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Cloning, Sequencing and Characterization of Acyltransferase Gene Involved in Exopolysaccharide Biosynthesis of *Zoogloea ramigera* 115SLR

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Abstract The recombinant plasmid pLEX2FP complements the mutation in *Zoogloea ramigera* 115MM1, and the complemented mutant produces an exopolysaccharide that shows higher affinity for the calcofluor dye than the exopolysaccharide from *Z. ramigera* 115SLR, resulting in higher fluorescence intensity under UV light. A compositional and structural analysis of the exopolysaccharide from *Z. ramigera* 115MM1 showed that the different fluorescent properties were due to a lower content of acetyl groups when compared with *Z. ramigera* 115SLR exopolysaccharide. These results were in agreement with a sequence analysis of the gene carried in the plasmid pLEX2FP, which appeared to encode an *O*-acyltransferase highly homologous to the 3-*O*-acyltransferase of *Streptomyces mycarofaciens*. The gene encoding the acyltransferase from *Z. ramigera* 115SLR was expressed as a GST-fusion protein with 70,000 daltons in *E. coli*.

Key words: *Zoogloea ramigera* 115SLR, exopolysaccharide, acyltransferase, expression

Zoogloea ramigera 115, a Gram-negative rod-forming bacterium, produces an extracellular polysaccharide with metal-binding properties [2, 6] and unique rheological behavior [2]. Although the complete structure of this exopolysaccharide is not yet known, the proposed structure contains glucose and galactose in a 2:1 ratio, in a main chain predominantly β(1–4) linked. The exopolysaccharide is modified by the presence of pyruvate, acetate, and succinate as side groups [5, 11, 17, 23].

Calcofluor white M2R is a fluorescent brightener that binds to β-linked polysaccharides such as cellulose and chitin [8]. In addition a number of bacterial polysaccharides have also been shown to bind this dye, which has been used to study bacterial polysaccharide [15, 16, 22]. Easson [4] investigated the activity of calcofluor dye using various polysaccharides with different glycosidic linkages and established that the presence of β(1–4) or β(1–3) in a particular polysaccharide was required to bind the dye. However, the presence of these linkages does not always provide calcofluor-binding properties, since xanthan gum obtained from *Xanthomonas campestris* is a β(1–4)-linked polysaccharide, yet shows no affinity for the calcofluor dye [4].

Although there is relatively little information on the exopolysaccharide biosynthetic pathway in *Z. ramigera* 115, the gene involved in morphological change of *Z. ramigera* 115SLR was recently reported [14]. The cloning and characterization of the gene involved in exopolysaccharide biosynthesis is necessary to produce a novel exopolysaccharide through metabolic engineering. However, the presence of a capsular polysaccharide in *Z. ramigera* 115 makes genetic manipulation difficult, so a slime-forming mutant, 115SL, has been derived using nitrosoguanidine (NTG) mutagenesis. To facilitate further manipulations, a spontaneous rifampicin-resistant strain, designated 115SLR, was also isolated [4], and mutant strains constructed using NTG treatment [13].

Z. ramigera 115SLR fluoresce under UV light on medium containing the calcofluor dye, and this characteristic was used to screen for mutants derived from *Z. ramigera* 115SLR using Tn5 mutagenesis. *Z. ramigera* 115MM1 was isolated on the basis of its failure to fluoresce under UV light on the calcofluor medium [4]. However, this mutant strain still produces a significant level of polysaccharide in contrast with other calcofluor negative mutants in which there is no exopolysaccharide biosynthesis. Accordingly, this paper

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reports on the cloning and characterization of the gene that complements *Z. ramigera* 115MM1 mutation and is involved in the restoration of the fluorescence properties of its exopolysaccharide. In addition, a description is given of the differences between the exopolysaccharides from *Z. ramigera* 115SLR and 115MM1.

E. coli DH5α [F-Ø80 *lac* M15 (*lacZYA-argF*)U169 *recA1endA1*] was grown in LB or 2×YT, whereas the *Z. ramigera* 115SLR was grown in Trypticase Soy Broth (TSB, BBL, Cockysville, MD, U.S.A.). The medium used for the exopolysaccharide production was prepared as described by Norberg and Enfors [17], with the following antibiotic concentration: 10 μg/ml tetracycline, 50 μg/ml rifampicin, 50 μg/ml kanamycin, 50 μg/ml ampicillin. For fluorescence studies, 0.02% (w/v) calcofluor white M2R (Sigma, Louis, MO, U.S.A.) was added to TSB agar.

The plasmid DNA was isolated from overnight cultures of *E. coli* using the alkaline lysis method [19]. The restriction enzyme digestions and ligations were performed according to the manufacturer's instructions (New England Biolabs). The recombinant plasmid pLEX1F, complementing the 115MM1 mutant, was isolated from a recombinant cosmid library [4]. It was confirmed that the functional gene was located in a 2-kb DNA fragment obtained from *Eco*RI restriction of the plasmid pLEX1F. To construct the subclone, the plasmid pLEX1F was digested with an *Eco*RI restriction enzyme, and then the DNA fragment was ligated into a pUC19 vector. Next, the ligated DNA was transformed into *E. coli* DH5α cells and plated on LB agar plates containing ampicillin and X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) (50 μg/ml). The recombinant plasmid pUEX2F was isolated by screening the white colonies, and the 2-kb DNA fragment of the plasmid pUEX2F then isolated and cloned into the broad host range vector pLAFR3 (tetracycline-resistant) to generate the plasmid pLEX2F. A restriction map of the plasmid pLEX2F was constructed using various restriction enzymes (Fig. 1). A 1.35-kb *Pst*I fragment was then isolated from the plasmid pLEX2F and ligated into plasmid pLAFR3 to generate the plasmid pLEX2FP. To construct the plasmid pLEX2FX, the plasmid pLEX2F was digested with *Xmn*I, modified with an *Eco*RI linker, and then the DNA fragment was ligated into the pLAFR3 vector.

The plasmid pUEX2F was digested with an *Eco*RI restriction enzyme and the 2-kb DNA fragment subcloned into a M13mp18 vector. The unidirectional deletion of the target DNA was accomplished using an Erase-a-Base TM System (Promega, Madison, WI, U.S.A.). The complete nucleotide sequence of the 2-kb DNA fragment was determined by the dideoxy chain termination method using a Sequence Kit (United State Biochemical, Cleveland, OH, U.S.A.). The DNA sequence was analyzed using the BLAST program [1].

The plasmid pLEX2F and other derived plasmids were transferred into *Z. ramigera* 115MM1 by conjugation

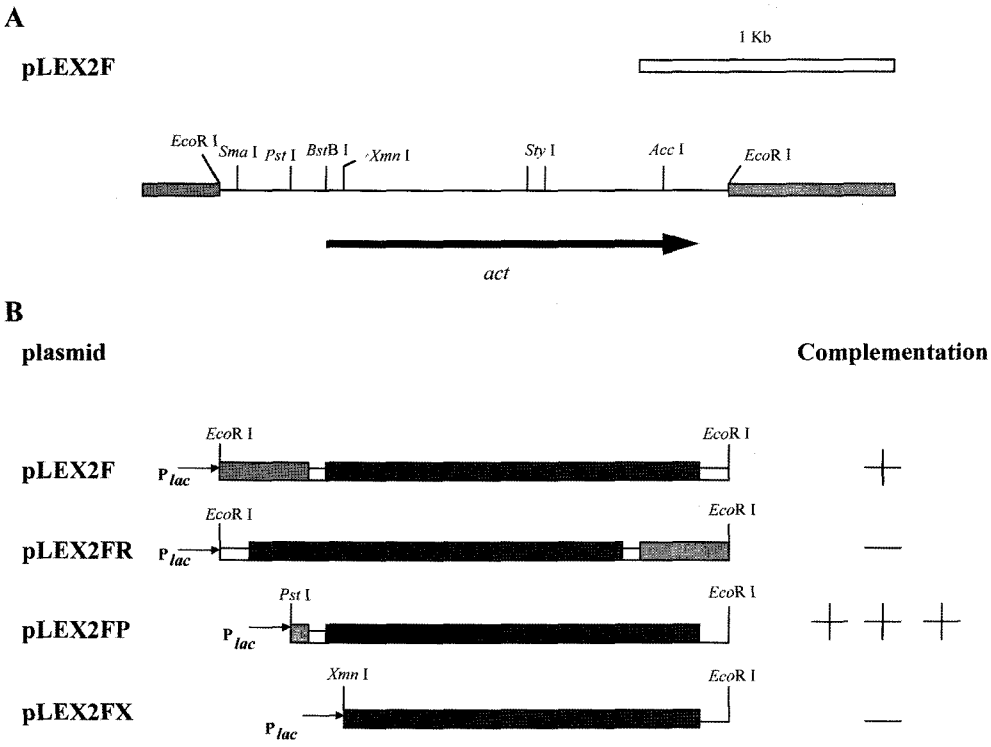

Fig. 1. Restriction map of plasmid pLEX2F and subclones constructed in plasmid pLAFR3.

using *E. coli* S17-1 [20]. The transformants grown on TSA agar plate were recovered and plated on TSA agar plate containing tetracycline and rifampicin. The complementation of transformants was confirmed by comparing the fluorescence intensity of microbial polysaccharide in the transformants grown on TSA agar plate containing calcofluor dye with that of *Z. ramigera* MM1. The fluorescence of exopolysaccharide was examined using a hand-held UV lamp (long wavelength, Fisher Scientific, Pittsburgh, PA, U.S.A.).

The *Z. ramigera* 115SLR and mutant strains were grown in the defined media as described by Norberg and Enfors [17] at 30°C for 3 days. The whole culture was diluted with 2 volumes of hot water (50°C), and the cell pellet removed by centrifugation at 25°C for 25 min (11,000 rpm, GSA rotor, Sorval RC5B centrifuge). Therefore, the EPS was precipitated with 2 volumes of isopropanol at −20°C for 6 h and the recovered EPS dissolved in water and lyophilized (Freeze Dryer 4.5, Labcono). Prior to the analytical procedures, EPS samples were dissolved in distilled water. The exopolysaccharides were dissolved in 20 mM sodium phosphate buffer, pH 7, containing 0.125 mM calcofluor dye in a concentration range of 0.025 to 0.2 mg/ml. The emission fluorescence was scanned at 30 nm per min within a range 420–550 nm, using a fluorescence spectrophotometer (F-3010, Hitachi, Japan) at an excitation wavelength of 410 nm.

The lyophilized exopolysaccharides (10 mg) were dissolved in 5 ml of 0.1 M trifluoroacetic acid (EM Science, Gibbtown, NJ, U.S.A.), hydrolyzed at 120°C for 6 h, filtered using Millipore 0.22 μm Millex-GV filters (Millipore, Bedford, MA, U.S.A.) and analyzed using an HPLC (Hewlett Packard 1050) connected to an Aminex HPX-87H column (Bio-Rad, Richmond, CA, U.S.A.) [14]. The exopolysaccharides were methylated following Hakomori's [9] and Prehm's [18] methylation procedures, as described by Troyano *et al* [23].

The plasmid pGEX2FX containing the *Z. ramigera* 115SLR *act* gene was constructed using a pGEX-2T vector containing the glutathione-S-transferase (GST) gene [21]. As such, the 1.3-kb DNA fragment isolated from the plasmid pUEX2F by digestion with *Xmm*I and *Eco*RI was treated with the klenow enzyme and cloned into the *Sma*I site of a pGEX-2T vector to create an in-frame GST-fusion protein. The transformants of *E. coli* DH5α harboring the plasmid pGEX2FX or pGEX2FXR were grown in 2×YT broth containing ampicillin (100 μg/ml) at 37°C. The cells were induced with 0.2 mM IPTG at $O.D._{600\,nm}$=0.5, grown for 3 more hours, and the cells were then harvested at 7,000 rpm for 10 min. Next, the cell pellet was suspended in 5 ml of 10 mM Tris-HCl, pH 8.0, and sonicated (Ultrasonic Processor XL, Farmingdale, NY, U.S.A.) in an ice bath for 10 min. The total protein of the cell lysate was analyzed by discontinuous SDS-PAGE [12].

Previously, the plasmid pLEX1F, isolated from a library of *Z. ramigera* 115 DNA, was shown to complement the mutation in the *Z. ramigera* 115MM1 strain. In the present study, the pLEX2F derived from plasmid pLEX1F was also found to restore the fluorescent phenotype by complementation. When determining the complete nucleotide sequence of the 2-kb DNA insert of this plasmid, it was shown to contain a complete open reading frame (Fig. 1). The target gene nucleotide sequence of *Z. ramigera* 115SLR has been submitted to the GenBank nucleotide sequence database and is available under accession number AY515303. The function of the target gene was confirmed by the simple complementation of the *Z. ramigera* MM1 mutant, resulting in a higher intensity of fluorescence. However, plasmid pLEX2FR, with a reverse insert to that in plasmid pLAFR3, did not complement the mutation of *Z. ramigera* MM1 (Fig. 1). Thus, after eliminating the unnecessary upstream region of the plasmid pLEX2F, the resulting subclone pLEX2FP produced brighter colonies than those of *Z. ramigera* 115SLR in the calcofluor medium, indicating that the plasmid strongly complemented the mutation in *Z. ramigera* 115MM1. However, since the subclone pLEX2FX containing the truncated ORF did not complement the mutation in the *Z. ramigera* 115MM1 mutant, the N-terminal sequence including 11 amino acids appeared to be important for the enzyme activity.

The protein encoded by the open reading frame in the plasmid pLEX2FP had a predicted molecular weight of 43,000 daltons. When searching for protein homology with this polypeptide, a significant homology was found with the 3-*O*-acyltransferase in *Streptomyces mycarofaciens* [10]. A BESTFIT alignment of the overall protein sequence of the *O*-acyltransferase gene is shown in Fig. 2, which indicates 53% similarity and 30% identity when comparing the amino acid sequence. In addition, a hydrophobic plot of the protein revealed many prominent hydrophobic regions (data not shown), and this hydrophobic profile has previously been reported for other *O*-acyltransferases, such as the *Exo* 2 involved in the acetylation of the succinoglycan in *Rhizobium meliloti* [7] or the *O*-acyltransferase of the bacteriophage SF6 involved in the conversion of *Shigella flexneri* group 3, 4 antigens to a group 6 antigen [24].

Whereas the exopolysaccharide from *Z. ramigera* 115MM1 had a slight affect on the fluorescent intensity of the calcofluor dye, the exopolysaccharide from *Z. ramigera* 115SLR strongly increased the fluorescent intensity of the dye. The exopolysaccharide from *Z. ramigera* 115MM1 after complementation with the plasmid pLEX2FP also increased the fluorescent intensity of the calcofluor dye, and its effect was even stronger than that of the *Z. ramigera* 115SLR exopolysaccharide.

As shown in Fig. 3, in the range of 25–200 μg/ml exopolysaccharide, the relative fluorescence of the calcofluor was dependent on the concentration of exopolysaccharide

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 12 PTISKIEELESLRGLAALLVVFFHLPKWN.........PVLHVGFIDSGY  52
  3 PRVVRLPSLTGLRWFAALAVFAGHIAQQQFFADQQVGTALLHITTLGS..  50

 53 LMVQLFFVLSGFVIYNAYATRISSGMDLVKFQFLRFGRLYPVHFLFLMAY 102
 51 IAVSVFFLLSGFVL..AWSARDKDS..VTTFWRRRFAKIYPLHLVTFL..  94

103 LSIEIAKYIAATKFGVSGPNARPFEENTVQALVQQIFLLQA.IGPTGNAT 151
 95 ....IAGVIIFSLAEPTLPGGSVWD.....GLVPDLLLVQSWLPEPTIIA 135

152 TFNIPAWSISTEFYTYLLFALILLFAKK.........AKDIVFLVIVV.. 190
136 GFNTPSWSLSCEFAFYLTFPLWYRLVRKIPVRRLWWCAAGIAAAVICVPF 185

191 VSLFMLASGQTFGTVEL....LRCF......AGFFLGCLTAKLMNKLTFQ 230
186 VTSQFPASAETAPGMPLNELWFACWLPPVRMLEFVLGIVMALILRTGVWR 235

231 VPGY..CAYIVILIIGIYLQFKPLGSYDLFIYLLTVLLILSLVLAKDGPA 278
236 GPGVVSSALLLAAAYGVTQVVPPMFTIAACSIVPAALLITALANADVQGL 285

279 KEMLRAKVLIWLGSISYALYMSHTIVIWVANQVFRFVLKRPEIMIEGKSY 328
286 RTGLRSAVLVRLGEWSFAFYLVHFMVIRYGHR.........LMGGELGY 325

329 P.QLSTVETGVAIVIVMAAVLVLSQIVYVVVEKPMREKSRRVVSVKAKPA 377
326 ARQWSTASAGALALAMLAVAIVAGGLLHTVVE.............NPG 360

378 RRLPHRRRCHRGTPPRSPRTHRRDT 402
361 MRLLGRRRPVATAPDPATDEAPKLT 385
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Fig. 2. Alignment of deduced amino acid sequences for *O*-acyltransferase from *Z. ramigera* 115SLR (upper line) and *O*-acyltransferase from *S. mycarofaciens* (lower line).

obtained from *Z. ramigera* 115SLR and MM1/pLEX2FP. As a general assay for determining the polysaccharide concentration, the hexose/phenol-sulfuric acid method has been used [4], where the polysaccharide is hydrolyzed under acidic condition at high temperature, followed by a color reaction of hexose with phenol in conc. sulfuric acid. Although this assay method is available within 200 μg/ml of sugar concentration, it involves a long assay time and is hazardous because of the use of concentrated sulfuric acid. Conversely, a fluorescence assay of polysaccharide in the presence of calcofluor is a novel alternative method for quantifying intact polysaccharides. When the exopolysaccharides from *Z. ramigera* 115SLR, MM1, and MM1 strains complemented with plasmid pLEX2FP were treated with KOH beforehand, the fluorescence intensity of the calcofluor dye was markedly enhanced in comparison with that of the native polymers (data not shown). This may imply that the pH of the environment affects the fluorescence intensity of calcofluor dye. Thus, based on the binding ability of exoplysaccharide with calcofluor dye, the fluorescence intensity can be utilized to determine the concentration of exopolysaccharide as well as elucidate the conformation change.

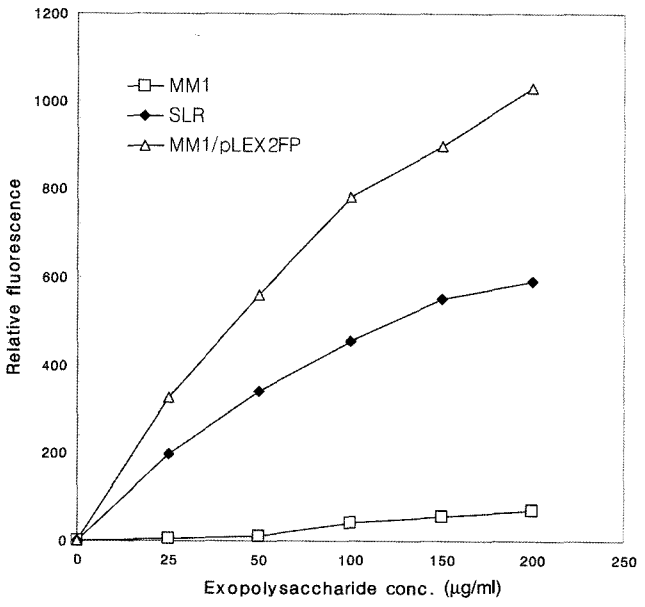

Fig. 3. Effect of exopolysaccharides produced from *Z. ramigera* 115SLR, MM1, and MM1/pLEX2FP on the relative fluorescence intensity of calcofluor dye.
Excitation: 410 nm; emission: 446 nm

As expected, acid hydrolysis and HPLC analysis of the exopolysaccharide from the 115MM1 mutant revealed a lower content of acetate than that in 115SLR. After complementing the 115MM1 mutant strain with the plasmid pLEX2FP, the acetate content in the exopolysaccharide increased above that in the 115SLR exopolysaccharide (Table 1). As such, the presence of a higher number of acetyl groups in the exopolysaccharide from *Z. ramigera* 115MM1 after complementation with the plasmid pLEX2FP may explain the enhanced fluorescent properties of this exopolysaccharide.

Hakomori's methylation [9] and gas chromatography/mass spectrometry (GC/MS) analysis of the exopolysaccharides from *Z. ramigera* 115 MM1 before and after complementation with the plasmid pLEX2FP showed the same percentages of partially methylated alditol acetates as the previously reported value for the exopolysaccharide from 115SLR [23]. Since Hakomori's methylation procedure takes place under alkaline conditions that remove the *O*-acyl groups from the exopolysaccharide, this confirmed that the difference between the two exopolysaccharides was due to an *O*-acyl substitution.

Table 1. Compositional analysis of EPS samples isolated from *Z. ramigera* 115 SLR, MM1, and MM1/pLEX2FP.

μmol/mg Exopolysaccharide

Compound	115SLR	MM1	MM1/pLEX2FP
Glucose	1.82	1.82	1.82
Galactose	1.08	0.95	1.10
Pyruvate	0.68	0.53	0.71
Acetate	0.95	0.66	1.11
Succinate	0.55	0.09	0.10
Glc: Acetate	1.89	2.75	1.63

Conditions: Aminex HPX-87H column (organic acid analysis column, 8% cross-linked cation exchange resin, 300×7.8 mm) mobile phase: 5 mM sulfuric acid; temperature: 40°C; flow rate: 0.6 ml/min; sample injection volume: 30 μl.

When the exopolysaccharides were methylated under neutral conditions [18], the exopolysaccharide from 115MM1 showed a slightly lower content of 2,3-di-*O*-methylglucose than the exopolysaccharide from MM1 after complementation with the plasmid pLEX2FP. This result corresponded with a higher content of 2,3,6-tri-*O*-methylglucose in EPS from 115MM1. As such, these results may indicate that the *O*-acyl substitution occurs at position 6 in the nonreducing glucose already substituted at position 4. However, even though the same results were obtained for two different samples of the exopolysaccharides, the Prehm methylation procedure gives rise to undermethylated products, thus the position of the acetate group could not be confirmed.

Although the 2-*O*-methylglucose residue, corresponding to a 3-substituted glucose with a pyruvate substitution at positions 4 and 6 [23], was found after Hakomori's methylation of the exopolysaccharide from *Z. ramigera* 115MM1, this residue was not present in the samples after the Prehm methylation procedure, whereas a new compound identified as a totally acetylated glucose residue was found. This could indicate an increase of the intramolecular associations in the polymer impelled by the presence of the *O*-acetyl substitution in the exopolysaccharides from 115SLR and 115MM1 after complementation with the plasmid pLEX2FP. This increase in the intramolecular associations could explain the slight effect of the 115MM1 exopolysaccharide on the fluorescent intensity of the calcofluor dye, due to a reduced number of hydroxyl groups available to interact with the dye.

To study the expression of the *Z. ramigera* 115SLR *act* gene in a heterogeneous *E. coli* genetic background, crude extracts from cultures of *E. coli* harboring the plasmid pGEX-2T or recombinant plasmids were analyzed by SDS-PAGE. Although the GST protein, with a molecular weight of 27,000 daltons, was overproduced, the GST-ACT fusion protein with an expected molecular weight of 70,000 daltons was weakly produced under the same conditions (data not shown). *E. coli* harboring the plasmid pGEX2FXR with a reversely oriented insert did not produce the target protein. Therefore, it was presumed that the gene cloned into pGEX2T did not include the promoter for transcription because of the deletion of the upstream region of the *act* gene. In addition, the reduced expression of the *act* gene may have been due to its high hydrophobic profile, as described above.

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