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Mutants of *Xanthomonas campestris* defective in secretion of extracellular enzymes

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SUMMARY

Mutants of *Xanthomonas campestris* B1459 were isolated that are defective in secretion of both cellulase and amylase. Both enzymes accumulated in the periplasmic space. The defects in secretion of cellulase or amylase were partly overcome by introducing into the mutants specific multiple copies of DNA cloned from *X. campestris*, and presumed to code for cellulase or amylase enzymes. The mutant strains also showed reduced amounts of extracellular pectinase and protease activities, as if the mutants were generally defective for secretion of extracellular enzymes. The mutants showed reduced pathogenesis for turnip seedlings. The secretion-defective mutants may allow production of xanthan gum with reduced cellulose, pectin, protein and starch-degrading enzyme activities, thereby allowing more widespread mixing of microbially produced xanthan gum with these commercially important water-soluble polymers.

INTRODUCTION

Mutations in *Xanthomonas campestris* that decrease secretion of hydrolytic enzymes from these phytopathogenic bacteria may lead to improved qualities in xanthan gum, a commercially useful polysaccharide that is also secreted from *X. campestris*. Our long-range objective has been to develop strains of *X. campestris* to improve both the utility of the water-soluble polysaccharide and to make incremental improvements in the productivity of the bacteria [13,17,18]. Toward this end, we have borrowed information and techniques from those studying *X. campestris* and related bacteria as plant pathogens. One way to improve the utility of xanthan would be to generate production strains that secreted reduced levels of enzymes that hydrolyze other polymers, such as cellulose, starch, pectin and protein. This could lead to more widespread use of xanthan in mixtures containing these components. In a survey of users of xanthan, we learned that the

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pasteurization step in current production schemes is not sufficiently reliable (especially for removal of cellulase) and therefore precludes mixed formulations. Following chemical mutagenesis we isolated two independent derivatives of *X. campestris* B1459 that were pleiotropically deficient in secretion of more than one extracellular enzyme, but did not completely block the synthesis of the enzymes. Production of xanthan gum remained normal for one mutant, but was deficient or altered for the other. Preliminary mixing experiments with carboxymethylcellulose and starch demonstrated the potential utility of the 'low enzyme' xanthan. In addition, we cloned putative cellulase and amylase genes from *X. campestris*. These will be useful for construction of new strains specifically and completely defective in synthesis of each enzyme, as well as for testing the individual roles of cellulase and amylase in plant pathogenesis.

Not only is *X. campestris* cultivated industrially for production of xanthan gum [11], but it also causes black rot of crucifers [15,20]. Although it is not clear just how the plant disease comes about, some of the more obvious possible contributing factors include compounds secreted from the bacteria: pectinase, protease, cellulase, toxic acidic metabolites and xanthan gum itself. Early work with variants of *X. phaseoli* suggested that the extent of pathogenic symptoms might be determined by the amount of polysaccharide synthesized and secreted [5]. Mutant strains are being isolated to begin to examine more closely the role of xanthan and other determinants in the disease. Mutants of *X. campestris* that are defective in production of xanthan 'ex planta' appear indistinguishable from wild-type bacteria in a test of pathogenesis 'in planta' [2], suggestive, but not conclusive, of a lesser role for xanthan in the disease. Another mutant of *X. campestris* that was isolated because it failed to give a pathogenic response in plants was later found to have reduced levels of pectinase and protease, and the three phenotypes were reversed by the introduction into the mutant of a cloned piece of wild-type DNA comprising about 27 kilobasepairs (kbp) [7]. However, the major secreted protease was not implicated in pathogenesis, since a cloned protease gene from wild-type *X. campestris* failed to restore pathogenicity to this mutant [16]. Furthermore, when the wild-type *X. campestris* protease gene is replaced by marker exchange 'in vivo' with a mutant form of the same gene, the strain retains pathogenicity in the absence of protease accumulation [16]. Thus, like xanthan, normal amounts of the protease seem nonessential for development of the disease. The genetic studies of this and related plant pathogens are still at an early stage and the secretion mutants described in this report may also contribute to the study of bacterial pathogenesis. We show here that a general secretion defect interfered with the pathogenicity of *X. campestris* upon injection of turnip seedlings.

MATERIALS AND METHODS

Bacterial strains and plasmids

X. campestris B1459S-4L-II (our strain X55) was obtained from the Northern Regional Research Center and was the 'wild-type' parent of all *X. campestris* strains used in this study [4]. Strain X59 was a spontaneous rifampicin-resistant derivative of X55 that grows on nutrient-agar plates or liquid medium supplemented with rifampicin at 60 μg/ml [17]. *Escherichia coli* HB101 (Lac^+), JM109 (Lac^-) and vector pUC13 [19] were obtained from Bethesda Research Laboratories. The transferable broad host-range cosmid vector pRK311 (RK2 origin, Tra^+, Mob^- Tet^r λ cos *lacZα*) [8] and mobilizing narrow host range helper plasmid pRK2013 (ColE1 origin, Imm^+, Amp^r Tra^+ Mob^+ Kan^r) [10] were from D.R. Helinski.

Growth media

X. campestris and *E. coli* were grown for enzyme assays in liquid 'YCS' medium containing (per liter of deionized H_2O): 1 g Bacto yeast extract (Difco), 1 g casamino acids (Difco), 3.5 g K_2HPO_4, 2.6 g KH_2PO_4, 0.26 g $MgSO_4 \cdot 7H_2O$, 6 mg H_3BO_3, 6 mg ZnO, 2.6 mg $FeCl_3 \cdot 6H_2O$, 20 mg $CaCO_3$ and either 2 g glucose or potato starch (Difco). For plasmid isolation, *X. compestris* was grown in YT medium, containing tryptone (16 g/l; Difco), yeast

extract (10 g/l; Difco) and NaCl (5 g/l). *E. coli* was grown in LB broth containing (per liter of deionized H_2O):10 g Bacto tryptone (Difco), 5 g Bacto yeast extract (Difco) and 10 g NaCl. Antibiotics were included as needed: rifampicin (100 μg/ml), tetracycline (10 μg/ml for *E. coli* and 7.5 μg/ml for *X. campestris*) and ampicillin (40 μg/ml). All nutrient agar plates contained tryptose blood agar base (TBAB; Difco). Special additions to the plates included potato starch (Difco) at 1% (w/v), carboxymethylcellulose (low viscosity; Sigma) at 0.5% (w/v), nonfat dry milk (Carnation) at 0.5% (w/v), polygalacturonic acid (85–90% pure; Sigma) at 0.7% (w/v) and antibiotics, as above.

Mutagenesis of X. campestris

About $2 \cdot 10^9$ freshly grown cells (A_{600} of 2) were suspended in 2 ml of minimal salts medium containing (per liter): 3.5 g K_2HPO_4, 2.6 g KH_2PO_4 and 0.26 g $MgSO_4 \cdot 7H_2O$ at a final pH of 7.0. The suspension was shaken at 30°C with 0–40 μl of ethylmethanesulfonate for 1, 2, or 3 h. Samples of 0.5 ml were taken from each treatment, washed twice with YT medium, suspended in 2 ml of YT medium, and shaken overnight at 30°C. Dilutions were spread on TBAB plus 1% (w/v) starch plates. After 3 days, non-wild-type colonies (about 1% of the total) were observed. Most of the mutants were nonmucoid. The two mutants described in this work (m60 and m205) were mucoid and had narrow to nonexistent zones of starch hydrolysis (halos) surrounding their colonies. Only one mutant was selected from each treatment with ethylmethanesulfonate, unless colony morphology was clearly distinctive. The mutants were tested for retention of the Rif^r marker of the parent X59. Mutagenesis of plasmid DNA with bacteriophage λ carrying Tn5 was as previously described [14,17].

Cell fractionation and enzyme assays

To determine enzyme localization, log-phase cultures were separated into three fractions. The first 'medium', was the culture fluid after two centrifugation cycles at 10 000 × *g* to remove cells. The second, 'periplasm', was obtained by gently treating washed cells with lysozyme and EDTA. After removing the medium, cell pellets were resuspended at a final concentration of 10^{10} cell/ml in a solution containing 30 mM Tris-HCl (pH 7.0), 3 mM EDTA, 20% (w/v) sucrose and lysozyme (1 mg/ml), and incubated for 30 min at 37°C. This converted the rod-shaped cells to spherical protoplasts. The treated cells were pelleted by centrifugation in an Eppendorf microfuge and the supernatant was taken as the periplasm. The third fraction, 'cell', was prepared by resuspending the protoplasts in 1 ml of cold deionized H_2O and then lysing them with a cycle of freezing and thawing. In some cases the cellular debris was also collected by centrifugation for assay of enzymatic activities.

Cellular fractions were assayed for amylase and cellulase activities by measuring the release of dye from insoluble dye-polymer substrates. Usually 1 volume of sample was mixed with 1 volume of a 0.5% (w/v) solution of either cellulose-azure type II (Sigma) or amylose-azure (Sigma) in 50 mM NaCl, 40 mM potassium phosphate buffer (pH 7). The substrates were washed before use to remove soluble dye. The reactions were at room temperature and stopped by adding 4 volumes of 0.1 N HCl. The reaction mixes were then centrifuged to pellet the unreacted substrate and the absorbance at 595 nm for amylase and 545 nm for cellulase was measured. These were the wavelengths for maximum absorbance. Enzyme activity was calculated as the absorbance per 10^{10} cell equivalents per hour of reaction. Cell equivalents in fractions were determined by prior counting of cells in fixed volumes with a Petroff-Hausser counter and phase contrast microscope.

DNA isolation and recombinant DNA techniques

Total DNA from strain X59 (Xgs^+) was prepared by the boiling method [12] or the Birnboim and Doly procedure [3]. Plasmids were further purified by equilibrium sedimentation in density gradients of CsCl containing ethidium bromide [12]. Restriction enzymes (from Boehringer Mannheim GmbH) were used according to the instructions of the manufacturer. Fragments of DNA were separated by electrophoresis through agarose gels (0.6–1.0% (w/v)) in Tris acetate buffer [12].

Construction of libraries of fragments of chromosomal DNA from *X. campestris* has been described [17]. A complete library (or a specific element of the library) was transferred from *E. coli* to *X. campestris* by a triparental mating scheme [9]. From fresh overnight cultures, 10^9 recipient cells (*X. campestris* Xgs^- mutants), $5 \cdot 10^8$ donor cells (JM109 containing L[X59], the library), and $5 \cdot 10^8$ helper cells (*E. coli* HB101 containing plasmid pRK2013) were mixed and passed through a type HA 0.45-μm-pore-size Millipore filter. The filters were incubated on TBAB plates overnight at 30°C, and the cells were then washed into 2 ml of selecting medium (TBAB plus tetracycline at 7.5 μg/ml and rifampicin at 50 μg/ml). The cells were diluted 10^4–10^5-fold and spread on selection plates containing antibiotics. Exconjugants were purified, and the recombinant plasmid was isolated and transferred back to *E. coli* JM109 for storage and further purification.

Pathogenesis determination with turnip seedlings

The pathogenic phenotype of *X. campestris* was determined essentially as described by Daniels et al. [6] with the following modifications. We used Purple-top White Globe turnip seeds germinated on water-saturated Whatman paper under a 150 watt General Electric 'Plant Gro N Show' lamp. Seedlings of similar appearance and having about 1–2 cm of stem were segregated into lots for injection with viable bacteria. Bacterial cultures were grown on TBAB plates containing rifampicin, resuspended in 100 volumes of LB broth and centrifuged to concentrate the cells. About 10^8 cells were applied to each seedling stem in about 1 μl and then a needle was passed through the adsorbed cells into each stem. Alternatively, the needle was stabbed into a bacterial colony on a growth plate before piercing the stem.

RESULTS

Isolation of secretion mutants of X. campestris

X. campestris mutants defective in secretion of extracellular hydrolytic enzymes were obtained by chemical mutagenesis of strain X59 with ethylmethanesulfonate. Mutagenized cells were spread onto nutrient agar plates containing potato starch. Wild-type *X. campestris* and rifampicin-resistant strain X59 colonies were surrounded by a clear zone, where the insoluble and slightly opaque starch had been hydrolyzed. The diameter of the cleared zone was about 2–3 times the colony diameter. The concentration of mutagen and duration of treatment were chosen to yield about 5% survivors. About 1% of the survivors of mutagenesis were nonmucoid: deficient in synthesis of xanthan gum. Of about 10 000 colonies surveyed on the starch-containing plates only two, m205 and m60, appeared to have small or nonexistent halos, respectively. Each came from a different mutagenesis treatment and each retained the rifampicin-resistance trait and mucoid appearance of the parent X59. Primary screening was not attempted with plates containing either carboxymethylcellulose, pectin or milk protein.

Hydrolysis of polymeric substrates in growth plates

Cells of strain X59, m60 and m205 were spotted onto nutrient agar plates containing either starch, carboxymethylcellulose, pectin or milk protein, and grown for 3 days at 30°C. The patterns of halos, indicating extracellular hydrolysis, are shown in Fig. 1. The breadth of the halo surrounding each bacterial spot indicates the extent of hydrolysis of each substrate. The two mutants both show a similar pattern of deficient hydrolysis for each polymer tested, as if the secretion of different hydrolytic enzymes were coincidently affected by single mutations. Since we have not isolated either mutation away from the mutagenized genetic backgrounds, the possibility remains that the apparent pleiotropy is due to multiple mutations. However, that would be unlikely for four traits.

Accumulation and cellular localization of hydrolytic enzymes in X. campestris

Amylase and cellulase activities in culture medium, periplasm and periplasm-depleted cells were measured for the parental and mutant strains. The results are summarized in Table 1. About 80% of

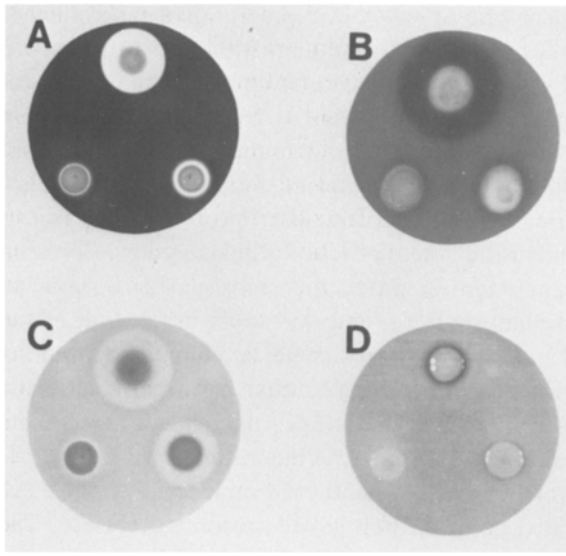

Fig. 1. Plate assay for polymer hydrolysis. Nutrient agar plates containing either (A) potato starch, (B) carboxymethylcellulose, (C) pectin or (D) milk protein were spotted with about 10^8 cells from cultures of the parental X59 (top) or the mutants, m205 (right) or m60 (left). The same pattern is maintained for each plate.

Table 1

Localization of amylase and cellulase activities in *X. campestris*

Strain	Fraction[a]	Percent of total amylase activity[b]	Percent of total cellulase activity[b]
X59	medium	84 ± 8	78 ± 6
	periplasm	13 ± 6	14 ± 5
	cell	3 ± 4	8 ± 2
m205	medium	42 ± 18	48 ± 20
	periplasm	52 ± 19	42 ± 17
	cell	6 ± 5	10 ± 4
m60	medium	36 ± 23	44 ± 5
	periplasm	50 ± 12	37 ± 2
	cell	14 ± 16	19 ± 5

[a] The cell fractionation scheme is given in Materials and Methods.

[b] Reported are means and standard deviations from independent determinations. Enzyme assays are described in Materials and Methods. The enzyme activity in each fraction was expressed as the absorbance per 10^{10} cell equivalents per hour of reaction before calculating percentages.

each hydrolytic activity was found in the medium for wild-type X59. In contrast, both mutants retained significant amounts of amylase and cellulase within the cell or periplasm, and to about the same extent. Although the zones of clearing surrounding colonies of strain m60 on polymer-containing plates were barely detectable and smaller than those for m205, this mutant and m205 were less distinguishable for localization of either enzyme following cell fractionation. However, the total amount of either amylase or cellulase activity in the culture for m60 was about 2–4-fold lower than m205, which in turn was about 2-fold lower than the X59 parent. This analysis would be improved if we possessed proven enzymatic markers for the cytoplasm and periplasm, as are available for *E. coli*. In a parallel experiment we fractionated *E. coli* by the same lysozyme-EDTA method and found 95% of the β-galactosidase activity in the 'cell' fraction. This was the expected distribution for a cytoplasmic enzyme. When we tried to localize the weakly active β-galactosidase synthesized by *X. campestris*, we found that it was periplasmic, quite unlike the cytoplasmic nature of the *E. coli* enzyme. By microscopic analysis of the resulting protoplasts, the effect of the lysozyme-EDTA treatment on *E. coli* and *X. campestris* appeared indistinguishable: protoplasts were formed for each species. Still, it is possible that for *X. campestris* some of the periplasmic activity may originate from the cytoplasm during the lysozyme-EDTA treatment. Even if this is the case, the mutants were defective in secretion of at least two different enzymes into the culture medium.

For amylase measurements, we have grown cells with glucose or with starch. The latter appeared to specifically increase the amount of amylase activity. Cellulase activity was not affected (data not shown). We think this was the result of gene induction rather than protection of the amylase enzyme through binding to substrate, since the amylase activity was stable without added starch and since all of the starch was hydrolyzed by the time the cultures were sampled.

Viscosity of carboxymethylcellulose or starch after mixing with samples taken from X. campestris cultures

Another way to quantitate hydrolytic enzymes is to follow degradation of the viscosity of water-soluble polysaccharides through time after mixing with a test sample. The test sample can be either *X. campestris* cells, cell extracts, culture medium or partially purified xanthan gum. Cultures of X59, X59m205 and X59m60 were grown for 24 h in TS medium plus 2% (w/v) glucose to obtain viscous broths containing xanthan gum at about 1.0–1.6% (w/v). The xanthan was partially purified by removing cells by centrifugation after diluting the viscous cultures 3-fold with water, and then precipitating the polysaccharides with 2 vol. of isopropyl alcohol. The precipitate was dried and ground before resuspending in 0.1% (w/v) NaCl at a final concentration of 0.5% (w/v) xanthan. (Actually about 30% of this solution on a dry weight basis is the added NaCl, contaminating protein and ash.) To 10 ml of 1% (w/v) high-viscosity carboxymethylcellulose was added the equivalent of 1.25 mg xanthan prepared from each culture. After brief mixing, the viscosity was measured at intervals between 1 and 30 min. The amount of carboxymethylcellulose was chosen to give an initial viscosity of about 1000 cps at a shear rate of 4 s^{-1}. A representative experiment is shown in Fig. 2. Although this assay showed no significant difference between the cellulase content of xanthan gum prepared from the parent strain X59 and the mutant m205, the xanthan made from the m60 mutant appeared more deficient in cellulase. For comparison, the lower curve in Fig. 2 shows the potent effect of cellulase from *Trichoderma viridae* (Sigma) on the viscosity of carboxymethylcellulose. We found that most but not all of the decrease in viscosity could be eliminated by prior heat treatment of the xanthan samples. Treatments of 3 h at 60°C and 100°C inactivated most of the cellulase. Similar experiments with xanthan gum from strains X59 and m60 mixed with boiled 2% (w/v) potato starch gave less dramatic results: viscosities of starch remained 2-fold higher in mixtures with m60 xanthan than with X59 xanthan. This was consistent with m60 being deficient in extracellular amylase activity.

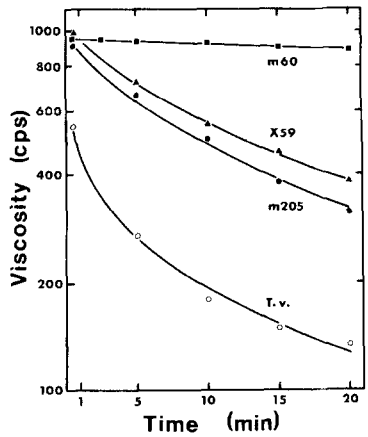

Fig. 2. Decay of viscosity of carboxymethylcellulose after mixing with samples of xanthan gum. The equivalent of 1.25 mg of xanthan gum prepared by isopropyl alcohol precipitation of cultures of X59, m205 or m60 was added to 1% (w/v) high-viscosity carboxymethylcellulose. 'T.V.': 0.5 unit of cellulase prepared from *Trichoderma viridae* (Boehringer Mannheim).

Cloning of genes from X. campestris that code for hydrolytic extracellular enzymes

A library of cloned genes from *X. campestris* strain X59 was screened for sequences coding for cellulase, amylase, protease and pectinase. Generation of the library of clones in *E. coli* JM109 was previously described [17]. Briefly, total DNA from strain X59 was incompletely digested with *Sau*3A restriction endonuclease and inserted into the *Bam*HI site of the broad host-range cosmid pRK311, a vector constructed by Ditta et al. [8]. The vector coded for resistance to tetracycline. The library exceeded 1000 clones, each carrying an insert of about 20–25 kbp. The screening and analysis of each clone is described separately below.

To isolate an amylase clone, the library contents were spread on plates containing 1% (w/v) potato starch and tetracycline. Two colonies of about 1000 examined showed zones of clearing around the colonies within 2–3 days of plating. The host strain by itself, JM109, acquired narrower halos after 5 days. The recombinant plasmids carried in each of these two clones were identical by restriction fragment analysis. One was retained as pSY1494. The DNA insert in pSY1494 included fragments totaling

about 20 kbp in size, following digestion with a mixture of *Eco*RI and *Hin*dIII enzymes. A fragment of about 2.7 kbp and flanked by *Hin*dIII restriction sites was subcloned from pSY1494 into pUC13 and pRK311. *E. coli* JM109 carrying either of these recombinant plasmids produced halos on starch plates, with the halos for pUC-amy being larger. A restriction map of the subcloned 2.7 kbp *X. campestris* DNA is given in Fig. 3. When the 2.7 kbp segment was separated into two *Hin*dIII-*Sma*I subclones, both failed to code for active amylase. Also shown on the map are three insertions of transposon Tn5 that inactivated the amylase activity. However, the precise gene limits are not yet known.

Screening for clones of cellulase genes was as described above for amylase, but with nutrient plates containing 0.5% (w/v) carboxymethylcellulose and tetracycline. In this case, zones of clearing were observed after immersing the plate surface in Congo red, which stains the remaining carboxymethylcellulose. Halos were barely detectable for four colonies out of about 1000 screened. Two clones carrying overlapping pieces of *X. campestris* DNA were isolated and referred to as cel1 and cel10. As for the amylase clone, an identical sibling of each cellulase clone was found. Restriction maps for the cel1 and cel10 cloned inserts are given in Fig. 3. Also shown is a *Sal*I-*Bam*HI subclone ('cel-sal') of about 4.5 kbp that was inserted into pUC13 and that retained the cellulolytic activity. This smaller construction produced more enzyme activity in *E. coli*, as judged by halo size. We speculate that this was due to a higher plasmid copy number.

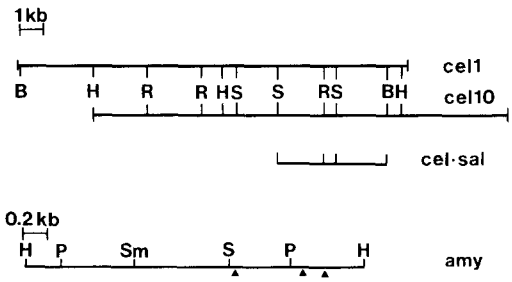

Fig. 3. Map of cleavage sites of restriction endonucleases for cloned fragments of DNA from *X. campestris*. B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; S, *Sal*I; P, *Pvu*II; and Sm, *Sma*I. Arrowheads indicate insertion sites for transposon Tn5 that eliminate amylase activity.

We wondered whether the narrow zones of polymer hydrolysis surrounding the *E. coli* colonies carrying the *X. campestris* amylase or cellulase genes reflected specific secretion or whether the halos were due to nonspecific partial cell lysis. We separated cultures into 'medium', 'periplasm' and periplasm-depleted 'cell' fractions and assayed each for hydrolytic activity. As a control to detect nonspecific lysis, we measured β-galactosidase activities in each fraction. From 93 to 98% of the β-galactosidase remained in the 'cell' fraction, as expected for a cytoplasmic enzyme in the absence of cell lysis. Most of the cellulase and amylase activity also remained cell-bound, indicating that the thin halos on plates were likely due to cell lysis.

A gene believed to code for a protease of *X. campestris* was also isolated. In this case the library was screened on nutrient plates containing milk protein and tetracycline. One clone was found to cause halos around colonies of host *E. coli*, but has not been further analyzed.

Pathogenic effect of X. campestris strains on turnip seedlings

The seedling assay of Daniels et al. [6] was used with minor alterations to measure the destructive effect of wild-type and mutant strains of *X. campestris* for plant stems. The turnip seedlings were 'Purple-Top White Globe' from Burpee. A pathogenic response was scored when the stem darkened and shriveled above and below the point of inoculation within 5 days. This usually caused the stem to collapse and the cotyledons to darken and shrivel as well. In the first test, 8 of 22 seedlings were destroyed by the wild-type strain X59, 3 of 21 by m205 and only 1 of 24 by m60. In an independent follow-up test, 9 of 24 seedlings were destroyed by X59, 0 of 24 by m205, 0 of 28 by m60 and 1 of 22 by *E. coli*.

DISCUSSION

Our overall objective has been to improve the utility of xanthan gum by modifying the production

strains used to make this important water-soluble polysaccharide. Industrial users of xanthan gum are reluctant to mix this preferred viscosifying agent with other water-soluble polysaccharides, such as those derived from cellulose, because of the possibility of introducing hydrolytic enzymes produced by *X. campestris* along with the xanthan gum. Our initial approach to alleviate this problem was to isolate mutants of *X. campestris* that were singly deficient in the production of extracellular hydrolytic enzymes. Unexpectedly, the two independent mutants that we isolated were both deficient for more than one extracellular enzyme activity, among them the hydrolysis of starch, carboxymethylcellulose, pectin and protein. Although some residual hydrolytic activities were present, we found that xanthan gum prepared from these mutant strains contained lower amounts of the enzyme activities than the parental source.

The Food and Drug Administration of the United States stipulates (21 CFR 172.695) that food-grade xanthan gum must be partially purified by recovery with isopropyl alcohol and be free of viable cells. The latter is accomplished by pasteurization. The heat treatment also reduces the activities of contaminating enzymes. A combination of the existing heat treatment with new enzyme-deficient production strains, such as described here, may deplete enough enzyme activity to allow mixing of xanthan with cellulose, starch, pectin or protein, so that the beneficial attributes of each are stably maintained. However, the two mutants reported here will probably not be used to produce xanthan gum. Although the m205 mutant appears to make normal amounts of xanthan gum, it still produces some hydrolytic enzymes, and even though mutant m60 is even more deficient in enzyme production, it undesirably appears to make less xanthan and with an altered quality. Using recombinant DNA techniques, as a secondary strategy, we thought that we could use these original enzyme-deficient mutants as recipients to help screen for clones of the structural genes that actually coded for the hydrolytic enzymes. However, early on we found that it was easier simply to screen libraries of *X. campestris* genes directly in *E. coli*. This works if the genes are expressed in *E. coli*. By looking for zones of hydrolysis surrounding recombinant *E. coli* colonies on culture plates containing a specific polymer, we identified different cloned genes coding for cellulase, amylase and protease activities. The cloned genes were then transferred by conjugation into either mutant *X. campestris*, m60 or m205, to verify the initial observation in *E. coli* of hydrolysis of polymer on plates. The additional copies of the cloned genes on plasmids appeared to partially overcome the secretion defects of the *X. campestris* mutants. We have partly characterized and subcloned two of the enzyme-coding DNA sequences. Assuming that these DNA fragments represent the structural genes for each enzyme activity, we can now mutate the DNA 'in vitro' or 'in vivo' with transposon Tn5 and introduce it back into *X. campestris* to create mutant strains defective in single genes. Recently, we constructed a plasmid vector that can be mated into *X. campestris* but which cannot replicate in that host [18]. Using this vector to carry the mutated DNA into *X. campestris*, we can isolate a mutated recipient as a result of marker exchange between homologous DNA segments. Such gene-specific mutations should not have pleiotropic effects on other hydrolytic activities nor be associated with undesirable additional mutations from chemical mutagenesis. Furthermore, this will allow us to determine whether *X. campestris* has one or multiple enzymes for each hydrolytic activity.

The apparent pleiotropic effects displayed by the new *X. campestris* mutants, m205 and m60, could be the result of either a common element in control of synthesis of the enzymes, or alternatively, a shared secretory mechanism or route. It is possible that an as yet undiscovered global control mechanism operates to switch on or elevate the synthesis of several secreted hydrolytic enzymes. However, if such a mechanism exists, it is distinct from the substrate-specific response of *X. campestris* to starch that leads to elevated amylase activity, but not cellulase. An alternative to the common synthesis argument is the notion of a common secretory mechanism. We favor the common secretion idea because both mutants appeared to accumulate amylase and cellulase in the periplasm, rather than secrete the

enzymes into the medium. Mutations affecting the secretion of more than one enzyme appear to be quite common. Earlier studies with other gram-negative microorganisms, *Pseudomonas aeruginosa* and *Erwinia chrysanthemi*, revealed pleiotropic mutations that affected the secretion of several extracellular enzymes [1,21,22]; and the culture medium from cultures of *X. campestris* mutant strain 8237, isolated by Daniels et al. [7], is reported to be deficient in both protease and polygalacturonate lyase activities.

The question remains as to whether the periplasmic accumulation for the mutant strains reflects a part of the normal secretory pathway but with a defect in a later step in secretion, or alternatively, an artificial rerouting of the enzymes. The low but significant amount of enzyme activity in the periplasm for the parental strain suggests that the enzyme probably moves through the periplasm on its normal secretory route. However, definition of the actual route must await physical identification of the enzymes (e.g. with specific antisera) and quantitative pulse-chase labeling experiments.

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