

THE ROLE OF A CLONED α -AMYLASE GENE IN FERMENTATION BY *Xanthomonas campestris*

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ABSTRACT

A hybrid plasmid (pAP1) containing the α -amylase gene from *Bacillus subtilis* was introduced, by transformation, into amylolytic and non-amylolytic *Xanthomonas campestris* cells (Stripecke *et al.*, Appl. Microbiol. Biotechnol. 31: 512-517, 1989). Transformants from both strains were used in fermentation and analysed for the viscosity produced in the fermentation broth. Sucrose and starch in different combinations were utilized as carbon sources. Although complete substitution of glucose by starch was not attained, the combination of 1% glucose plus 1% starch enabled the non-amylolytic strain to produce viscosity.

INTRODUCTION

Xanthomonas campestris is a phytopathogenic bacteria (Williams, 1980) and producer of an extracellular polysaccharide (EPS) which is excreted by the cells into the medium as a slime (Jeanes, 1961). This biopolymer, also called xanthan gum, is thought to be related to the pathogenicity process (Sutton and Williams, 1970; Ramirez, 1989) but its role is not yet well established. The xanthan gum has unique rheological characteristics which are useful for industrial purposes such as for food, cosmetics, improved oil recovery and pharmaceutical areas (Baird *et al.*, 1983). The chemical structure of xanthan gum (Jansson *et al.*, 1975; Melton *et al.*, 1976) and the biosynthetic process (Ielpi *et al.*, 1983) have been extensively studied. Nevertheless it is still far from being fully understood. Recently the use of molecular techniques has

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allowed the definition of some genes involved in xanthan gum production (Barrère *et al.*, 1987; Harding *et al.*, 1987; Thorne *et al.*, 1987).

The use of xanthan gum is increasing each year and it is traditionally obtained from broths which contain glucose or sucrose as main carbon sources (Sow and Demain, 1979). Other alternative substrates have been neglected, although Walsh *et al.* (1984) have developed a new strain containing a β -galactosidase gene suitable for cheese whey-containing media. The use of starch as a carbon source for xanthan gum production has been reported (Sow and Demain, 1979). It has usually been isolated from maize but other plants such as cassava and potatoes are also good producers. However the yield of xanthan gum has been considered low compared to that obtained with glucose or sucrose. The starch has also been used for alcoholic fermentation by *Saccharomyces cerevisiae* (Cole *et al.*, 1988). According to some authors (Schenberg and Costa, 1987) the cassava crop can be considered advantageous over sugar-cane in Brazil because it is less susceptible to diseases, shows better tolerance to climatic variations and demands less amounts of water and fertilizers.

The majority of the *X. campestris* strains are considered to be capable of degrading starch (Dye and Lelliott, 1974), however most of *X. campestris* pv *manihotis* strains are non-amylolytic (Stripecke *et al.*, 1989). One of these strains proved to be a good exopolysaccharide producer. In this report we describe the role of a cloned α -amylase gene in the amylolytic *X. campestris* pv *campestris* and in the non amylolytic *X. campestris* pv *manihotis*, using starch and glucose as a carbon courses.

MATERIALS AND METHODS

Strains

The strains used are shown in Table I. The chloramphenicol resistant mutant (Cm^r) was isolated after mutagenesis of the NRRL-B-1459 strain of *X. campestris* pv *campestris* (hereafter called Ca1120). The strain nalidixic acid resistant (Ma 3281) is a spontaneous mutant obtained from the non-amylolytic strain Ma 3281 (*X. campestris* pv *manihotis*) isolated by the Biological Institute of Campinas, SP, Brazil.

Media

The strains were maintained in yeast maltose medium YM (Haynes *et al.*, 1955). For fermentation the cells were grown in YM broth and 10% of inoculum was added to MPI medium (Sow and Demain, 1979). Carbon sources were used at the following concentrations: sucrose 2%, sucrose 1%, starch 2% and sucrose 1% plus

starch 1%. Tetracycline and ampicillin (10 and 100 μ g/ml, respectively) were added in the YM medium and omitted in the MPI medium.

Table I - *Xanthomonas campestris* strains used.

Strain	Characteristics	Source or reference
Ca1120	Chloramphenicol resistant mutant from NRRL B-1459 strain	Lanza and Rosato (1986) Peoria, IL, USA
Ca1120/pMFY40	Ca1120 containing the pMFY-40 plasmid (Fukuda and Yano, 1985)	Stripecke <i>et al.</i> (1989)
Ca1120/pAP1	Ca1120 containing the amy ⁺ gene into pMFY-40 plasmid	Stripecke <i>et al.</i> (1989)
Ma 3281	Non-amylolytic, nalidixic acid resistant	Stripecke <i>et al.</i> (1989)
Ma3281/pMFY40	Ma 3281 containing the pMFY-40 plasmid	Stripecke <i>et al.</i> (1989)
Ma3281/pAP1	Ma 3281 containing the amy ⁺ gene into pMFY-40 plasmid	Stripecke <i>et al.</i> (1989)

Abbreviation: amy⁺ gene: α -amylase.

Fermentation

125 ml flasks containing 40 ml of YM broths supplemented with antibiotics as required, were inoculated with fresh colonies from YM plates and incubated for 24 h/28°C/180 rpm. 2 ml of these cultures were used to inoculate 125 ml flasks containing 20 ml of MPI medium plus carbon source. Incubation was at 28°C/180 rpm for 72 h.

Viscosity measurements

Viscosity was measured using a RVT Brookfield viscometer, spindle 21, at 20 and 100 rpm, at room temperature.

Plasmid stability in fermentation broth

Samples were taken from starch-containing fermentation broth inoculated with Ma 3281/pMFY40 or Ma 3281/pAP1 after 72 h of incubation and plated into antibiotic-containing and antibiotic-free medium.

RESULTS AND DISCUSSION

The viscosity measurements of the fermentation broth for the original strains, mutants and derivatives harbouring either pMFY40 or pAP1 of *X. campestris* pv *campestris* and *X. campestris* pv *manihotis*, are presented in Tables II and III, respectively.

Table II - Viscosity measurements (cPS) in the fermentation broth inoculated with *X. campestris* pv *campestris*.

Carbon source	Ca 1120		Ca 1120 /pMFY40		Ca 1120 /pAP1	
	20 rpm	100 rpm	20 rpm	100 rpm	20 rpm	100 rpm
Sucrose 2%	293,8	128,8	303,1	139,4	270,8	117,2
	±	±	±	±	±	±
	52,5	20,2	49,3	29,0	7,2	2,5
Sucrose 1%	78,1	43,1	68,8	40,0	81,3	45,0
	±	±	±	±	±	±
	23,7	8,3	7,2	3,5	7,2	0
Sucrose 1%	190,6	85,6	162,5	79,4	253,1	111,9
Starch 1%	±	±	±	±	±	±
	27,7	5,5	20,4	5,1	27,7	20,9
	204,1	89,2	159,4	76,3	215,6	96,3
Starch 2%	±	±	±	±	±	±
	28,9	7,2	18,8	7,8	12,0	4,8

Average of 3 repetitions.

Table III - Viscosity measurements (cPS) from the fermentation broth inoculated with *X. campestris* pv *campestris*.

Carbon source	Ma 3281		Ma 3281 /pMFY40		Ma 3281 /pAP1	
	20 rpm	100 rpm	20 rpm	100 rpm	20 rpm	100 rpm
Sucrose 2%	970,8	250,8	586,8	173,8	643,8	196,9
	±	±	±	±	±	±
	97,1	24,5	12,5	12,5	11,1	
Sucrose 1%	300,0	92,5	150,0	58,1	253,1	82,5
	±	±	±	±	±	±
	10,2	2,0	10,2	2,4	15,7	3,5
Sucrose 1%	268,8	85,0	171,9	66,9	446,9	145,6
Starch 1%	±	±	±	±	±	±
	16,1	5,4	12,0	4,3	70,2	29,9
	9,4	6,9	< 5,0	5,6	21,9	13,8
Starch 2%	±	±	±	±	±	±
	6,3	1,3		1,3	6,3	1,4

Average of 3 repetitions.

For *X. campestris* pv *campestris* the best results were obtained with 2% sucrose whereas 1% sucrose gave the lowest values. In starch-containing broth (sucrose + starch or starch) the viscosity values obtained were similar for all strains tested; the presence of pAP1 seems to result in slightly higher values. As discussed by Stripecke *et al.* (1989) it is not known to what extent each α -amylase gene (the original and the cloned one) is being expressed in the recombinant strain. In a comparison of the viscosity of the original strain Ca1120 and Ca1120/pAP1 we found an increase of around 30% in the viscosity produced by the strain harbouring pAP1, at 20 rpm. A comparison between Ca1120 and Ca1120/pMFY40 (2% starch, 20 rpm) indicates a better viscosity for Ca1120. This may mean that the presence of the antibiotic/plasmid could be detrimental and therefore there is a beneficial effect exerted by the cloned amylase gene in the Ca1120/pAP1.

The role of the cloned amylase can be better understood with the *X. campestris* pv *manihotis*, which does not produce amylase (Table III). The worst

results were obtained in the 2% starch medium showing that under the conditions used here, starch alone is not efficiently converted to gum production, even by the strain harbouring the cloned α -amylase. However in the combined sucrose + starch substrate, the strain Ma 3281/pAP1 showed the highest value. Why there is no conversion of starch to gum in media containing starch alone we can not be sure but some possibilities may be considered. The process of fermentation utilized here is a two-step procedure: first, cellular growth is obtained by using nitrogen and carbon sources in a complex composition, and second, there is induction of polysaccharide production by maintenance of a high proportion of carbon source. In the second step metabolic activity is low and therefore there is little or no enzyme production, resulting in a low degree of starch degradation. When glucose is present, this is easily taken up and metabolized, and consequently the amylase enzyme production could occur and trigger all subsequent reactions. A second possibility which could explain why there is no gum production in starch 2% medium is related to the action of α -amylases which only break the α -1,4 glycosidic bounds of starch, resulting in branched oligosaccharides and a few monosaccharides. Glucose is the preferential substrate entering the xanthan biosynthetic pathway (Ielpi *et al.*, 1983). Thus most of the oligosaccharides would be neglected. The presence of another enzyme such as (glucoamylase) could help by completely breaking down and this might improve the gum production. Finally, fructose can act as an inducer of xanthan production (Sow and Demain, 1979) and it can be obtained breaking down sucrose, but not starch.

During the period of fermentation, both plasmids, pMFY40 and pAP1 were maintained in the Ma 3281 strain at frequencies of 57% and 80%, respectively. Although no antibiotic was added to be fermentation medium, the rate of maintenance of the plasmids was higher than in the LB medium (Stripecke *et al.*, 1989) due probably to the low rate of cellular growth. Experiments are underway to ligate the *par B* locus (Gerdes, 1988) to the pAP1 plasmid to achieve better stabilization of the system.

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RESUMO

O plasmídeo híbrido pAP1, contendo o gene da alfa-amilase de *Bacillus subtilis* inserido no plasmídeo pMFY-40, foi introduzido em células amilolíticas e não amilolíticas de *Xanthomonas campestris*

pv. *campestris* e pv. *manihotis*. Os transformantes obtidos foram analisados quanto à viscosidade produzida em caldos de fermentação contendo sacarose e/ou amido como fontes de carbono. Os valores de viscosidade mais altos foram obtidos em meio de sacarose 2% e os mais baixos em meio de sacarose 1% para as linhagens Ca1120 e meio de amido 2% para as linhagens Ma3281. O gene da alfa-amilase clonado apresentou boa expressão em linhagens não amilolíticas e o aumento da viscosidade foi altamente significativo em meio de sacarose + amido mas não em meio contendo apenas amido.

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