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Variations in xanthan production by antibiotic-resistant mutants of *Xanthomonas campestris*

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Abstract Mutants resistant to different antibiotics (streptomycin, tetracycline, ampicillin and penicillin) were obtained from several strains of *Xanthomonas campestris* and evaluated for xanthan production. Most of the mutants showed alterations in their polysaccharide production, either increasing, decreasing or totally losing their polymer-production capacity. The existence of two types of antibiotic-resistance mechanisms for the assayed drugs is suggested: one that affects xanthan production and another that does not. Differences in outer-membrane protein patterns of mutants that were simultaneously altered in antibiotic resistance and xanthan production were found, in comparison with their parental strains. These findings suggest the existence of a genetic relationship between antibiotic-resistance mechanisms and xanthan production. Some of the mutants obtained showed significant increases in broth viscosity and xanthan concentration. These results suggest that resistance to streptomycin and ampicillin can be used to obtain improved strains in plate screening assays.

Introduction

Xanthan gum is an exopolysaccharide produced by the bacterium *Xanthomonas campestris* showing a wide range of applications in several industries (Baird et al. 1983). Therefore, searching for mutants with increased production or gum quality is of economic interest. However, this has been counteracted by the lack of suitable screening techniques (Torrestriana et al. 1990; Rodríguez and Aguilar 1997). Sensitivity to some antibiotics is among the characteristics of some wild-type *Xanthomonas* strains with good production properties (Kidby et al. 1977; Torrestriana et al. 1990). In contrast, other authors have found improvements in xanthan production by some bacitracin- and rifampicin-resistant mutants (Sutherland 1977; Marquett et al. 1989). Such evidence suggests the necessity to study the possible relationship between antibiotic resistance mechanisms and xanthan production.

A relationship between multiple antibiotic resistance and changes in outer-membrane protein patterns has been demonstrated in several gram-negative bacteria (Gutman et al. 1985; Legakis et al. 1989; Lecso-Bornet et al. 1992). In *Escherichia coli*, the genes responsible for multiple antibiotic resistance associated with membrane protein changes have been sequenced and characterized (Cohen et al. 1993; Seoane and Levy 1995). Gene products involved in exopolysaccharide production are membrane-bound in *Rhizobium* (Latchford et al. 1991; Muller et al. 1993; Reuber and Walker 1993). In the present paper, mutants from several strains of *X. campestris* were selected for resistance to different antibiotics and evaluated for xanthan production. The outer-membrane protein patterns of some mutants and wild-type strains were analysed. The possibility of a genetic relationship between antibiotic-resistance and xanthan production in *X. campestris* is indicated.

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Materials and methods

Strains and cultivation conditions

The strains and their relevant characteristics are summarized in Table 1. They were maintained in YM/agar medium (in g/l: glucose 20, yeast extract 3, peptone 5, malt extract 3, Oxide no. 2 agar 20, pH 7.0). Shake cultures were carried out in YM broth (YM medium without agar), and in the "production medium" described by Shu and Yang (1990) (in g/l: glucose 20, yeast extract 3, K_2HPO_4 2,

Table 1 Strains of *Xanthomonas campestris* pv. *campestris* and their relevant characteristics. *Str* streptomycin, *Amp* ampicillin, *Tet* tetracycline, *Pen* penicillin, μ mutant

Strain	Relevant characteristic	Reference
SAV	Wild type	Valdés et al. 1992
FAT	Wild type	Valdés et al. 1992
5270	Wild type	Valdés et al. 1992
NRRL B-1459	Wild type	Northern Regional Research Laboratory
M-11	μ from strain SAV	Rodríguez and Aguilar 1997
ST-2	μ from strain SAV, Tet^r	This work
SP-2	μ from strain SAV, Pen^r	This work
11S-1	μ from strain M-11, Str^r	This work
11S-2	μ from strain M-11, Str^r	This work
11S-3	μ from strain M-11, Str^r	This work
11T-1	μ from strain M-11, Tet^r	This work
11T-2	μ from strain M-11, Tet^r	This work
11P-2	μ from strain M-11, Pen^r	This work
11P-5	μ from strain M-11, Pen^r	This work
11A-1	μ from strain M-11, Amp^r	This work
FAT A-1	μ from strain FAT, Amp^r	This work
FAT A-2	μ from strain FAT, Amp^r	This work
5270 A-1	μ from strain 5270, Amp^r	This work
5270 A-2	μ from strain 5270, Amp^r	This work
5270 S-1	μ from strain 5270, Str^r	This work
5270 S-2	μ from strain 5270, Str^r	This work
NRRL B A-1	μ from strain NRRL B-1459, Amp^r	This work
NRRL B A-2	μ from strain NRRL B-1459, Amp^r	This work
NRRL B S-1	μ from strain NRRL B-1459, Str^r	This work
NRRL B S-2	μ from strain NRRL B-1459, Str^r	This work

$MgSO_4 \cdot 7H_2O$ 0.1, KH_2PO_4 2, tap water to 1 l, pH 7.0), using 500-ml conical flasks containing 70 ml medium. The flasks were incubated in an orbital shaker at 30 °C and 250 rpm for 72 h. The experiments were carried out in triplicate.

Analysis

Growth was measured by the absorbance at 600 nm. The viscosity of the culture broth was assayed in a Haake VT/181 rotational viscosimeter at 7.3 s^{-1} and at 26 °C. Xanthan concentration was estimated in the supernatant after dilution and centrifugation of the culture at 20 000 *g* for 40 min, using ethanol precipitation (2:1 v/v) in the presence of 1% KCl and dry-weight determination (Rodríguez and Aguilar 1997). Proteins were determined by the Lowry method (Lowry et al. 1951).

Isolation of mutants

Aliquots of 0.1 ml from overnight cultures were plated in YM/agar medium plus the antibiotic and incubated at 30 °C for 24 h. Spontaneously resistant colonies were isolated and transferred several times onto plates of YM/agar plus the corresponding antibiotic in order to verify purity and the maintenance of the antibiotic-resistant phenotype. The stability of the 11S-1 mutant was further verified as follows. A culture in YM broth was inoculated with a single colony, shaken to the late-exponential growth phase and diluted 1/2000 into fresh broth in the absence of the antibiotic. Growth was continued to the late-exponential phase and the dilution/growth procedure repeated for seven cycles. The culture was sampled at different times and plated onto non-selective medium. About 200 single colonies from each sample were screened for the streptomycin-resistance character by replica plating. The experiment was carried out in triplicate.

Antibiotic concentrations were as follows (μg/ml): ampicillin 100, streptomycin 500, penicillin 50, tetracycline 25.

Outer-membrane proteins extraction

The extraction of outer-membrane protein was performed according to the procedure described by Gutman et al. (1985), with some modifications. Cultures in late-exponential growth in YM medium, or YM medium plus the corresponding antibiotic in the case of the resistant mutants, were centrifuged at 23 000 *g* for 10 min, washed twice with 50 mM TRIS/HCl buffer pH 7.5, and resuspended in the same buffer. The cell suspensions were submitted for sonication at an amplitude of 10 μm for 10 min in an MSE 150-W sonicator with a 9.5-mm probe (MSE, Manor Royal, Sussex, England). The remaining whole cells were discarded by centrifugation at 10 000 *g* for 10 min. The supernatant was subsequently centrifuged at 42 000 *g* for 60 min at 4 °C, for the isolation of cellular debris. The pellet was redissolved (0.5 g/l) in 50 mM phosphate buffer, pH 7.0, containing 0.3% *N*-lauroylsarcosine (Sigma), and incubated at 28 °C for 30 min without stirring. The outer-membrane proteins fractions were obtained by centrifugation at 45 000 *g* for 60 min at 4 °C. The pellet was resuspended in sample buffer and aliquots containing 20–40 μg proteins were submitted to discontinuous sodium dodecyl sulphate/polyacrylamide gel electrophoresis, according to Laemmli (Laemmli 1970), using 12% separation gels. These were stained with Coomassie brilliant blue R-250. Low-molecular-mass protein markers were from Pharmacia LKB (Upsala, Sweden).

Results

Xanthan production by antibiotic-resistant mutants

The xanthan production and growth of the mutants and their parental strains are shown in Table 2. Several of the antibiotic-resistant mutants were simultaneously altered in their capacity for xanthan production. An improvement of 15% in viscosity development was achieved by a streptomycin-resistant (Str^r, 11S-1) mutant, while two ampicillin-resistant (Amp^r) mutants (5270 A-1 and 5270 A-2) exhibited 15% and 19% improvements respectively, over their parental strains. However, other Str^r and Amp^r mutants showed different responses in relation to exopolysaccharide production:

Table 2 Xanthan production by the antibiotic-resistant mutants and their parental strains. Results are average values of three replicate flasks ± SD. *cP* centipoise = 1 mPa s, *ND* not determined

Strain	Growth (A_{600})	Viscosity (cP)	Xanthan (g/l)
SAV	4.3 ± 0.3	2045 ± 85	9.5 ± 0.4
ST-2	4.0 ± 0.0	0	0
SP-2	4.6 ± 0.0	35 ± 15**	ND
M-11	3.6 ± 0.4	2863 ± 402*	13.5 ± 0.5*
11S-1	4.1 ± 0.6	3344 ± 273*	13.8 ± 1.7
11S-2	5.2 ± 0.8*	2689 ± 206	12.4 ± 0.9
11S-3	5.5 ± 1.0*	2662 ± 400	11.9 ± 1.1
11A-1	3.7 ± 0.1	0	ND
11T-1	3.5 ± 0.1	0	ND
11T-2	3.3 ± 0.2	0	0
11P-2	4.0 ± 0.2	287 ± 26**	ND
11P-5	4.0 ± 0.3	278 ± 79**	ND
FAT	3.9 ± 0.1	1870 ± 171	6.6 ± 0.5
FAT A-1	3.5 ± 0.3	1984 ± 99	7.8 ± 0.3*
FAT A-2	3.4 ± 0.1	2012 ± 130	8.7 ± 0.7*
5270	3.7 ± 0.1	2353 ± 130	8.5 ± 0.7
5270 A-1	3.6 ± 0.2	2721 ± 0*	8.2 ± 0.1
5270 A-2	3.7 ± 0.1	2806 ± 170*	9.1 ± 0.5
5270 S-1	3.4 ± 0.3	1417 ± 98**	6.0 ± 1.6
5270 S-2	3.2 ± 0.2	2409 ± 98	8.6 ± 0.1
NRRL B-1459	6.8 ± 1.0	2806 ± 595	11.7 ± 1.3
NRRL A-1	7.3 ± 0.2	3061 ± 294	12.9 ± 0.7
NRRL A-2	6.7 ± 0.2	2891 ± 126	12.6 ± 1.7
NRRL S-1	6.6 ± 0.4	2891 ± 510	13.5 ± 1.4
NRRL S-2	6.3 ± 0.2	2806 ± 85	12.5 ± 1.6

* Significant higher value
** Significant lower value, in comparison with the parental strain (Student *t*-test, 95% reliability)

the 11S-2, 11S-3 and the NRRL B-1459 derivatives did not show any change, while 11A-1 lost the capacity to produce polymers. Tetracycline resistance always resulted in the total loss of viscosity in the culture broth, while the Pen^r mutants showed significantly diminished polymer production.

After 50 generations of growth in non-antibiotic medium, 100% of the assayed colonies from the 11S-1 mutant retained the streptomycin-resistance trait (data not shown), showing the good stability of this mutant. This strain showed the best results among the mutants obtained, in relation to xanthan production and broth viscosity.

All the mutants were further characterized for possible alterations in other phenotypic properties. Their cell morphology and ability to grow in minimal medium remained unaltered. The colony morphology was similar in all gum-producing strains (intense yellow, convex, mucoid colonies about 4 mm in diameter, after 48 h growth in YM/agar medium), while non-mucoid, pale yellow and less convex colonies of similar size were observed in the non-producing strains. Besides this, a dark-brown pigment was detected in the tetracycline-resistant mutants.

Outer-membrane proteins pattern

Electrophoretic patterns of the outer-membrane proteins extracted from some mutants and their wild-type strains are shown in Fig. 1. They revealed no changes for the mutants that were only altered in xanthan production (M-11) or resistance to antibiotics (11S-2). However, alterations in the protein patterns were detected for mutants affected simultaneously in both characteristics (11S-1 and 11A-1).

Discussion

The antibiotic-resistance mutations gave rise to three different responses in relation to xanthan production: mutants with increased exopolysaccharide production, mutants with decreased concentration and those that

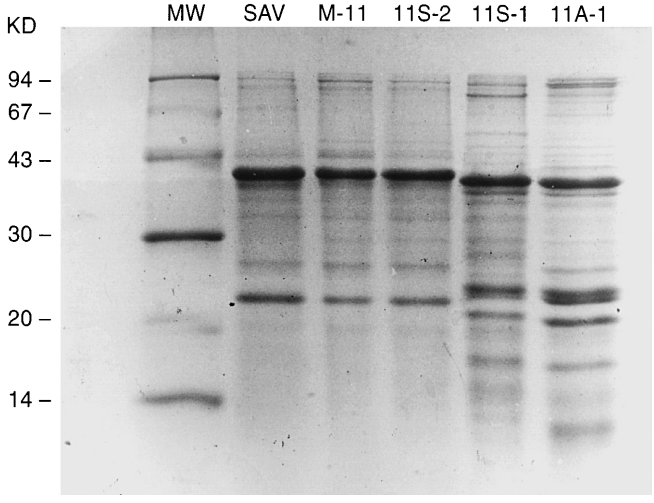

Fig. 1 Electrophoretic pattern of outer-membrane proteins from antibiotic-resistant mutants and their parental strains. *SAV* parent strain for M-11, *M-11* parent strain for 11S-1, 11S-2 and 11A-1

were not affected. We infer from these results that at last two types of antibiotic-resistance mechanisms for the drugs assayed are evident in these strains: one that simultaneously affects xanthan production and another that is not related to this character.

These results also show that the relationship between antibiotic resistance and xanthan production can not be explained by the specific mechanisms responsible for the resistance to one given antibiotic, as was postulated by Sutherland (1977) for the bacitracin-resistant mutants and by Marquett et al. (1989) for their rifampicin-resistant strains. This suggests that an unspecific antibiotic-resistance mechanism could be involved.

Taking into account the previously reported relationship between outer-membrane proteins and resistance to different antibiotics in various gram-negative bacteria (Gutman et al. 1985; Legakis et al. 1989; Lecso-Bornet et al. 1992), as well as the membrane association of polysaccharide gene products in *Rhizobium* (Latchford et al. 1991; Muller et al. 1993; Reuber and Walker 1993), we considered the hypothesis that the observed relationship between xanthan production and antibiotic resistance in these strains of *X. campestris* could be associated with variations in the outer-membrane proteins. The observed changes in outer-membrane protein patterns from the mutants altered in both xanthan production and antibiotic resistance suggest that the mutation confering resistance to the antibiotic and alterations in xanthan production simultaneously could be associated with changes in the protein composition of the outer membrane. Such a mutation could be involved in alterations of the membrane permeability that would affect antibiotic uptake and xanthan export.

The viscosity and xanthan production shown by mutant 11S-1 compared favourably with values for the standard commercial strain NRRL B-1459 (Table 2), and with other reports at the shake-flask level (Torrestriana et al. 1990; Tseng et al. 1992), making this mutant an attractive strain for the production process. The increase in polymer production exhibited by mutants resistant to streptomycin and ampicillin, reported here, also shows the feasibility of using resistance to these antibiotics as a new screening criterion for the selection of more effective strains for xanthan production.

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