

EFFECT OF TRANSPOSON Tn5 ON EXOPOLYSACCHARIDE PRODUCTION BY *Xanthomonas campestris*

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ABSTRACT

Tn5-induced mutants were produced in *Xanthomonas campestris* pv. *campestris* by mating a streptomycin resistant mutant with an *E. coli* WR 6016 strain containing the lac Ts ::Tn5 plasmid. This plasmid showed suicidal behaviour and it was transferred at a frequency of approximately 3×10^{-5} per recipient cell. 90 mutants were selected according to mucoid morphology and the viscosity of the culture broth was measured. These selected mutants showed high values of viscosity, when compared to the original Sm^r strain. Crude gum was extracted from five mutants and the gum solution (1%) was analysed in comparison with commercial xanthan gum. The viscosity values measured at different rotations showed similarities except for the original Sm^r strain which showed the lowest values. High temperatures decreased viscosity in all samples. The 228 and Sm^r strains gave the greatest reduction. Viscosity was also reduced under acidic conditions, whereas at an alkaline pH it was generally higher.

INTRODUCTION

Xanthomonas campestris is a phytopathogenic bacteria with over 120 different pathovars (Bradbury, 1984). The pathovar *campestris* is the most studied; it causes black rot in crucifers (Williams, 1980) and is the producer of xanthan gum (Jeanes *et al.*, 1961). Xanthan gum is the commercial name of an exopolysaccharide produced by *X. campestris* pv. *campestris* that is used for food, industrial and oil field applications (Baird *et al.*, 1983). Some genes related to pathogenicity and xanthan gum biosynthesis have been cloned (Barrere *et al.*, 1986; Thorne *et al.*, 1987) but for

only a few are their roles established (Harding *et al.*, 1987; Hotte *et al.*, 1990). The cloned genes were identified through the complementation of defective mutants by the transfer of genomic libraries. The isolation of defective mutants is an important step towards the identification of genes related to pathogenicity and gum production. Transposon mutagenesis, which inactivates a gene by insertion of a drug-resistance transposable element is a suitable method for the generation of selectable, single-site mutations in bacteria. Mutants obtained by transposon mutagenesis are also useful for providing physical markers for the target region.

The transposon Tn5 bears the kanamycin resistant gene (Berg *et al.*, 1975) and it transposes at variable frequencies depending on the transfer system and physiological factors (Kim *et al.*, 1988). It has been used in *E. coli* (Shaw and Berg, 1979) and in phytopathogenic bacteria successfully to isolate avirulent and auxotrophic mutants (Turner *et al.*, 1984; Cuppels, 1986; Steinberger and Beer, 1988).

During isolation of defective xanthan gum producers in *X. campestris* pv. *campestris*, it was found that Tn5-induced mutants also showed variability in shape, size and colony appearance, which allowed for the isolation of colonies with increased viscosity. In this report we describe the isolation and analysis of Tn5-induced viscosity mutants of *X. campestris* pv. *campestris*.

MATERIAL AND METHODS

Bacterial strains and culture media

X. c. pv. *campestris* strain NRRL-B 1459 and one streptomycin-resistant (Sm^r) mutant were used. The Tn5 donor strain was *E. coli* WR 6016 F⁺ts 114 lac :: Tn5 (Sansone *et al.*, 1982). For growth and maintenance of the *X. c.* strain the media YM (3 g malt extract, 3 g yeast extract, 5 g peptone, 10 g sucrose per liter) and YMA (YM plus 1.5% agar) were used. Antibiotics were added at the following concentrations (in $\mu\text{g/ml}$): kanamycin (Km) 20 and streptomycin (Sm) 100. For *E. coli* the LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) was used.

Matings

The parental strains, *X. campestris* Sm^r and *E. coli* WR 6016, were grown overnight at 28 and 37°C, respectively. The donor strain (*E. coli*) was diluted (1:100) and grown until log phase. The donor and recipient cells were mixed (ratio 3:1) and incubated overnight at 28°C, without agitation. Samples were plated in selective medium.

Colony hybridization

Freshly grown $Km^r Sm^r$ *X. campestris* recombinants were transferred to membranes, denatured (0.5 M NaOH, 1.5 M NaCl), neutralized (1.0 M Tris-HCl pH 8.0, 1.5 M NaCl), washed in SSC (2X) and dried. Pre-hybridization (1% sarcosyl, 30 μ g/ml tRNA, SSC 6X) was done at 65°C for 2 h. Tn5 probe was nick-translated with (32 p) dATP and used for hybridization (100 μ g/ml tRNA, 1% sarcosyl, SSC 6X) at 65°C for overnight incubation. The membranes were washed (1% sarcosyl, SSC 2X) twice at 65°C for 30 minutes, dried and autoradiographed using X-ray film.

Fermentation

The inoculum was prepared on YM medium (overnight incubation) and used at 10% (v/v) to seed the production medium which contained 5 g KH_2PO_4 , 0,2 g $MgSO_4 \cdot 7H_2O$, 2 g $(NH_4)_2SO_4$, 2 g citric acid, 6 mg H_3BO_3 , 6 mg ZnO, 2,4 mg $FeCl_3 \cdot 6H_2O$, 20 mg $CaCO_3$ and 20 g sucrose. In the preliminary assays 20 ml of medium was used and in the final experiments, 500 ml of production medium in 3,000 ml Fernbach flasks. The temperature was kept at 28°C for 72 h in a incubator shaker (New Brunswick, model Psychroterm) at 180 rpm. Viscosity was measured using a Brookfield RVT viscometer, spindle 21 at 5, 20, 50 and 100 rpm, at room temperature.

Recovery of the xanthan gum

The biopolymer was recovered by precipitation with ethanol (2 volumes). The wet precipitate was dried at 37°C and milled in a mortar. One percent solutions were made from the extracted gum. pH alterations were done with 6 N HCl or NaOH. For the temperature test the solutions were kept for 15 minutes at the respective temperatures.

RESULTS AND DISCUSSION

Transfer of Tn5 to X. campestris

581 mutants of *X. campestris* showing resistance to streptomycin and kanamycin were isolated. Streptomycin was used at a high level (250 μ g/ml) because Tn5 confers resistance to Sm at a low level (Turner *et al.*, 1984). There was no background mutation in the 1×10^9 cells observed. The frequency of transfer was approximately 3×10^{-5} per recipient cell, which is higher than has been obtained in

Erwinia amylovora (Steinberger and Beer, 1988), *Rhizobium* (Berg and Berg, 1983; Kim *et al.*, 1988), *Pseudomonas* (Cuppels, 1986) and *X. campestris* (Turner *et al.*, 1984). The differences in the efficiency of Tn5 insertions has been attributed mainly to the host vector system used. F⁺lac::Tn5 was considered efficient in *X. campestris* and is easy to use. It behaved as a suicide vector since the replication system cannot operate outside *E. coli* (Guiney, 1982) and therefore there was no need of using its thermosensitivity at 42°C. Some mutants (50) were analysed in agarose gel electrophoresis to check for plasmid transfer and none was detected. It was concluded that insertion of Tn5 into the chromosome had occurred since there also was positive colony hybridization.

Fermentation

Preliminary selection for mutants producing higher viscosity was done by inspecting the mucoid appearance and size of the colonies. 90 mutants were selected and tested for viscosity of the culture broth. Six independent experiments (three repetitions) were set up and the original Sm^r strain was used as a control. Variance analysis and the Tukey test were used to detect the best producers. Among 90 mutants tested, 29 showed significantly higher values of viscosity when compared to the original strain (Sm^r). Although selection of mutants according to colony appearance was a simple way to isolate strains for polysaccharide production, the results obtained from fermentation confirmed higher viscosity in only 29 out of 90 mutants. Probably the selection efficiency could be increased by also considering the convexity of the colonies, as suggested by Ramirez *et al.* (1988). A summary of the results showing the viscosity of the five best producers (228, 432, 434, 501 and 526) is given in Table I. The percentage increase varied from 50 to 90%, when compared to the Sm^r strain. The Sm^r strain was highly unstable in terms of viscosity, ranging from 325 to 862 cps at 20 rpm in different experiments. Therefore in most experiments a high number of repetitions was used for this strain.

Higher production and high quality of xanthan gum are desired characteristics for industry and considerable effort has been directed towards the improvement of the production strains and the optimization of fermentation and recovery processes. In a few cases *X. campestris* has also been genetically manipulated to allow use of alternative substrates as a carbon source (Walsh *et al.*, 1984; Stripecke *et al.* (1989). The use of Tn5 to induce variability in xanthan gum production was considered useful because high and low (not shown) viscosity strains were isolated. The non-xanthan gum producer strains could also be used in complementation tests to identify genes related to xanthan gum biosynthesis (Barrère *et al.*, 1986; Thorne *et al.*, 1987; Harding *et al.*, 1987).

Table I - Viscosity values (cps) of selected mutants and the original Sm^F strain.

Strain	20 rpm	100 rpm
Sm ^F	515.84 ± 41.76	149.58 ± 14.48
228	795.84 ± 41.67	230.84 ± 11.58
432	945.84 ± 46.40	265.84 ± 13.41
434	945.84 ± 29.17	283.34 ± 3.00
501	862.50 ± 12.5	264.17 ± 5.84
526	970.84 ± 36.32	235.00 ± 21.65

* Average of 18 repetitions. Viscosity was measured at room temperature, spindle 21.

Analysis of the gum produced

The xanthan gum yield as a fraction of the amount of sucrose added was around 55% (g xanthan/g sucrose) for all strains. The productivity of xanthan gum varies depending on the fermentation conditions and strains used, and high yield of 80% have been registered (Moraine and Rogovin, 1966; Marquet *et al.*, 1989). Though our yields were lower, they could be optimized by alterations in the fermentation conditions for these strains. Since the yield among the mutants was similar, the differences in the viscosity shown by the mutants could be due to the quality of the gum produced. Therefore the crude gum extracted from the best five producers was analysed at different rotations, temperatures and pHs. Commercial Keltrol gum (Food grade) was used as a reference gum. The results were analysed statistically using the Friedman test. At the various rotations, the commercial gum showed the highest values although they were not significantly different from those produced by most strains. The Sm^F and 501 strains showed the lowest values (Table II). At high temperatures (100°C) all mutants analysed showed a decrease in viscosity and the Sm^F again showed the lowest stability and viscosity (Table III). The pH effect was more significant under acidic conditions in which viscosity was reduced more than 50%. At alkaline pH, however, there was a higher value of viscosity in most cases (Table IV). The control strain Sm^F showed a higher increase in viscosity and also a lower resistance to shearing and temperature effects. The increase in the viscosity under alkaline conditions is known and it has been attributed to deacetylation (Information CA, 1959). It might have some advantages over unmodified xanthan gum.

Table II - Viscosity values (cps) of gum solution (1%) measured at different rotations.

Gum from	5 rpm	20 rpm	50 rpm	100 rpm	Final Friedmann**	
					Score*	Test
Reference	3250.00 (5)	1012.50 (7)	487.50 (7)	323.75 (7)	28	a
432	3625.00 (7)	1025.00 (6)	450.00 (6)	233.75 (5)	24	a
434	3525.00 (6)	943.75 (5)	412.50 (5)	238.75 (6)	22	a b
526	2475.00 (4)	787.50 (4)	342.50 (3)	208.75 (4)	15	a b c
228	2375.00 (3)	775.00 (3)	350.00 (4)	190.00 (3)	13	a b c
501	2150.00 (2)	662.50 (2)	320.00 (2)	186.25 (2)	08	b c
Sm ^r	1900.00 (1)	600.00 (1)	280.00 (1)	168.75 (1)	04	c

The values are an average of two repetitions. The number in parentheses indicates, the increasing order (points) in the Friedman test.

* Indicates the sum of the points.

** The same letter indicates no significant difference (5%).

Table III - Viscosity values (cps) of gum solution (1%) measured at 100 rpm at different temperatures.

Gum from	Temperature				Friedmann	
	100°C	75°C	50°C	30°C	Score*	Test**
Reference	207.50 (7)	250.00 (7)	271.66 (7)	325.83 (7)	28	a
432	101.67 (6)	111.67 (6)	135.00 (6)	160.84 (6)	24	a
526	78.34 (3)	98.34 (5)	116.67 (4)	147.50 (5)	17	a b
501	82.50 (4)	89.17 (3)	120.00 (5)	140.84 (4)	16	a b
434	85.00 (5)	90.00 (4)	103.34 (2)	133.34 (3)	14	a b
228	73.34 (2)	86.67 (2)	105.84 (3)	126.67 (2)	09	b
Sm ^r	61.67 (1)	78.34 (1)	91.67 (1)	120.00 (1)	04	b

The values are an average of three repetitions. The number in parentheses indicates the increasing order (points) in the Friedman test.

* Indicates the sum of the points.

** The same letter indicates no significant difference (5%).

Table IV - Viscosity values (cps) of gum solution (1%) measured at different pHs.

Gum from	1.0		6.5	11.0	
	cps	%*	cps	cps	%*
Reference	298.75	92.2	323.75	266.26	82.2
432	161.25	69.0	233.75	242.50	103.7
526	135.00	64.7	208.75	236.25	113.2
501	86.25	46.3	186.25	193.75	104.0
434	91.25	38.2	238.75	176.25	73.8
228	156.25	82.2	190.00	238.75	125.6
Sm ^r	127.50	75.5	168.75	208.75	123.7

Measured at 100 rpm, spindle 21. Average of two repetitions.

* Percentage in relation to pH 6.5 (100%).

Although an increase in xanthan gum yield was not detected in the Tn5-induced mutants, the quality of the gum produced showed some physical alterations. Other tests, such as chemical composition analysis could detect other differences. In spite of these slight modifications, the use of transposons may lead to the selection of strains producing xanthan gum with different properties or with improved productivity. These type of chromosomal mutants might show advantages over recombinants with cloned genes because of their higher stability in the fermentation process.

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RESUMO

A partir da conjugação entre um mutante Sm^r de *Xanthomonas campestris* pv. *campestris* e uma linhagem de *Escherichia coli* contendo o transposon Tn5, foram isolados 581 mutantes apresentando resistência aos antibióticos kanamicina e estreptomicina. Foram selecionados 90 mutantes pela viscosidade aparente e tamanho das colônias. Estes mutantes foram analisados quanto à viscosidade em seis experimentos independentes de fermentação. A análise dos resultados obtidos através do teste estatístico de Tukey permitiu diferenciar 29 mutantes que apresentaram valores de viscosidade significativamente

maiores que a linhagem original Sm^r. Dentre estes foram selecionados os 5 melhores mutantes. Uma solução (1%) da goma produzida por estes foi analisada à diferentes rotações, temperaturas e pHs tendo sido utilizada a goma comercial Keltrol como referência. Os resultados foram analisados através do teste de Friedman e verificou-se que nos testes à várias rotações não houve diferença significativa entre as linhagens testadas e a goma Keltrol, com exceção da linhagem Sm^r que apresentou os menores valores de viscosidade. Nos testes à altas temperaturas, todas as linhagens apresentaram um decréscimo na viscosidade; em pH ácido a redução foi de aproximadamente 50% e em pH alcalino as linhagens apresentaram valores de viscosidade maiores que em pH normal.

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