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Control of Pierce's Disease through Degradation of Xanthan Gum

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The diseases caused by *Xylella fastidiosa* are associated with aggregation of the bacteria in xylem vessels, formation of a gummy matrix and subsequent blockage of water uptake. In the closely related pathogen, *Xanthomonas campestris*, xanthan gum is known to be an important virulence factor, probably contributing to bacterial adhesion, aggregation and plugging of xylem. Xanthan gum, produced by *X. campestris*, is an extracellular polysaccharide consisting of a cellulose backbone (β-1,4-linked D-glucose) with trisaccharide side chains composed of mannose, glucuronic acid and mannose attached to alternate glucose residues in the backbone. We had constructed a mutant of *X. campestris* lacking *gumI* gene that is responsible for adding the terminal mannose for producing modified xanthan gum which is similar to xanthan gum from *X. fastidiosa*. The modified xanthan gum degrading endophytic bacterium *Acinetobacter johnsonii* GX123 isolated from the oleander infected with leaf scorch disease.

Keywords **:** *Acinetobacter johnsonii*, *Xylella fastidiosa*, xanthanase

Xylella fastidiosa colonizes the xylem of various host plants, causing economically important diseases such as Pierce's disease (PD) in grapevine and citrus variegated chlorosis (CVC) in sweet oranges. In addition, it has been related to alfalfa dwarf and peach leaf scorch, among other diseases in trees such as almond, elm, oak, oleander, plum, maple and sycamore (Hopkins, 1989; Purcell and Hopkins, 1996; Wells et al., 1987). Pierce's disease of grapevine has been known in California for over 100 years, but the recent arrival of a much more efficient vector, the glassy-winged sharpshooter, has greatly increased the threat of this pathogen to the grape industry (Gardner and Hewitt, 1974; Blua et al., 1999).

The diseases caused by *X. fastidiosa* are associated with aggregation of the bacteria in xylem vessels, formation of a gummy matrix and subsequent blockage of water uptake. In the closely related pathogen, *Xanthomonas campestris*, xanthan gum is known to be an important virulence factor, probably contributing to bacterial adhesion, aggregation and plugging of xylem (Katzen et al., 1998). The recently published genome sequence of the citrus variegated chlorosis strain of *X. fastidiosa* revealed that this pathogen also has genes for xanthan gum production (Simpson et al., 2000). In Pierce's disease, xanthan gum is likely to contribute to plugging of the grapevine xylem and possibly to the aggregation of the bacterium in the mouthparts of an efficient vector, the glassy-winged sharpshooter (Keen et al., 2000; Blua et al., 1999).

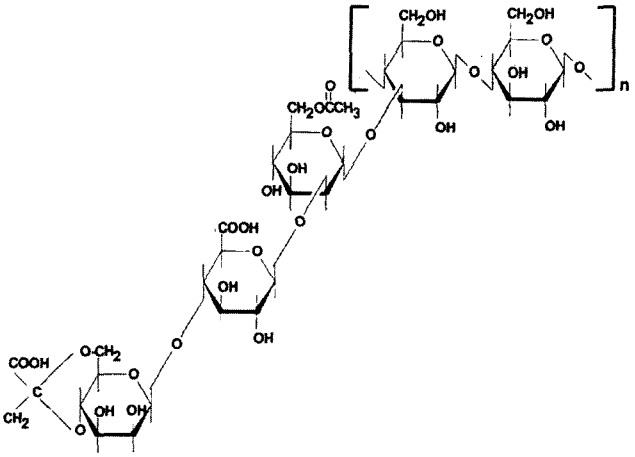

Fig. 1. Structural unit of xanthan gum. The structure of the xanthan repeating unit is according to Jansson et al. (1975). The number of substituents is variable. Some external mannose contain a second *O*-acetyl substituent (Stankowsky et al., 1993).

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Xanthan gum, produced by *X. campestris*, is an extracellular polysaccharide consisting of a cellulose backbone (β-1,4-linked D-glucose) with trisaccharide side chains composed of mannose, glucuronic acid and mannose attached to alternate glucose residues in the backbone (Fig. 1., Jannson et al., 1975; Rogovin et al., 1961). The internal and terminal mannosyl residues of the side chain are frequently acetylated and pyruvylated, respectively, depending on both growth conditions and the bacterial strains (Sandford et al., 1977). Variant xanthan gums with truncated side chains, consisting of tetrasaccharide or trisaccharide repeating units, are produced by *X. campestris* mutants (Bradshaw et al., 1983; Tait and Sutherland, 1989). Truncation of the side chain affects the viscometric properties of xanthan gum. Compared to the polypentamer, the acetylated polytetramer is a weak viscosifier, whereas the polytrimer is reported to be a superior viscosifier on a weight basis (Levy et al., 1996; Shatwell and Sutherland, 1990; Hassler and Doherty, 1990).

To determine whether the xanthan gum from the PD strain of *X. fastidiosa* is likely to be structured as we had predicted from the CVC strain sequence, we compared the available genomic sequences of the closely related almond leaf scorch strain and the more distantly related oleander leaf scorch strain. All of these *Xylella* strains have the same nine gum genes conserved, and they all lack *gumG, gumI* and *gumL* that are found in *Xanthomonas* (Fig. 2., Simpson et al., 2000; Bhattacharyya et al., 2002; Da Silva et al., 2001). In *Xanthomonas*, *gumI* is thought to add the terminal mannose residue on the sugar side chains of the xanthan polymer. *gumG* and *gumL* add acetylate and pyruvate to the terminal mannose (Fig. 3., Becker et al., 1998). The lack of these three genes in *Xylella* suggests that its xanthan gum lacks the terminal mannose on the side chain, which is predicted to reduce its viscosity slightly. In *Xanthomonas*, mutations in *gumL* had no effect on virulence, and mutations in either *gumG* or *gumI* only slightly reduced virulence (Katzen et al., 1998). This suggests that *Xylella* xanthan gum, while lacking the acetylated and pyruvated terminal mannose on the side chains, is still a significant virulence factor. The distinction between the forms of xanthan from *Xanthomonas* and *Xylella* is important, however, since some xanthan-degrading enzymes show specificity in their recognition of different forms of xanthan, such as pyruvated or non-pyruvated forms (Ruijssenaars et al., 1999)

Purifying significant quantities of xanthan gum from the slow-growing *X. fastidiosa* is not practical. Therefore, we had constructed a mutant of *Xanthomonas campestris* lacking *gumI* gene that is responsible for adding the terminal mannose. A large section of the *gum* operon was cloned from *Xanthomonas*, and an antibiotic resistance cassette was inserted into a deletion in the *gumI* gene. This construction was used to introduce the mutation into wild type *X. campestris* by homologous recombination.

For xanthan gum production, a full loop of 2 days old bacteria in YM agar medium was transferred to a 125 ml

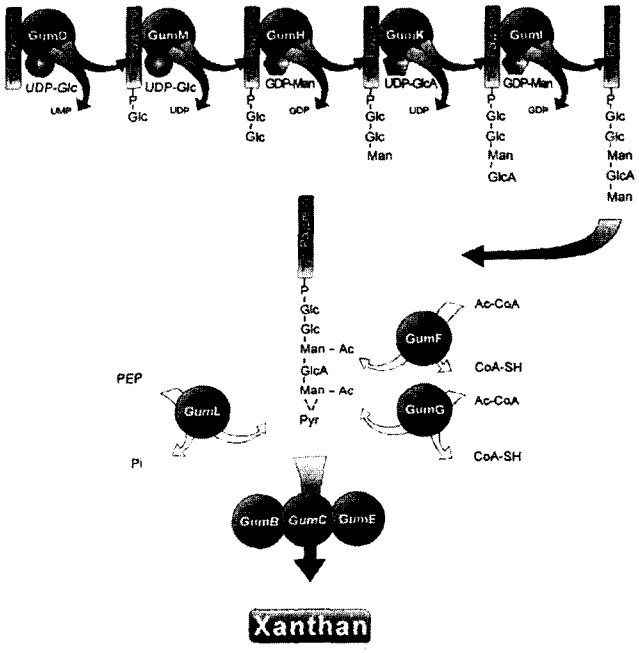

Fig. 3. Proposed pathway for the synthesis of the xanthan gum. *Glc* glucose, *Man* mannose, *GlcA* glucuronic acid, *Ac* acetyl, *Pyr* pyruvyl (Becker et al., 1998).

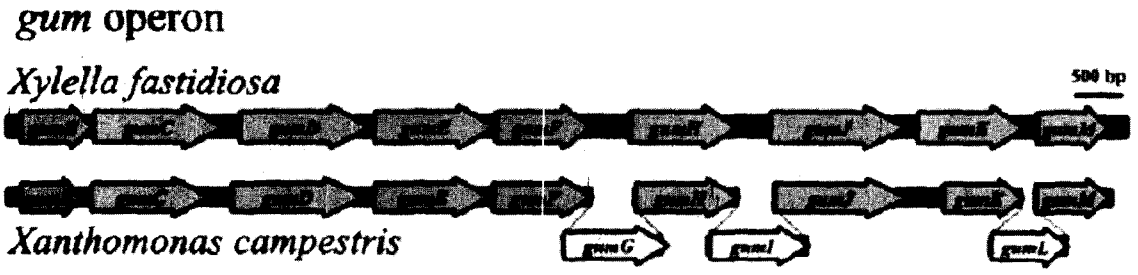

Fig. 2. Genetic map of the *X. campestris gum* operon compared to the region of the homologous genes in the *X. fastidiosa* genome. The black bar represents the DNA sequence and the overlying arrows represent the genes. The white genes in the *X. campestris* operons are not found in *X. fastidiosa* and are drawn shifted out of the sequence in order to maintain alignment (Da Silva et al., 2001).

Erlenmary flask containing 25 ml of YM-T medium and incubated overnight in an orbital shaker at room temperature and 200 rpm (Casas et al., 2000; García-Ochoa et al., 1992, 1995). Five milliliter of the culture were transferred to a 500 ml Erlenmary flask containing 90 ml of OP20 medium and incubated for 4 days in orbital shaker at room temperature and 200 rpm (Casas et al., 2000; García-Ochoa et al., 1992, 1995). Xanthan gum was recovered by the method of García-Ochoa et al. (1999, 2000). The grown culture was diluted 8 times with 0.9% NaCl solution, shook with stirrer overnight and centrifuged at 12,000 rpm for 90 min. The supernatant was removed to the clean bottle and precipitated with 3 volumes of isopropanol. The precipitated xanthan gum was filtrated by 80ìm nylon net filter with vacuum and purified by dissolving it in 0.1% NaCl solution and repeating the precipitation step before drying. The viscosities of xanthan gum solutions were measured according to the method of Shoemaker et al. (1989) with Ostwald capillary viscometer at 25C. The modified xanthan gum was still viscous, but had a measurable decrease in viscosity compared with xanthan gum from the wild type strain of *Xanthomonas.*

Scientists interested in the industrial production of xanthan gum have characterized several xanthan-degrading enzymes. Xanthan gum is used as food additive in drinks, ice cream, salad dressings, syrups and other food products. However, most of the 10,000-20,000 tons of xanthan gum produced per year worldwide is used for oil recovery and in the manufacture of pharmaceuticals, cosmetics, paper, paint, adhesives and textiles (Becker et al., 1998). The features that make xanthan gum such as attractive industrial product include its high viscosity at low concentrations, tolerance for a wide range of pH and temperature conditions and a pseudoplasticity property. These are probably also the reasons why it is an effective virulence factor in plant pathogens by promoting bacterial colonization and adherence and blocking of water-conducting tissues in plants under a wide range of environmental conditions. A major problem in production of large amounts of xanthan gum is the extreme viscosity of culturings during fermentation, which reduces yield by affecting oxygen and nutrient availability. Enzymatic modification of xanthan polysaccharide has therefore been studied to modify its viscosity in controlled ways. Xanthan-degrading enzymes have been isolated from microorganisms discovered through enrichment of environmental samples with growth media containing xanthan gum as the sole carbon source (Ahlgren, 1991; Cadmus et al., 1982, 1989; Hashimoto et al., 1998; Hou et al., 1986; Lesley, 1961; Sutherland, 1982, 1987; Ruijssenaars et al., 1999). Several hydrolytic enzymes that cleave the cellulosic main chain of xanthan gum have been described and are generally referred to as xanthanases. Common cellulases, such as those produce from fungi, can also cleave xanthan gum, but only slowly and only under restricted conditions (Sutherland, 1984). Recognition of the side chain sugars appears to be responsible for the specificity of the xanthan specific enzymes. In addition to xanthanases that cleave the main chain, other enzymes called xanthan lyases, remove the side chain sugars from the xanthan polymer, which also decreases viscosity.

We were interested in exploring application of both xanthanases and xanthan lyases. Unfortunately, many of these strains that have xanthanases or xanthan lyases are no longer available or were never obtained in pure culture; much of the work on xanthan-degrading enzymes was done with mixtures of uncharacterized bacteria. In addition, all of the individual strains that were characterized are gram positive soil bacteria. Bacteria isolated from the soil would be unlikely to be useful as systematic colonists as grape endophytes, and gram positive bacteria are much less amenable to genetic analysis for obtaining cloned genes encoding xanthan-degrading enzymes. Two such genes have been cloned and recently sequenced (Ruijssenaars et al., 2000; Hashimoto et al., 2001). However, these genes encode a xanthan lyase that only recognizes the pyruvated form of xanthan gum, which *Xylella* is not expected to produce.

The modified xanthan gum from the *Xanthomonas* mutant described above was used as the sole carbon source for enrichment culture from Pierce's disease infected grapevines. One hundred samples of oleander infected with leaf scorch disease in Riverside and two hundreds of samples of grapevine infected with Pierce's disease in Southern California were collected. Samples were washed in running tap water. Individual 2-3cm tissues (stem, petiole, leaf vein) were cut with a razor blade from the samples and were placed into sterile test tubes with 10 ml of 1% NaOCl solution amended with 0.1% tween 20 for 5 min. Surface-disinfected pieces were aseptically transferred through three washes of 10 ml of sterile phosphate buffered saline (PBS) for 3 min each. To check for surface contamination, 0.1ml of the third wash for each tissue was transferred to 5ml of Tryptic Soy Broth (TSB) and was incubated at room temperature on a rotary shaker for 2 days. Surface-disinfected pieces were macerated with 2 ml of PBS using mortars and pestles. One ml of resulting suspension was transferred 25 ml of mineral salts medium, pH 6.9, with modified xanthan gum and incubated at 28 °C on rotary shaker for 5-7 days to allow growth of endophytic bacteria. Mineral salts medium contained the following (in milligrams per liter): EDTA, 10.0; $ZnSO_4 \cdot 7H_2O$, 2.0; $CaCl_2 \cdot 2H_2O$, 1.0; $FeSO_4 \cdot 7H_2O$, 5.0; $Na_2MoO_4 \cdot 2H_2O$, 0.2; $CuSO_4 \cdot 5H_2O$, 0.2; $CoCl_2 \cdot 6H_2O$, 0.4; $MnCl_2 \cdot 4H_2O$, 1.0; $(NH_4)_2SO_4$, 2,000; $MgCl_2 \cdot 6H_2O$, 100; K_2HPO_4, 1,550; and $NaH_2PO_4 \cdot H_2O$, 850.

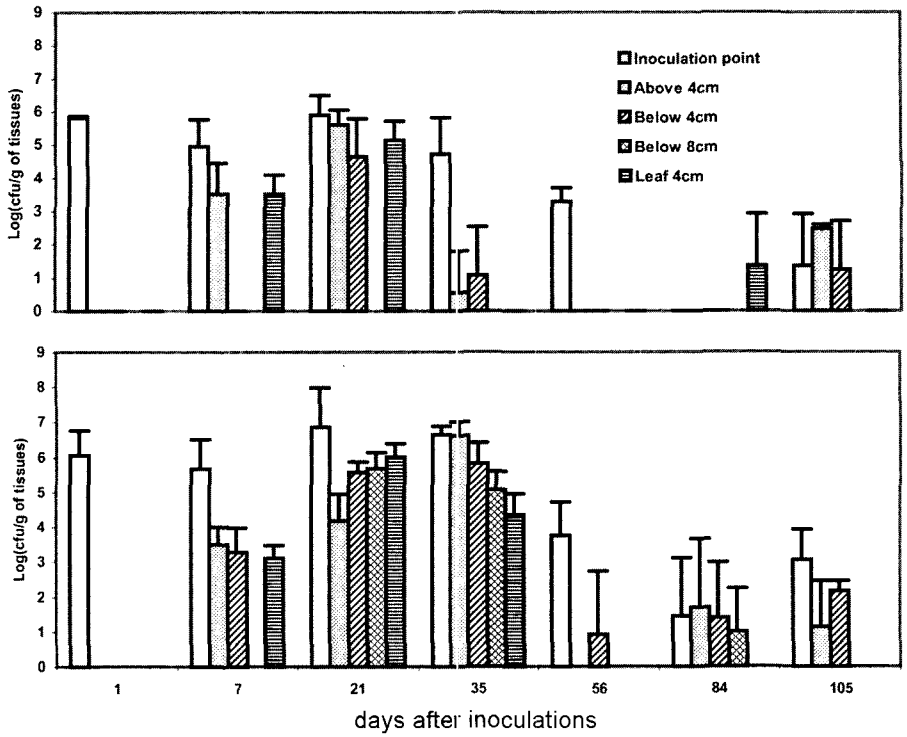

Fig. 4. Populations of *Acinetobacter johnsonii* GX123 in the stem and leaf of the oleander at the various weeks after needle inoculation alone (above) and with *Xylella fastidiosa* (below).

Cultures were centrifuged, and the viscosity of their supernatant was measured. Cultures that had a decreased viscosity were transferred to fresh media and incubated for 3 days. The enrichment step was repeated twice. Cultures were finally spread on solid media with modified xanthan gum as the sole carbon source, and individual colonies were streaked to purify on fresh plates. Pure cultures were tested for reduction of viscosity of modified xanthan gum as measured with an Ostwald capillary viscometer. Over 100 bacterial isolates were initially recovered from these enrichment experiments, and 11 isolates were subsequently confirmed to effectively degrade modified xanthan gum. These isolates were then tested for cellulase activity. Degradation of the cellulosic backbone of xanthan polymer would be desirable, but we do not want enzymes that recognize and degrade plant cellulose. Six of the isolates had low or non-detectable cellulase activity.

Identification of strain GX123 isolated from oleander was carried out by Biolog automated microbial identification system, computer-assisted fatty acid profiling and 16S rDNA analysis. On Biolog MicroLog2 version 4.20, GX123 was identified to *Acinetobacter johnsonii* with 83.0% similarity and fatty acid analysis showed the pattern for *A. johnsonii* with 61.2% similarity. 16S ribosomal DNA sequencing showed 99.0% similarity to *A. johnsonii.*

One year-old oleander plants were inoculated in the green house (20-32°C). Needle inoculation was done by probing five times with a number 0 insect pin through a 10 µl drop of a log 8 CFU/ml bacterial suspension of strain GX123 in PBS into young stems of the test plants. Plants were sampled destructively 1 day after inoculation, and 1, 3, 5, 8, 12, and 15 week later. In each evaluation, five samples were taken from each plant at the following locations: inoculation point (area of stem in which inoculation puncture was done), first leaf distal to the inoculation point and 1-cm sections of stem 4 cm above, 4 cm below and 8 cm below that area. The sections were surface-sterilized in 1% NaOCl for 5 min and rinsed in three changes of sterile distilled water and ground in 2 ml of PBS with a mortar and pestle. The serial dilutions of the suspension in PBS were completed and aliquots plated onto LB medium with the antibiotics (Bacitracin, 50 µg/ml; Spectinomycin, 50 µg/ml and Streptomycin 50 µg/ml).

Systemic movement was shown by culturing of *A. johnsonii* GX123 from leaves, following inoculations of the stem, and from parts of the stem 4cm above and below the inoculation point 1 to 3 weeks. Population of *A. johnsonii* GX123 was high on the inoculation with *X. fastidiosa* in the inoculation points. *A. johnsonii* GX123 spread rapidly in oleander and reached populations of up to log 7 CFU/g of tissue by 3 weeks after inoculation with *X. fastidiosa*. After 8 weeks, *A. johnsonii* GX123 was not detected in the leaves

and the stems except on the inoculation point, but after 15 weeks, bacteria were detected in the stems again.

References

Ahlgren, J. A. 1991. Purification and characterization of a pyruvated-mannose-specific xanthan lyase from heat-stable, salt-tolerant bacteria. *Appl. Environ. Microbiol.* 57:2523-2528.

Becker, A., Katzen, F., Pühler, A. and Ielpi, L. 1998. Xanthan gum biosynthesis and application: a biochemical/genetic perspective. *Appl. Microbiol. Biotechnol.* 50:145-152.

Bhattacharyya, A., Stilwagen, S., Ivanova, N., Mark, D., Bernal., A., Lykidis, A., Kapatral, V., Anderson, I., Larsen, N., Los, T., Leznik, G., Selkov., E. Jr., Walunas, T. L., Feil, H., Feil, W. S., Purcell, A., Lassez, J.-L., Hawkins, T. L., Haselkorn, R., Overbeek. R., Predki, P. F. and Kyrpides, N. C. 2002. Whole-genome comparative analysis of three phytopathogenic *Xylella fastidiosa* strains. *Proc. Natl. Acad. Sci. USA* 99:12403-12408.

Blua, M. J., Phillips, P. A. and Redak, R. A. 1999. A new sharpshooter threatens both crops and ornamentals. *California Agriculture* 53:22-25.

Bradshaw, I. J., Nisbet, B. A., Kerr, M. H. and Sutherland, I. W. 1983. Modified xanthan: its preparation and viscosity. *Carbohydr. Polym.* 3:23-28.

Cadmus, M. C., Jackson, L. K., Burton, K. A., Plattner, R. D. and Slodki, M. E. 1982. Biodegradation of xanthan gum by *Bacillus* sp. *Appl. Environ. Microbiol.* 44:5-11.

Cadmus, M. C., Slodki, M. E. and Nicholson, J. J. 1989. High-temperature, salt-tolerant xanthanase. *J. Ind. Microbiol.* 4:127-133.

Casas, J. A., Santos, V. E. and García-Ochoa, F. 2000. Xanthan gum production under several operational conditions: molecular structure and rheological properties. *Enzyme Microb. Technol.* 26:282-291.

Da Silva, F. R., Vettore, A. L., Kemper, E. L., Leite, A. and Arruda, P. 2001. Fastidian gum: the *Xylella fastidiosa* exopolysaccharide possibly involved in bacterial pathogenicity. *FEMS Microbiol. Lett.* 203:165-171.

García-Ochoa, F., Santos, V. E. and Alcón, A. 1995. Xanthan gum production: an unstructured kinetic model. *Enzyme Microb. Technol.* 17:206-217.

García-Ochoa, F., Santos, V. E. and Fritsch, A.P. 1992. Nutritional study of *Xanthomonas campestris* in xanthan gum production by factorial design of experiments. *Enzyme Microb. Technol.* 14:991-996.

García-Ochoa, F., Santos, V. E., and Casas, J. A. 1999. Production and isolation of xanthan gum, In Bucke, C. (ed), *Methods in biotechnology*, Vol. 10. Humana Press Inc., NJ. p. 7-21.

García-Ochoa, F., Santos, V. E., Casas, J. A. and Gómez, E. 2000. Xanthan gum: production, recovery and properties. *Biotechnol. Adv.* 18:549-579.

Gardner, M. W. and Hewitt, W. B. 1974. Pierce's disease of the grapevine: *The Anaheim disease and the California vine disease*. Univ. of California Press, Berkley, CA 225pp.

Hashimoto, W., Miki, H., Tsuchiya, N., Nankai, H. and Murata, K. 1998. Xanthan lyase of *Bacillus* sp. Strain GL1 liberates pyruvated mannose from xanthan side chains. *Appl. Environ. Microbiol.* 64:3765-3768.

Hashimoto, W., Miki, H., Tsuchiya, N., Nankai, H. and Murata, K. 2001. Polysaccharide lyase: Molecular cloning, sequencing, and overexpression of the xanthan lyase gene of *Bacillus* sp. Strain GL1. *Appl. Environ. Microbiol.* 67:713-720.

Hassler, R. A. and Doherty, D. H. 1990. Genetic engineering of polysaccharide structure: production of variants of xanthan gum in *Xanthomonas campestris*. *Biotechnol. Prog.* 6:182-187.

Hopkins, D. L. 1989. *Xylella fastidiosa*: A xylem-limited bacterial pathogen of plants. *Ann. Rev. Phytopathol.* 27:271-290.

Hou, C. T., Barnabe, N. and Greaney, K. 1986. Biodegradation of xanthan by salt-tolerant aerobic microorganisms. *J. Ind. Microbiol.* 1:31-37.

Jannson, P. E., Kenne, L. and Lindberg, B. 1975. Structure of the extracellular polysaccharide from *Xanthomonas campestris*. *Carbohydr. Res.* 45:275-282.

Katzen, F., Ferrerio, D. U., Oddo, C. G., Ielmini, M. V. Becker, A., Pühler, A. and Ielpi, L. 1998. *Xanthomonas campestris* pv. campestris *gum* mutants: effects on xanthan biosynthesis and plant virulence. *J. Bacteriol.* 180:1607-1617.

Keen, N. T., Dumenyo, C. K., Yang, C-H. and Cooksey, D. A. 2000. From rags to riches: insights from the first genomic sequence of a plant pathogenic bacterium. *Genome Biology* 1:1019.1-1019.4.

Lesley, S. M. 1961. Degradation of the polysaccharide of *Xanthomonas phaseoli* by an extracellular bacterial enzyme. *Can. J. Microbiol.* 7:815-825.

Levy, S., Schuyler, S. C., Maglothin, R. K. and Staehelin, L. A. 1996. Dynamic simulations of the molecular conformations of wild type and mutant xanthan polymers suggest that conformational differences may contribute to observed differences in viscosity. *Bioploymers* 38:251-272.

Purcell, A. H. and Hopkins, D. L. 1996. Fastidious xylem-limited bacterial plant pathogens. *Ann. Rev. Phytopathol.* 34:131-151.

Rogovin, S. P., Anderson, R. F. and Cadmus, M. C. 1961. Production of polysaccharide with *Xanthomonas campestris*. *J. Biochem. Microbiol. Technol. Eng.* 3:51-63.

Ruijssenaars, H. J., Hartmans, S. and Verdoes, J. C. 1999. A pyruvated mannose-specific xanthan lyase involed in xanthan degradation by *Paenibacillus alginolyticus* XL-1. *Appl. Environ. Microbiol.* 65:2446-2452.

Sandford, P. A., Pittsley, J. E., Knutson, C. A., Watson, P. R., Cadmus, M. C. and Janes, A. 1977. Variation in *Xanthomonas campestris* NRRL B-1459: characterization of xanthan products of differing pyruvic acid content, In Sandford, P. A. and Laskin, A. (ed), *Extracellular microbial polysaccharides*. American Chemical Society Symposium Series, no. 45. American Chemical Society, Washington, D.C. p. 192-210.

Shatwell, K. P. and Sutherland, I. W. 1990. Influence of acetyl and pyruvate substituents on the solution properties of xanthan polysaccharide. *Int. J. Biol. Macromol.* 12:71-78.

Shoemaker, D. P., Garland, C. W. and Nibler, J. W. 1989. Experi-

ments in physical chemistry, 5th ed. McGraw-Hill Book Company, NY.

Simpson, A. J. G., Reinach, F. C., Arruda, P., Abreu, F. A., Acencio, M., Alvarenga, R., Alves, L. M. C., Araya, J. E. Baia, G. S. Baptista, C. S., et al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* 406:151157.

Stankowski, J. D., Mueller, B. E. and Zeller, S. G. 1993. Location of a second *O*-acetyl group in xanthan gum by the reductive-cleavage method. *Carbohydr. Res.* 241:321-326.

Sutherland, I. W. 1982. An enzyme system hydrolyzing the polysaccharides of *Xanthomonas* species. *J. Appl. Bacteriol.* 53:385-393.

Sutherland, I. W. 1984. Hydrolysis of unordered xanthan in solution by fungal cellulases. *Carbohydr. Res.* 131:93-104.

Sutherland, I. W. 1987. Xanthan lyase - novel enzymes found in various bacterial species. *J. Gen. Microbiol. 1*33:3129-3134.

Tait, M. I. and Sutherland, I. W. 1989. Sysnthesis and properties of a mutant type of xanthan. *J. Appl. Bacteriol.* 66:457-460.

Von Stettern, F., Francis, K. P. Lechner, S., Neuhaus, K. and Scherer, S. 1998. Rapid discrimination of psychrotolerant and mesophilic strains of the *Bacillus cereus* group by PCR targeting of 16S rDNA. *J. Microbiol. Methods* 34:99-106.

Wells, J. M., Raju, B. C., Huang, H. Y. Weisberg, W. G., Mandelco-Paul, L. and Brenner, D. J. 1987. *Xylella fastidiosa* gen. Nov., sp. Nov.: Gram negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. *Int. J. Syst. Bacteriol.* 37:136-143.