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Cloning and Analysis of a 35.3-Kilobase DNA Region Involved in Exopolysaccharide Production by *Xanthomonas campestris* pv. *campestris*

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Cosmid clones able to restore exopolysacoharide production in possibly insertion sequence element-induced surface mutants of *Xanthomonas campestris* pv. *campestris* were isolated. By fragment-specific Tn*5-lac* mutagenesis of one of the cosmids, pXCB1002, a new DNA region which is involved in exopolysaccharide biosynthesis and which is organized into at least 12 complementation groups was identified.

Because of its rheological properties, the exopolysaccharide (EPS) xanthan is of great industrial importance, and strains of *Xanthomonas campestris* pv. *campestris* are used industrially for xanthan production (1). Although the chemistry of xanthan has been extensively studied (e.g., 14, 15, 21, 27) and clustered genes involved in the synthesis of xanthan have been identified (2, 5, 12, 28), the gene functions and control mechanisms are poorly understood. A detailed genetic analysis of xanthan biosynthesis requires the isolation of mutants that have an altered EPS phenotype. In general, one has to be aware of the fact that particular mutations may have pleiotropic effects on the bacterial envelope components, because EPSs and lipopolysaccharides are partially composed of the same sugar subunits; therefore, the same precursors are utilized in the synthesis of both compounds (8, 30). Pleiotropic mutants have already been isolated from *Xanthomonas*, *Rhizobium*, and *Pseudomonas* strains (e.g., 13, 19, 29, 30). In this paper, we describe the cloning and characterization of a new gene region involved in xanthan gum synthesis. The cloned DNA was physically mapped and arranged into groups according to complementation ability.

Following Tn*5* mutagenesis with pSUP102-Gm as the transposon carrier (24, 25), different colony morphology mutants of *X. campestris* pv. *campestris* B100, a Sm^r derivative of the wild-type strain DSM1526, were isolated on TY medium (5 g of tryptone–3 g of yeast extract–0.7 g of $CaCl_2$ per 1 liter of H_2O) supplemented with 2% sucrose. The mutants were also tested for autoagglutination in TY liquid medium and for motility on TY soft agar, since data from other gram-negative bacteria have shown that defects in lipopolysaccharide production are correlated with a number of other mutant phenotypes, such as rough colony surface, autoagglutination, and loss of motility (e.g., 7, 18, 26). Three EPS-deficient mutants were investigated: B100-152 had a smooth colony surface, whereas mutants B100-13 and B100-22 were characterized by rough colony types, autoagglutination in TY liquid medium, and lack of motility on TY soft agar plates.

A cosmid library of wild-type *X. campestris* pv. *campestris* was constructed with the mobilizable cosmid vector pSUP205 (24), which cannot replicate in *X. campestris* pv. *campestris*. Recombinant cosmids contained inserts ranging in size from 30 to 40 kilobases (kb) (*Eco*RI fragments). Bacterial matings on membrane filters (pore size, 0.2 μm) and genetic complementation analyses were performed as described elsewhere (22, 23). The cosmids were mobilized into B100-13, B100-22, and B100-152. Complemented colonies were identified on TY medium supplemented with tetracycline (10 μg/ml) and 2% sucrose. Different cosmids were isolated after retransfer to *Escherichia coli* and analyzed by restriction with *Eco*RI. All cosmids showed overlapping fragments. A restriction map of cosmid pXCB1002, which contains nine *Eco*RI fragments, was obtained for the enzymes *Eco*RI, *Bam*HI, *Hin*dIII, and *Xho*I (Fig. 1). After subcloning, plasmid pXCB3012 containing a 3.8-kb *Eco*RI fragment was shown to be sufficient to restore the wild-type phenotype in mutants B100-13 and B100-22, whereas B100-152 could only be complemented by a combination of 3.8-, 1-, and 0.5-kb fragments (pXCB2012).

For DNA isolation, *X. campestris* pv. *campestris* strains were grown overnight in Penassay medium (Difco Laboratories) and genomic DNA was isolated from about 10^9 cells (20). Total DNA from the *X. campestris* pv. *campestris* EPS mutants was digested with *Eco*RI and hybridized against DNA from cosmid pXCB1002 (Fig. 2). Mutants B100-13 and B100-22 showed different hybridization patterns, but both mutations were caused by a 1.7-kb insertion into the 3.8-kb fragment which introduced an additional *Eco*RI site. Mutant B100-152 carried an insertion of about 0.3 kb in the 1-kb *Eco*RI fragment. Therefore, the mutations could not be correlated to Tn*5* insertions. The possibility that the 3.8-kb fragment and neighboring DNA regions might be favored targets for insertion sequence element mutations is very interesting in view of the instability in EPS production which has been reported for several organisms, including *Pseudomonas aeruginosa* (9, 11), *Pseudomonas atlantica* (3, 4), *Zoogloea ramigera* (10), and *X. campestris* (17). To study the location and expression of EPS biosynthetic genes, we exposed pXCB1002 to transposon Tn*5-lac* (Tn*5-B20*; 25) mutagenesis in *E. coli* as described elsewhere (16, 25). After mapping the positions of the Tn*5-lac* insertions in the cloned *X. campestris* pv. *campestris* DNA, we recombined the mutagenized fragments into the *X. campestris* pv. *campes-*

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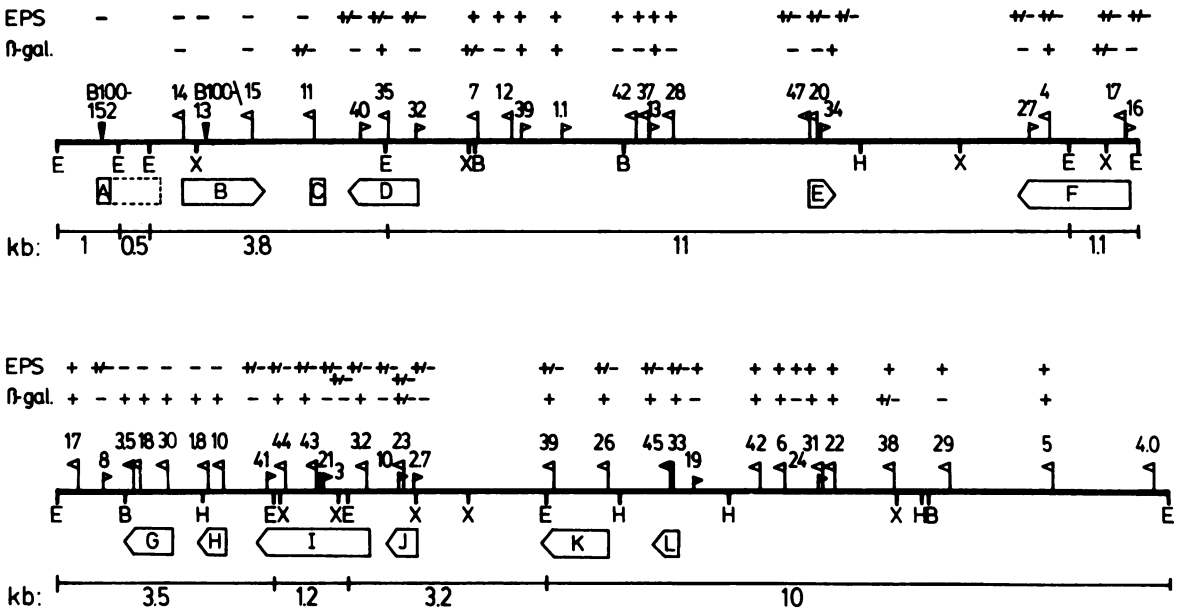

FIG. 1. Physical and genetic map of Tn*5-lac* and possible insertion sequence element positions in the *X. campestris* pv. *campestris* DNA cloned in pXCB1002 and associated phenotypes. Gene boundaries indicate the minimum extent and direction of transcription of the various complementation groups (A to L) established by cross-complementation as described in the text. EPS represents EPS production on TY plates supplemented with 2% sucrose: +, wild type; ±, intermediate phenotype; −, no visible EPS production. β-gal represents expression of β-galactosidase on 5-bromo-4-chloro-3-indolyl-β-D-galactoside medium: −, wild type; ±, intermediate expression; +, high expression. Symbols: ◥ and ▶, Tn*5-lac* insertions with different orientations of the *lacZ* gene; ▼, possible insertion sequence element. Abbreviations: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; X, *Xho*I.

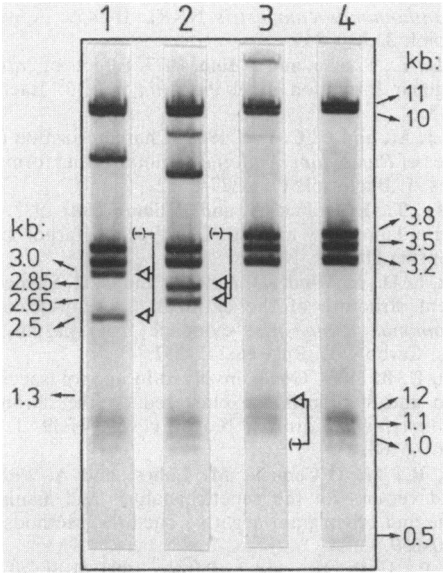

FIG. 2. Southern analysis of chromosomal DNA from some EPS mutants. Purified DNA from different mutants which could be complemented by pXCB1002 was digested with *Eco*RI and probed with biotin-labeled pXCB1002 DNA (Bethesda Research Laboratories, Inc.). Lanes: 1, B100-13; 2, B100-22; 3, B100-152; 4, wild-type *X. campestris* pv. *campestris*. Unmarked hybridization bands were due to insertions of the Tn*5* carrier vector into the chromosome, as determined by hybridization to labeled vector DNA (data not shown). Minus signs in parentheses indicate hybridization bands that are lacking in comparison with the wild type, and open arrows indicate new bands.

tris wild-type chromosome by marker exchange (22). The resulting transconjugants were tested for EPS production on TY agar supplemented with 2% sucrose. Figure 1 shows the positions of all insertion mutations and the different phenotypes of the resulting clones.

In cross-complementation experiments, cosmid pXCB 1002 containing the different Tn*5-lac* insertions was transferred into the Tn*5-lac*-induced mutants and tested for complementation (22). Selection for vector-encoded tetracycline resistance resulted in transconjugants carrying the cosmid integrated into the chromosome. By assessing the phenotypes of the merodiploids, we could identify at least 12 complementation groups (Fig. 1, A to L). The proposed directions of transcription were inferred from the data for β-galactosidase expression (measured on TY plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml). Table 1 shows the phenotypic characterization of the mutants belonging to the different complementation groups. Most mutants showed, in addition to an altered EPS phenotype, autoagglutination and reduced motility, suggesting that the affected genes might code for enzymes or regulatory factors involved in the synthesis of both EPS and lipopolysaccharide. The reduced EPS production of group A, E, and F mutants was not coupled to these altered phenotypes.

The cosmids identified in this work and total DNA from *X. campestris* pv. *campestris* (*Eco*RI digested) were hybridized against recombinant plasmids pIJ3040 and pIJ3041. These plasmids contain overlapping regions of the *X. campestris* pv. *campestris* genome and were found to restore in EPS-deficient mutants of *X. campestris* pv. *campestris*, *Rhizobium leguminosarum*, and *R. phaseoli* the ability to produce EPS (2, 6). While hybridization against wild-type *X. campestris* pv. *campestris* DNA resulted in signals identical to those obtained with *Eco*RI-digested plasmids pIJ3040 and pIJ3041,

TABLE 1. Phenotypic characterization of the Tn*5-lac* marker exchange mutants belonging to complementation groups A to L as compared with wild-type *X. campestris* B100

Complementation group	EPS production[a,b]	Colony morphology[c]	Agglutination[b]	Motility[b]
A	−	S	−	+
B	−	R	+	−
C	−	R	+	±
D	±	S	+	±
E	±	S	−	+
F	±	S	−	+
G	−	R	+	−
H	−	S	±	±
I	±	S	+	±
J	±	S	+	±
K	±	S	+	±
L	±	S	±	±
Wild type	+	S	−	+

[a] Relative amounts of EPS produced on TY with 2% sucrose.
[b] ±, Intermediate phenotype.
[c] Colony surface was smooth (S) or rough (R).

we detected no DNA homology with our cloned fragments (data not shown). Furthermore, the restriction patterns showed no similarities, and complementation of our mutants with these plasmids failed. The results indicate that we have isolated DNA fragments which are involved in EPS synthesis and which are different from the published ones. Work is in progress to analyze the gene functions encoded by pXCB1002.

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