.5 X TBE buffer (12) for 5 h at 70 V, stained with ethidium bromide, and photographed on a UV

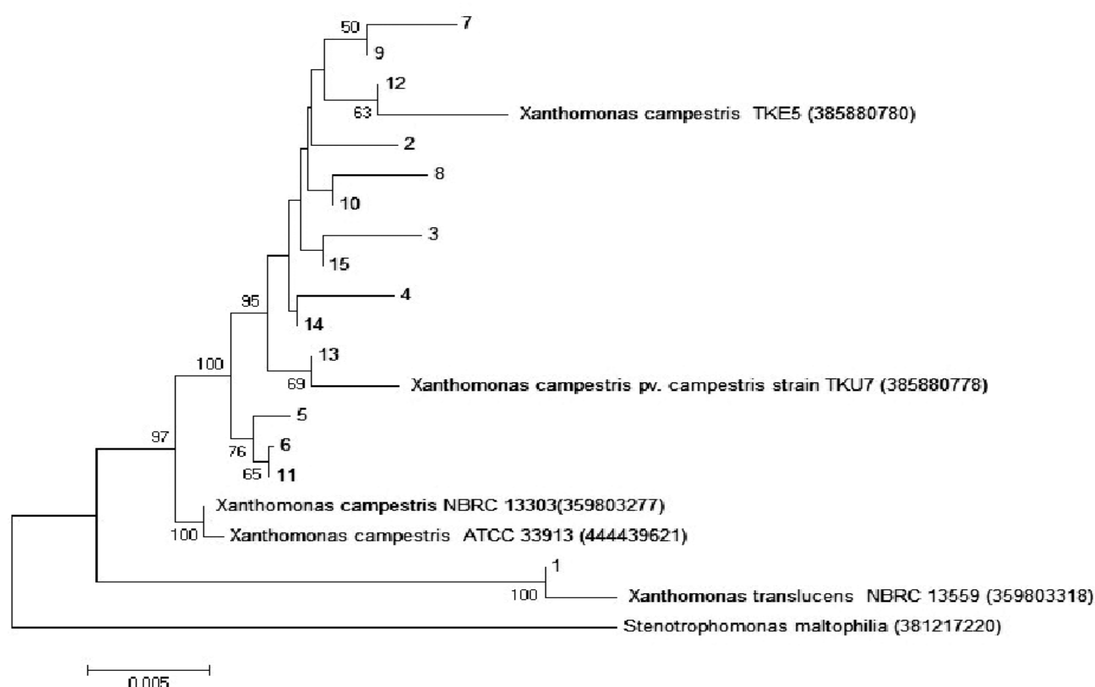

Fig. 1. Phylogenetic tree constructed with neighbor joining method. Our isolates were numbered 1 to 15 and *Stenotrophomonas maltophilia* numbered 16 and used as out group.

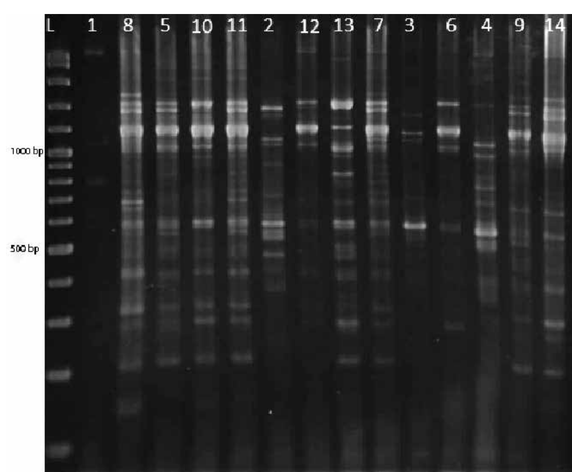

Fig. 2. Patterns of ERIC-PCR on agarose gel electrophoresis. Lanes 1-15 include the isolates of 1-15 and Lane L indicates DNA ladder 100bp plus.

transilluminator Ingenious LBR model.

The PCR reaction was carried out in two separate trials. Samples from each isolate loaded on gel electrophoresis plate and results were compared.

Bacterial identification. In this study *Xanthomonas* species were identified by sequencing of 16S rDNA region using universal primers including 27F and 1492R. Total genomic DNA was extracted by phenol – chloroform method. PCR was performed using universal primers and small amount of products were loaded on 1% agarose gel to detect a sharp single band, the remaining PCR product was sequenced. Sequencing results were assessed by bioEdit version 7.0.9.0 program and phylogenetic tree was constructed by MEGA5 program based on neighbor joining method (Fig. 1).

Data analysis. The results obtained from the xanthan production experiments were analyzed statistically by one-way analysis of variance and Tukey test with 95% confidence level using Minitab (version 15.2) software. ERIC-PCR amplification products were listed as discrete character states per strain (presence or absence of band). Jaccard's similarity coefficients between each strain were calculated using the SIMQUAL program in NTSYS-pc, version 2.02e, and these data were subjected to UPGMA cluster analysis by use of the SAHN program of NTSYS (13).

RESULTS

Average of apparent viscosity of culture broth and amount of raw xanthan obtained from the *Xanthomonas* isolates is shown in Table 1. From the xanthan production results, it is realized that the isolates can be placed in three statistically significant groups: isolate 1 not producing xanthan, isolates 2, 3, 4 and 10 which produced an average broth viscosity of 786 centipoises (cp) and average raw xanthan concentration of 8.96 g l^{-1}, and a third group with higher xanthan production capability. The last group includes isolates (5-9 and 11-15) which produced average broth viscosity and raw xanthan concentration of 1490 cP and 11.49 g l^{-1} respectively.

Result of 16S rRNA gene sequencing showed 14 isolates including isolates numbered 2 to 15 were *Xanthomonas campestris* and isolate 1 was *Xanthomonas translucens*. Similarity percent data from comparing to selected strain on NCBI data base are given in Table 2 and phylogenetic tree is shown in Fig. 3. The result of reproducibility tests confirmed the ability of this method as fingerprinting technique.

ERIC-PCR. ERIC-PCR yielded 5 to more than 21 PCR products, ranging in size from 100 bp to over 5 kb. There were differences in the concentration of some amplified fragments as well as in the occurrence of numerous polymorphic bands. Differences among strains were assessed visually on the basis of the migration patterns of PCR products (Fig. 2). UPGMA clustering separated all the isolates into different groups at a cut off of 86% similarity (Fig. 3). Three clusters were clearly separated with 40% similarity. This clustering was in agreement with xanthan production capability groups except for the isolate 10.

DISCUSSION

In this study we have demonstrated Entrobacterial Repetitive Intergenic Consensus as ERIC sequences are present in the genome of variant *Xanthomonas* strains, confirming and extending the conclusion of Versalovic et al.(5), de Bruijn (4), and Louws et al. (6). The *Xanthomonas* isolates used in this study were isolated previously from two distinct geographical locations with different cultivated crops. Based on 16S rDNA sequences, all the isolates are closely

Table 2. Results of genotypic and phenotypic characterization for identification of the isolates

Isolate number in this study	The most similar registered strain in NCBI database according to 16S rDNA identity	Max. Identity*	Nitrate reduction	Max. Temperature(°C)	Max. NaCl(%)	Citrate, Propionate	Acid from sucrose	Growth at %10 glucose	Acid from selected sugars **	Lactose, Raffinose	Manitol	Lecithinase
1	*X. translucens NBRC13559*	99	+	42	5	−	−	−	−	−	−	+
2	*X. campestris NBRC 13303*	100	−	39	5	+	+	+	+	+	−	+
3	*X. campestris TKU7*	99	−	39	5	+	+	+	+	+	+	+
4	*X. campestris TKU7*	100	−	38	5	+	+	+	+	+	+	+
5	*X. campestris NBRC 13303*	100	−	38	4	+	+	+	+	−	−	−
6	*X. campestris NBRC 13303*	99	−	38	4	+	+	+	+	−	−	−
7	*X. campestris TKU7*	100	−	38	4	+	+	+	+	−	−	−
8	*X. campestris TKU7*	100	−	38	4	+	+	+	+	−	−	−
9	*X. campestris TKU7*	100	−	38	4	+	+	+	+	−	−	−
10	*X. campestris TKE5*	100	−	38	4	+	+	+	+	−	−	−
11	*X. campestris NBRC 13303*	99	−	38	4	+	+	+	+	−	−	−
12	*X. campestris TKU7*	100	−	37	4	+	+	+	+	−	−	−
13	*X. campestris TKU7*	100	−	38	4	+	+	+	+	−	−	−
14	*X. campestris TKE5*	100	−	38	4	+	+	+	+	−	−	−
15	*X. campestris TKE5*	100	−	38	4	+	+	+	+	−	−	−

*max identity for 16S rRNA gene sequence similarity in NCBI data base
** D- Xylose, D- Mannose, D- Fructose, Cellobiose, Maltose

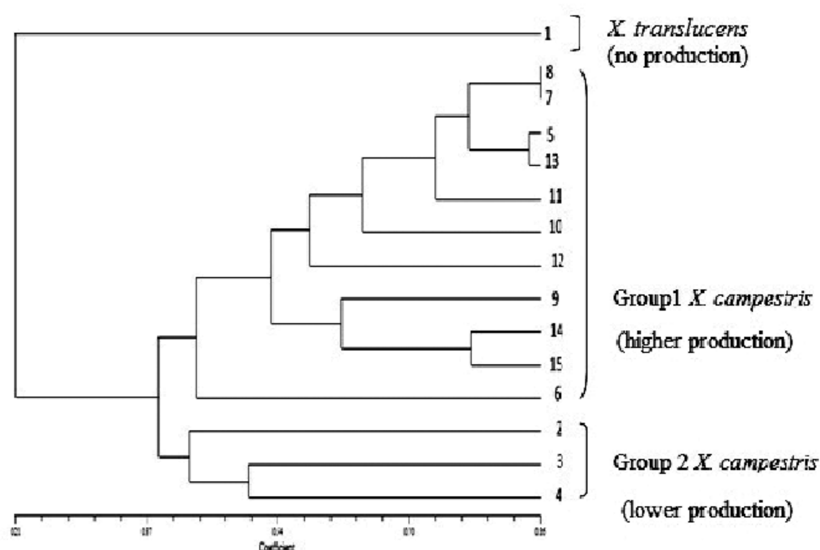

Fig. 3. Dendrogram based on the cluster analysis (UPGMA) of the estimate of genetic similarity by ERIC-PCR drawn with NTSYS-pc, version 2.02e. The numbers 1-15 indicates the isolates 1-15. Group 1 *X. campestris:* High and acceptable production. Group 2 *X.campestris* : less than 10 g/l production and less than 1×10^3cP viscosity. The strain 1 was, identified as *X. translucens*, isolated from the same place and used as negative control.

related to *X. campestris*, except for the isolate 1 that belongs to *X. translucens* (Table1). According to Louws et al. (6) and Barak and Gilbertson (14) rep-PCR can prove intraspecific variability among strains of heterogeneous pathovars of *X. campestris*. Thus, great genetic diversity among the isolates used in this study was expected and is consistent with previous studies and further confirms that *X. campestris* is heterogeneous.

After performing ERIC-PCR fingerprinting, a total of 21 distinctive bands ranging in size between 150 and 2500 bp was found among the analyzed isolates. Arshiya et al. (15) . Arshiya et al. (15) found bands ranging in size between 100 and 3,000 bp based on BOX, ERIC and REP profiles of 56 different *Xanthomonas axonopodis* pv. *citri*,, Although the number of distinctive bands found in our work is lower than that reported in other studies, we have to take into account that of the above authors worked with strains representing several *Xanthomonas* pathovars. We have also demonstrated that the ERIC-PCR protocols are rapid molecular characterization of *Xanthomonas* bacteria. The ERIC-PCR protocol clearly has the potential to differentiate *Xanthomonas* strains, including those pathovars not easily differentiated by other phenotypic and phylogenetic techniques (6). Our results showed partial relationship between ERIC-PCR patterns and xanthan production.. Isolate 1 placed in a separate group has no xanthan production. Isolates 2, 3 and 4 have similar xanthan production about 9.5 $g.l^{-1}$ and other isolates except isolate 10 have about 11.5 $g.l^{-1}$ xanthan productions according to different geographical regions (Table1). The results of this study were different with the results of Mayer et al. (1) that showed there were no relations between AFLP and xanthan production. This result can represent superiority of REP-PCR over AFLP method that was agreement with Rademaker et al resuts(16). The data presented here suggest that ERIC-PCR should also be a useful tool for assessment correlation between geographical region and xanthan production with ERIC-PCR patterns. Samples from the same or near sites showed similar patterns (Figure 1) of ERIC-PCR and clustered in the same group (Figure 2). ERIC-PCR analysis could cluster strains based on their geographical origin. Similar results were found by Massomo et al. (17) and Zhai et al. (18). However, contrary results have been obtained by Jensen et al. (19), Arshiya et al. (15) and Zaccardelli et al. (20).

CONCLUSION

ERIC-PCR was a good method for assessment of genetic diversity among *Xanthomonas* bacteria and the relation of them with xanthan production. If this method is developed by further strains it may predict ability of a strain to produce more and better quality of xanthan based on ERIC-PCR method.

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