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Localization of Genes Involved in Exopolysaccharide Biosynthesis in *Zoogloea ramigera* 115SLR

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Mutants having altered levels and/or types of EPS in exopolysaccharide biosynthesis were isolated after NTG mutagenesis of *Zoogloea ramigera* 115SLR. Mutant candidates were classfied with five groups based on the observed characteristics for EPS biosynthesis pattern. The recombinant plasmids pLEX3BS and pLEX3BM were constructed from pEX3B which was previously isolated from genomic DNA of *Z. ramigera* 115SLR. Plasmid pLEX3BM with a 7.8 kb insert DNA contains an additional 1.8 kb DNA fragment which is not present in pLEX3BS containing 13 kb insert DNA. Plasmid pLEX3BM was able to complement the mutation responsible for the changes in morphology of *Z. ramigera* 115SLR. However, the complementation of EPS negative mutant strains was not successful with pLEX3BM. Plasmid pLEX3BS on the other hand complemented the mutation responsible for the loss of EPS biosynthesis, resulting in the restoration of *Z. ramigera* 115SLR phenotype. But this plasmid was not able to complement the morphological mutant strain, *Z. ramigera* 115SLR.

Zoogloea ramigera 115 is a gram negative floc-forming bacterium and has an irregular form and a high dome shape with a bumpy surface (5). The exopolysaccharide (EPS) produced by this organism has interesting rheological behavior (17) and metal adsorption properties (3). The proposed EPS structure has a glucose-galactose chain linked predominantly by β (1-4) with branching occurring at the galactose units (9). Recently, the presence of acetate in EPS was also confirmed (18). Previously, *Z. ramigera* 115SL has been isolated as a EPS production strain because the EPS is released into the culture medium. A rifampicin resistant derivative of strain 115SL, *Z. ramigera* 115SLR has proven suitable for recombinant DNA experiments with this bacteria (3).

Microbial exopolysaccharides are complex and versatile bio-materials. The great diversity exhibited by exopolysaccharides can be attributed to differences in their composition and molecular weight. Because of their different structures and properties, exopolysaccharides have been used in a variety of applications such as rheological control, flocculating, gelling agents and adhesives.

Recent advances in microbial genetics, enzymology and material sciences now allow one to develop a strategy for the rational design of exopolysaccharides in biological systems to meet specific functions (14). Ultimately, the genetic manipulation of microbial EPS biosynthesis will lead to the development of unique, well-defined polymers for specific applications. In order to manipulate the microbial EPS structure genetically, genes involved in EPS biosynthesis should be cloned and characterized. For example, genes essential for xanthan gum biosynthesis in *Xanthomonas campestirs* were cloned by Harding *et al.* (8). The molecular mechanism for EPS biosynthesis in *Rhizobium meliloti* is now understood in detail (12). An initial genetic study on the EPS biosynthesis of *Z. ramigera* I-16-M showed a cluster of genes and genetic instability (4). A plasmid pEX3B containing a large piece of DNA was selected from a *Z. ramigera* 115 genomic library constructed in the cosmid vector pLAFR3 (3). The morphological mutation in *Z. ramigera* 115SLR was complemented with pEX3B. In addition, pEX3B turned out to have sequences homologous to plasmid pNH233 (8) which contained a gene encoding pyruvyl transferase of *X. campestris*. Therefore, as a means of isolating genes involved in the morphological change as well as EPS biosynthesis, the subclones from insert DNA of pEX3B were constructed. The function of the subclones was identified by complementing the various mutant strains of *Z. ramigera* 115SLR. Thus, this study was aimed at isolating genes involved in EPS biosynthesis as well as the morphological change of *Z. ramigera* 115SLR.

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Key words: gene cloning, *Zoogloea ramigera*, complementation, exopolysaccharide biosynthesis, morphology

MATERIALS AND METHODS

Strains and Plasmids

Z. ramigera 115SLR (ATCC 25935 variant) was used for EPS production and various mutant strains of *Z. ramigera* 115SLR were constructed for this study. Defined medium for EPS production consists of 25 g glucose, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g yeast extract, 2 g K_2HPO_4, 1 g KH_2PO_4 and 1 g NH_4Cl in 1 liter H_2O (13). For conjugation with recombinant plasmids, *E. coli* MM294A including pRK2013 and *E. coli* DH5α were used. The broad host range vector pLAFR3 (16) and plasmid pEX 3B were obtained from MIT (Prof. A.J. Sinskey' Lab.). Other subclones were constructed as described in Sambrook *et al.* (15).

Mutagenesis of *Z. ramigera* 115SLR

Z. ramigera 115SLR was grown in Trypticase soy broth (BBL, USA) medium with shaking at 30°C. Cells at a density of 2×10^8 per ml were exposed to 25 μg/ml nitrosoguanidine (6) for 30 min at 30°C. Cells with a survival rate of 30% were plated out on TS agar and were grown for three days. Mutant candidates were screened by examining the characteristics of stickiness and viscosity of microbial EPS.

Construction of Subclones

Plasmid pLEX3BS was obtained by digesting pEX3B with *Eco*RI. The linerized plasmid containing a 13 kb insert DNA fragment was self-ligated and transformed into *E. coli* DH5α. To construct pLEX3BM, pEX3B DNA was partially digested with *Sau*3A1. The 7.8 kb DNA fragments was isolated from 1% agarose gel and then ligated with vector pLAFR3 digested with *Bam*H1. After transforming *E. coli* DH5α cells, transformants containing clones were selected by complementing the morphological mutant or EPS negative mutant strains via conjugation. Because of the low copy number of recombinant plasmids in *Z. ramigera* 115SLR conjugants, the plasmids in *Z. ramigera* 115SLR conjugants were prepared by the SDS-alkaline lysis method (15). The crude plasmid mixture was transformed into *E. coli* DH5α for the purpose of amplifying plasmid DNA.

Southern Hybridization

To localize the insert DNA of pLEX3BM on pEX3B clone, pLEX3BM was digested with various restriction enzymes and Southern blot analysis was carried out using a probe pLEX3BS DNA labeled with α-^{32}P-dCTP by nick translation (10).

Complementation of *Z. ramigera* 115 SLR Mutants

Transfer of pLAFR3 and pLAFR3 recombinant DNA molecules into a *Z. ramigera* 115SLR mutant was accomplished by using pRK2013 as a helper vector (3). *Z. ramigera* 115SLR mutant was grown in TSB (50 μg/ml rifampicin). *E. coli* DH5α containing pLAFR3 was grown in LB (10 μg/ml tetracycline). *E. coli* MM294A containing pRK2013 was grown in LB (50 μg/ml kanamycine). After growing for 18 h, 1 ml of each culture broth was harvested and washed with 1 ml of TSB. The collected cells were resuspended with 100 μl of TSB. Each cell suspension (5 μl) was mixed and poured on TS agar and then incubated at 30°C for 18 h. To select transconjugants, cells including transconjugants were resuspended in 1 ml TSB and dilutions were plated on TS agar containing tetracycline (10 μg/ml) and rifampicin (50 μg/ml). Complementation of the morphological mutant strain was determined by examining the cell morphology affected by EPS. Complementation of the EPS negative mutant strain was also determined by measuring the level of EPS produced from the defined medium.

Isolation of EPS from *Z. ramigera* 115SLR

For EPS production, *Z. ramigera* 115SLR and mutant strains were grown in 100 ml of the defined media on a rotary shaker (200 rpm) at 30°C for 5 days. The whole culture was diluted with two volumes of hot water (50°C) and the cell pellet was removed by centrifugation at 25°C for 20 min (10,000 rpm, GSA rotor, Sorval RC5B). The EPS in the supernatant was precipitated with two volumes of isopropanol at -20°C for 6 h and then recovered by centrifugation (GSA rotor, 5,000 rpm for 10 min). The EPS pellet recovered by isopropanol precipitation was washed with ethanol and dried in a vacuum oven (55°C).

RESULTS AND DISCUSSION

Isolation and Characterization of *Z. ramigera* 115SLR Mutants

Z. ramigera 115SLR treated with NTG was plated on TS agar. After 3 days' growth, visual examination of the colonies was carried out to screen the mutated colonies. Those colonies which "looked" different were further examined for stickiness and/or viscosity by streaking with sterile toothpicks. In this way 38 mutant strains were isolated and separated into 5 groups (class A-E) based on the observed characteristics for deficiency and/or altered EPS biosynthesis (Table 1).

Class A strains were phenotypically identical to the *Z. ramigera* 115SLR whereas class B, D and E strains appeared to produce less or altered EPS and class C strains apparently no longer produce EPS. Mutant strains selected randomly from class A, B, D and E were assayed for EPS production. These data presented in Table 2 indicate the following: class A strains are indistinguishable from *Z. ramigera* 115SLR; class B strains produced around 10% of the wild type level of EPS; class D strains produced 30% of the wild type level of EPS. Class E strains were almost EPS free. Class C strains did not produce any EPS and were used for complementation analysis.

Table 1. Phenotypic classification of *Z. ramigera* 115 SLR mutant strains.

Class	Mutant strains	Characteristics of colonies (EPS/stickiness)
A	1, 3, 7, 8, 22, 26, 31, 34	+++/+++
B	2, 10, 27, 28, 29, 30	++/+
C	4, 5, 6, 9, 11, 13, 14, 16, 17, 24, 25, 32, 33, 35, 36, 37	−/−
D	23, 38	+++/+
E	12, 15, 18, 19, 20, 21	+/+

Table 2. Analysis of the level of EPS produced from the various mutant strains.

Class	Mutant strains of 115 SLR	EPS level (mg/100 ml)
A	M1	370.0
	M7	321.5
B	M10	38.7
	M27	23.7
	M28	22.5
	M29	46.8
D	M23	312.3
	M38	119.1
E	M12	11.1
	M15	14.9
	M18	6.2
	M19	12.7
	M20	6.5
	M21	16.9
wild type	*Z. ramigera* 115SLR	370.0

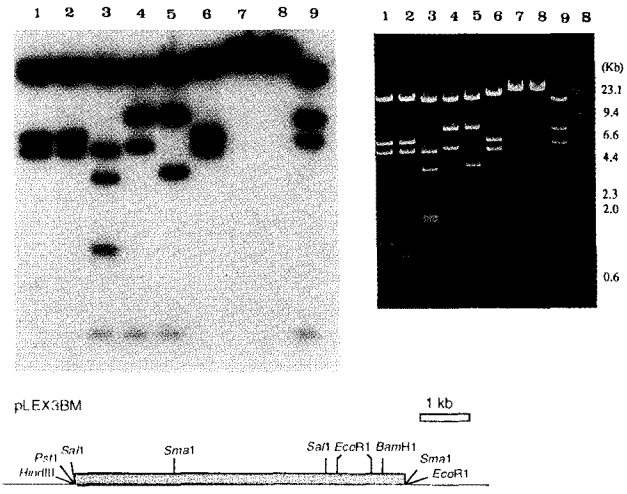

Fig. 1. Southern hybridization and restriction map of pLEX 3BM.

Top (left): autoradiograph of pLEX3BM digested with *Bam*H1/*Sal*1, *Eco*R1/*Sal*1, *Sma*1/*Sal*1, *Sma*1/*Bam*H1, *Sma*1/*Eco*R1, *Sal*1, *Eco*R1, *Bam*H1 and *Sma*1 (lanes 1-9, respectively). Lane S: *Hin*dIII digest of λ-DNA (size marker). Top (right): 1% agarose gel electrophoresis

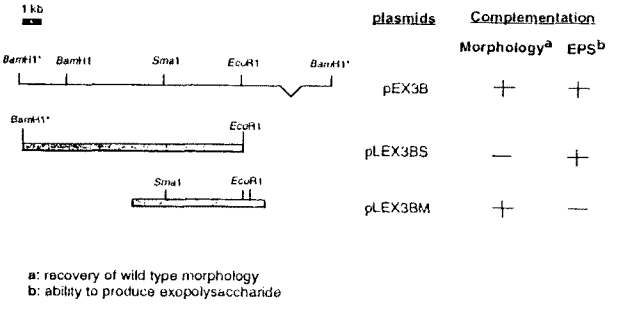

Fig. 2. Complementation analysis of *Z. ramigera* 115 mutant strains with subclones.

Host strains used for complementation: *Z. ramigera* 115SLR for morphology, *Z. ramigera* 115SLR (class C mutant) for EPS biosynthesis.

Construction of Recombinant Plasmids

The restriction map of pLEX3BM was determined by Southern hybridization analysis using a probe pLEX3BS (Fig. 1). Plasmid pLEX3BM showed three *Eco*R1 DNA fragments (28 kb, 1.3 kb and 0.5 kb). The *Eco*R1 DNA fragments of 1.3 and 0.5 kb did not show any homology with a probe pLEX3BS (Fig. 1, lane 7). It turned out that pLEX3BM contained a 1.8 kb *Eco*R1 DNA fragment unfound in pLEX3BS. Also, a 5.8 kb *Sal*1 fragment was derived from the insert DNA of pLEX3BM (lane 6) and the same DNA fragment was divided into 4 and 1.8 kb DNA after *Sal*1/*Sma*1 double digestion (lane 3).

From these results, we ascertained that pLEX3BM contained a 7.8 kb DNA fragment with *Sal*1 and *Sma*1 sites on the inserted DNA. When compared with the restriction map of pEX3B (10), it turned out that a DNA fragment of 7.8 kb was located in the middle of the pEX3B clone. However, pLEX3BS only contained a 13 kb *Eco*R1 fragment including the left region of the pEX3B clone (Fig. 2).

Complementation of Class C Mutant strains

In order to isolate genes involved in EPS biosynthesis of *Z. ramigera* 115SLR, class C mutant strains were tested for complementation with the recombinant plasmids. Previously, pEX3B was isolated by its ability to complement the morphological mutation in *Z. ramigera* 115SLR (3). The pLAFR3 vector and the recombinant plasmids (pLEX3BM and pLEX3BS) derived from pEX 3B were transfered by conjugation to the class C mutant strains to confirm genes involved in EPS biosynthesis. The introduction of pLAFR3 into the EPS negative mutant strains (class C) turned out to have no effect on the colony phenotype as well as EPS production. However, pLEX3BS complemented the mutation responsible for the loss of EPS biosynthesis, resulting in the restoration of a *Z. ramigera* 115SLR phenotype. The level of EPS produced by each mutant strains containing pLEX3BS was similar to that of *Z. ramigera* 115SLR (Table 3).

On the other hand, pLEX3BS was not able to com-

Table 3. Comparison of EPS content produced from class C mutant strains containing pLAFR3 and recombinant plasmids (mg/100 ml).

Strain	pLAFR3	pLEX3BS	pLEX3BM
M4	10.8	368.2	11.7
M5	10.3	450.0	12.6
M6	13.6	408.8	16.3
M9	6.6	319.1	19.3
M11	5.7	317.6	19.5
M13	13.2	421.0	8.5
M16	2.9	249.0	17.8
M17	10.1	453.4	19.6
M25	8.3	268.6	14.8
M32	6.2	288.6	8.1
M33	10.2	312.7	9.6
M35	2.2	344.9	18.6
M36	14.1	316.1	16.3
M37	2.9	362.3	8.4

plement the morphological mutant strains of *Z. ramigera* 115 (Fig. 2). Thus, pLEX3BS comprising the left end region of the pEX3B clone can only complement the mutations which eliminate EPS biosynthesis in all the EPS negative mutant strains tested. These data allow us to conclude that pLEX3BS contained the significant genes required for EPS biosynthesis in *Z. ramigera* 115SLR. It seems likely that the EPS biosynthesis genes are clustered in the genomic DNA of *Z. ramigera* 115 SLR. In fact, the clustering of genes for microbial exopolysaccharide biosynthesis is common to many bacteria such as *X. campestris* (8), *R. melilotti* (11) and *P. aeruginosa* (2).

In contrast to pLEX3BS, pLEX3BM did not complement EPS negative mutant strains (Table 3). pLEX 3BM was only able to restore the colony phenotype of mutant *Z. ramigera* 115SLR strains (Fig. 2). The restriction map of pLEX3BM showed the expanded right region with 1.8 kb DNA fragment compared with pLEX 3BS. The absence of this DNA sequence in pLEX3BS was confirmed by DNA hybridization analysis (Fig. 1). It is worth noting that at least a part of the sequences required for restoration of colony morphology are located in the 1.8 kb DNA fragment present in the pLEX3BM. Fig. 2 shows the complementation pattern of both the morphological mutant and EPS negative mutant strains with subclones. In some bacteria, the capsule polysaccharide is very firmly attached to the cell surface (1). Gotschlich *et al.* (7) reported that the presence of hydrophobic end (lipid) causes the polysaccharide to aggregate in a micellar form. It is still not clear which factor is involved in the morphological change of mutant *Z. ramigera* 115SLR. Further research will be necessary to characterize the individual genes involved in EPS biosynthesis as well as the morpholoical change in *Z. ramigera* 115SLR.

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