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Cloning and Sequencing of the Gene Involved in Morphological Change of *Zoogloea ramigera* 115SLR

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Abstract Plasmid pLEX3 isolated from the recombinant cosmid library of *Zoogloea ramigera* 115 was found to be responsible for the restoration of the rugose colony phenotype. To confirm the essential region responsible for the complementation, subclones were constructed from plasmid pLEX3 and transformed into mutant strain *Z. ramigera* 115SLR. The recombinant plasmids pLEX10 and pLEX11 were shown to complement the slime-forming property of *Z. ramigera* 115SLR. In a compositional analysis of the exopolysaccharides from *Z. ramigera* 115, *Z. ramigera* 115SLR, and *Z. ramigera* 115SLR harboring plasmid pLEX11, the exopolysaccharides showed a similar composition with glucose, galactose, and side chain groups. The complete nucleotide sequence of the 3.25 kb genomic DNA insert in plasmid pLEX11 was determined and its analysis identified two open reading frames which could encode two proteins. The gene products derived from the two open reading frames were confirmed by an *in vivo* transcription using a T7-RNA polymerase. The ORF1 produced a 30 kDa protein, whereas the ORF2 was found responsible for the complementation of the morphological mutation and produced a 14 kDa protein. An *in vivo* gene expression of plasmid pTEX10 showed another open reading frame encoding a 50 kDa protein. The gene products from ORF1 and ORF2 are regarded as novel proteins which do not show any homology with other proteins.

Key words: Gene cloning, morphology, exopolysaccharide, *Zoogloea ramigera.*

Zoogloea ramigera 115 is a gram-negative, floc-forming bacterium that produces a cell-bound exopolysaccharide (capsule-like matrix) with a strong affinity to several heavy metal ions [2, 5, 16] and unique rheological properties [22]. *Z. ramigera* 115 is also known to accumulate poly-β-hydroxybutyric acid (PHB) granules intracellularly, similar to *Alcaligenes eutrophus* [4, 20]. There have been several structural investigations of exopolysaccharides produced from *Z. ramigera* 115 [9, 17]. Recently, the presence of an acetate and succinate was confirmed in the highly branched exopolysaccharide (EPS) of *Zoogloea ramigera* 115 [25]. Previously, a mutant derived from *Z. ramigera* 115 by nitrosoguanidine mutagenesis was also isolated [4]. This mutant strain designated as *Z. ramigera* 115SL is non-floc forming and slime forming. It produces an exopolysaccharide that does not remain bound to the cell wall. To facilitate further gene manipulations in this microorganism, a spontaneous rifampicin resistant strain, *Z. ramigera* 115SLR, was derived from *Z. ramigera* 115SL [4].

Previously, the authors have isolated the genetic loci required for the production of EPS in order to produce a novel EPS through metabolic engineering [3, 13, 14]. The main effort was focused on plasmid pLEX3 that was constructed by cloning the *Z. ramigera* 115 chromosomal DNA insert into the broad host range vector pLAFR3. A 15 kb chromosomal DNA insert in plasmid pLEX3 exhibits a homology with the pyruvyltransferase gene of *Xanthomonas campestris* [7, 12]. The manipulation of EPS has been reported through genetic modification using a pyruvyltransferase gene [14]. In addition, the EPS negative mutant strain can be complemented by the plasmid pLEX3 [4]. A middle region of the clone is also able to complement the slime-forming property of mutant strain *Z. ramigera* 115SLR [10]. These data led us to conclude that plasmid pLEX3 contains a significant proportion of the *Z. ramigera* 115 genes required for the biosynthesis of EPS.

The polysaccharide produced by microorganims often functions as flocculant agents [26]. The morphological change of the *Z. ramigera* 115 strain resulted in a significant difference in the functional properties including the rheological properties of the EPS. For example, the cell-bound EPS produced from *Z. ramigera* 115 had

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Table 1. Bacterial strains and plasmids used.

Strain or plasmid	Relevant characteristics	Source or Reference
E. coli		
DH5α	F⁻Φ80d *lacZ*△M15△(*lacZYA-argF*)U169 *endA*1 *recA*1*hsdR*17 ($r_k^- m_k^+$)*deoRthi*-1*supE*44 λ^-gyrA96*relA*1	Gibco BRL
JM101	*supE*△(*lac-proAB*) F'*traD*36 *proAB*$^+$*lacI*^{q}*lacZ*△M15	Gibco BRL
S17-1	F⁻*recA hsd* [RRP4:: Amp^S. Tc^S. Km^S]	[19]
K38/pGP1	*In vitro* translation system	[24]
Z. ramigera		
115	Wild-type (ATCC 25935)	[2]
115SLR	Rif^R, derivatives of *Z. ramigera* 115	[4]
Plasmid		
pT7-7	T7 expression vector	[24]
pTEX10a	Amp^R, pT7-7:: 5.0 kb *Hin*dIII fragment in pUEX10	This work
pTEX10b	Amp^R, reverse orientation in pUEX10a	This work
pTEX11	Amp^R, pT7-7:: 3.3 kb *Hin*dIII fragment in pLEX11	This work
pTEX13	Amp^R. pT7-7:: 4.1 kb *Hin*dIII fragment in pLEX13	This work
pLAFR3	Tc^R, cosmid vector	[21]
pLEX3	Tc^R, pLAFR3 cosmid:: cloned exo genes, 15 kb	This work
pLEX5	Tc^R, pLEX3 derivative, 7.8 kb	This work
pLEX10	Tc^R. pLEX5 derivative, 5.0 kb	This work
pLEX11	Tc^R. pLEX10 derivative, 3.3 kb	This work
pLEX13	Tc^R. pLEX10 derivative, 4.1 kb	This work
pUC18	Amp^R	USB
M13mp19	Vector for DNA sequencing	Gibco BRL

Rif^R, rifampicin resistance. Amp^R, ampicillin resistance; Tc^R. tetracycline resistance, USB: United States Biochemical.

different properties for floc-formation compared with the slime EPS produced from *Z. ramigera* 115SLR [4]. The heavy metal binding properties of the slime EPS and cell-bound EPS also showed different patterns [11]. Based on these results, the modification of the functional properties of the EPS could be performed by manipulating genes involved in the morphological change. In order to carry out the genetic modification of EPS. it is necessary to characterize the gene involved in the morphological change and understand what produces the morphological change of *Z. ramigera* 115. This paper reports on the sequencing and characterization of a gene involved in the morphological change in *Z. ramigera* 115.

Materials and Methods

Bacterial Strains and Plasmids

Z. ramigera 115 (ATCC 25935) and other mutant strains were grown in a trypticase soy broth (TSB) (BBL, Cockeysville, U.S.A.) at 29°C with rotary shaking at 150 rpm. *Z. ramigera* 115SLR was grown in TSB containing rifampicin (50 µg/ml). The medium used for the EPS production was the same as described by Norberg and Enfors [15]. For the recombinant DNA procedures, *E. coli* DH5α was routinely grown in an LB medium [18]. The *E. coli* K38 strain. pLAFR3, pUC18, and pT7-7 vectors were all obtained from MIT (Prof. A. J. Sinskey's lab.). The M13 cloning vector and ^{35}S-methionine were purchased from Pharmacia Biotech. (Piscataway, U.S.A.) and the Amersham Corp. (Arlington Heights, U.S.A.), respectively. The genotypes of the strains and plasmids used in this work are described in Table 1.

DNA Manipulation and Subclones

Routine DNA manipulation, including plasmid purification and *E. coli* transformations were performed as described by Sambrook *et al.* [18]. The plasmid DNA was isolated from overnight cultures of *E. coli* by the alkaline lysis method [18]. The restriction and modifying enzymes were purchased from Promega (Madison. U.S.A.). The DNA sequencing kit and amino acid kit were purchased from United States Biochemical (Cleveland, U.S.A.). The restriction enzyme digestions and ligations were performed according to the manufacturers' instructions. The restriction fragments were electrophoresed in 1.0% agarose gels and isolated using a QIAGEN DNA extraction kit (QIAGEN Inc., Chatsworth, U.S.A.).

The subclones for complementing the slime-forming property of mutant strain were constructed from plasmid pLEX3. To construct plasmid pLEX3BM, a DNA fragment of 7.8 kb was isolated from the pLEX3 plasmid which was partially digested with *Sau*3AI and then ligated with a pLAFR3 vector digested with *Bam*HI. To construct plasmid pLEX10, plasmid pLEX3BM was digested with *Sma*I and then its DNA fragment was modified with a *Hin*dIII linker before being subcloned into a pUC18 vector. From the resulting pUEX10 plasmid, a 5.0 kb *Hin*dIII DNA fragment was isolated and subcloned into a pLAFR3 vector. In order to construct plasmid pLEX11, plasmid pUEX10 was digested with *Kpn*I and self-ligated to generate plasmid pUEX11. The plasmid pUEX11 digested with *Kpn*I was then modified with a Klenow fragment and *Hin*dIII linker. A 3.3 kb *Hin*dIII DNA fragment isolated from plasmid pUEX11 was finally cloned into a pLAFR3 vector. To construct plasmid pLEX13, plasmid pUEX10 was digested with an *Apa*I restriction enzyme and then modified with an *Hin*dIII linker after treatment with a mungbean nuclease and Klenow fragment enzyme. A 4.1 kb *Hin*dIII DNA fragment was finally isolated and cloned into a pLAFR3 vector. Figure 1 shows the overall diagram for the construction of subclones from plasmid pLEX3BM.

Complementation of the Slime-Forming Property of *Z. ramigera* 115SLR

After transforming plasmid pLEX10 and its derivatives into *E. coli* DH5α, the transformants were isolated and used for plasmid amplification. The *E. coli* S17-1 harboring plasmid pLEX10 or its derivatives was mated with *Z. ramigera* 115SLR by conjugation [19]. The transconjugants with a "rough" colony appearance were identified on a TSA agar containing rifampicin (50 μg/ml) and tetracycline (10 μg/ml).

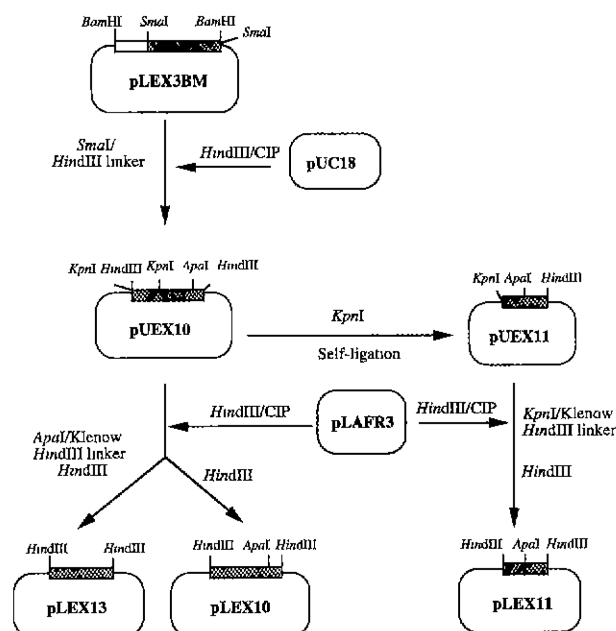

Fig. 1. Plasmids derived from plasmid pLEX3BM and cloning vectors.

Nucleotide Sequencing

The nucleotide sequence of the 3.3 kb *Hin*dIII DNA fragment in plasmid pLEX11 was determined using standard procedures. The 3.3 kb *Hin*dIII DNA fragment was isolated and subcloned into an M13mp19 cloning vector. After the double digestion of the clone with *Kpn*I and *Bam*HI, the subclone was deleted using an Erase-a-Base deletion kit (Promega, Madison, U.S.A.), ligated, and transformed into *E. coli* JM101 competent cells. The sequencing of each DNA fragment was performed by the dideoxy-mediated chain termination method using a Sequenase™ kit (USB, Cleveland, U.S.A.) and [α-^{35}S]dATP (Amersham, Arlington Heights, U.S.A.).

Production of EPS

Z. ramigera 115, *Z. ramigera* 115SLR, and *Z. ramigera* 115SLR/pLEX11 strains were grown in the defined media including glucose as a carbon source at 29°C for 5-6 days. The EPS content produced by the three strains was determined. The cell-bound EPS was extracted by treating the culture broth with 5 volumes of hot water (50°C) for 16 h. The whole culture producing slime EPS was diluted with 2 volumes of hot water. After the removal of the cells by centrifugation (10,000 rpm for 20 min, GSA rotor, Sorval RC5B), the EPS was precipitated with 2 volumes of isopropanol at -20°C for 6 h and then recovered by centrifugation. The EPS pellets were washed with water, recovered by isopropanol precipitation, and lyophilized.

Compositional Analysis of EPS

For an analysis of the sugar and organic acids, exopolysaccharide (10 mg) was dissolved in 5 ml of 0.1 M trifluoroacetic acid (TFA) and hydrolyzed at 120°C for 6 h. The hydrolyzate was filtered with a 0.22 μm Millex-GV filter (Millipore Corp., Milford, U.S.A.) and analyzed by high performance liquid chromatography (HPLC) equipped with an Aminex-87H column, using 5 mM sulfuric acid at a flow rate of 0.6 ml/min as the mobile phase. A sample analysis was performed at 40°C on a 1050 Hewlett Packard HPLC system equipped with a refractive index detector (model 1047A, Palo Alto, U.S.A.) for the sugar analysis and a U.V. detector (model 35900) at 220 nm for the organic acid analysis. The calibration curves were obtained by injecting increasing quantities of each sugar and organic acid.

In Vivo Gene Expression by T7 RNA Polymerase

The recombinant plasmids, pTEX10a, pTEX10b, pTEX11, and pTEX13, were constructed using the inserts of the

subclones and a pT7-7 vector. The genes in the recombinant plasmids were controlled by T7-RNA polymerase [24]. The *E. coli* K38/pGP1-2 strain harboring a recombinant plasmid was grown in LB containing ampicillin (50 µg/ml) and kanamycin (50 µg/ml). The cells were harvested at OD_{600} of 0.5 and then washed with 1 ml of the M9 medium. The cells were resuspended in 1 ml of the M9 medium containing 20 mg of thiamine and 0.01% of an 18 amino acid mixture (lacking methionine). In addition, ^{35}S-labeled methionine was added to the reaction mixture in order to label the foreign protein in the *E. coli*. The expression of the foreign gene fragments from the T7 promoter in the *E. coli* was performed essentially as described by Tabor and Richardson [24].

Nucleotide Sequence Accession Number

The nucleotide sequence of the gene that complements the slime-forming property of *Z. ramigera* 115SLR was deposited in the NCBI/GenBank Library under accession number AF181662.

Results and Discussion

Construction of Recombinant Plasmids

The recombinant plasmids derived from plasmid pLEX3BM which were able to complement the slime-forming property of mutant strain are shown in Fig. 1. Plasmids pLEX10, pLEX13, and pLEX11 were constructed with pLAFR3 vector and their restriction maps were determined using various restriction enzymes (Fig. 2). The genes involved in the exopolysaccharide biosynthesis of *Z. ramigera* 115SLR were previously isolated, and from them, plasmid pLEX3BM containing a 7.8 kb DNA fragment was identified as being able to complement the mutation responsible for the morphology changes in *Z. ramigera* 115SLR. The DNA sequence required for the restoration of the morphology was also found to be located in the 1.5 kb DNA present in the downstream region of plasmid pLEX3BM [10]. Accordingly, because of its essential role in the complementation of the slime-forming property of mutant strain, plasmid pLEX11 with a 3.3 kb DNA fragment including this region was constructed. Thereafter, plasmid pLEX11 and the other recombinant plasmids were used for the complementation of the slime-forming property of *Z. ramigera* 115SLR.

Complementation of the Slime-Forming Property of Mutant Strain

The subclones were transformed into *Z. ramigera* 115SLR by conjugation and their role in restoring a slime-type colony to a capsule-type colony was then determined. The restoration of the morphological mutant by recombinant plasmid pLEX11 is clearly shown on the TSB agar plate (Fig. 3). The recombinant plasmids pLEX10 and pLEX11 were able to complement the slime-forming property of mutant strain *Z. ramigera* 115SLR, as did plasmid pLEX3BM, however, plasmid pLEX13 was not able to

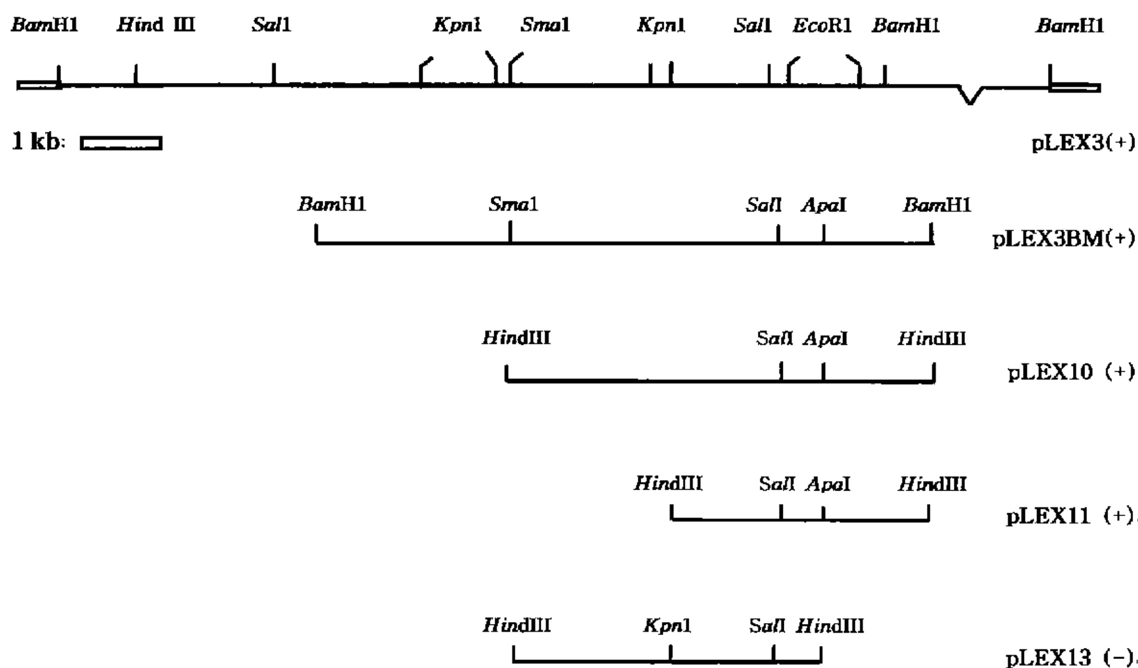

Fig. 2. Restriction map of subclones constructed from plasmid pLEX3 and their complementation patttern of the slime-forming mutant *Z. ramigera* 115SLR.

(+), capsule forming; (-), slime (non-capsule) forming.

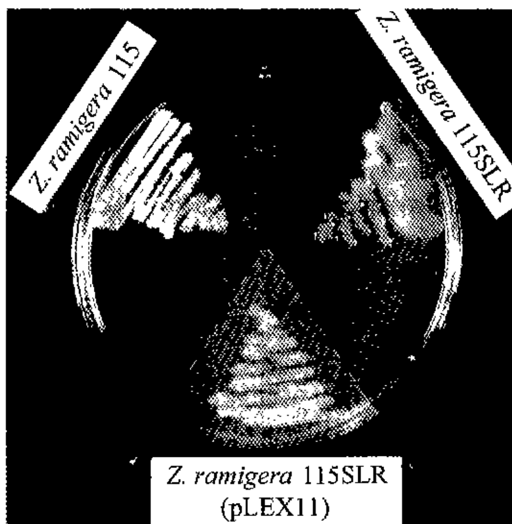

Fig. 3. Colony morphologies of *Z. ramigera* 115 and 115SLR, and strain 115SLR harboring plasmid pLEX11.
The strains were plated on TSB agar containing rifampicin and/or tetracycline

complement the *Z. ramigera* 115SLR strain (Fig. 2). Therefore, it can be concluded that the 1.1 kb *Apa*I-*Hin*dIII DNA fragment located in plasmid pLEX11 is the essential part for complementing the morphological mutation of *Z. ramigera* 115SLR.

Isolation and Characterization of EPS

Z. ramigera 115, 115SLR, and 115SLR/pLEX11 were grown in defined media, and then the level of bound and released EPS were determined. Figure 4 shows the pattern of both bound EPS and released EPS produced by *Z. ramigera* 115 and its mutant strains. As shown in Fig. 4, the *Z. ramigera* 115 strain produced mainly an EPS which remained bound to the cells, whereas, in the mutant strain

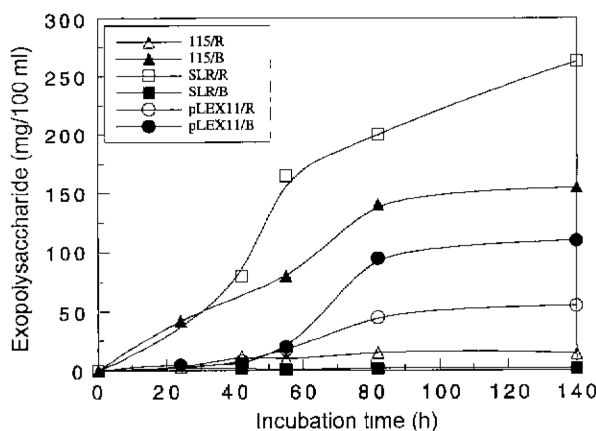

Fig. 4. Yield and type of exopolysaccharide produced from *Z. ramigera* 115 and derivative strains (R: released EPS; B: bound EPS).

115SLR, the EPS was released into the culture medium. The *Z. ramigera* 115SLR harboring plasmid pLEX11 showed an EPS distribution that lay somewhere between the wild-type and the 115SLR situation. After 80 h, 34% of the EPS was released into the medium and 66% remained bound to the cells. These results can be explained by the fact that strain 115SLR/pLEX11 contains both the mutant gene in the chromosome and the wild-type gene in plasmid pLEX11. The results also imply that the gene product is directly involved in the association of the EPS with the bacterial cell surface. It may function as the means of attachment on the cell surface. The EPS produced by all the strains gradually increased for 140 h. The EPS yield produced by *Z. ramigera* 115SLR was higher than that of the other strains.

Although plasmid pLEX11 complemented the slime-forming property of *Z. ramigera* 115SLR mutant, it is still not clear what makes the difference in the morphology of *Z. ramigera* 115. As a simple approach, the monosaccharide composition of the EPS isolated from *Z. ramigera* 115 and its mutant strains was determined by an HPLC analysis using the acid hydrolyzate of the EPS. The results are shown in Table 2. Glucose, galactose, and acetic acid were detected as the major EPS components. Succinic acid was also detected as a minor peak. The retention times of the sugars and organic acids were 9.36 min (glucose), 10.07 min (galactose), 13.5 min (succinic acid), and 16.2 min (acetic acid). The content of the monosaccharide, such as glucose and galactose, was similar between *Z. ramigera* 115 and its mutant strains. The pyruvate and acetate contents also had very similar values. In particular, the succinic acid content in the bound EPS of both *Z. ramigera* 115 and 115SLR/pLEX11 was higher than that of *Z. ramigera* 115SLR. However, according to the analysis of the HPLC fraction using GC/MS, this compound turned out to be butyric acid, which was probably derived from the acid hydrolyzate of the PHB contaminated from the cells. It was concluded that there were no detectable differences in the composition of the EPS produced by the different strains. Gotschlich *et al.* [6] reported that the

Table 2. Compositional analysis of exopolysaccharides isolated from *Z ramigera* 115, 115SLR, and 115SLR harboring plasmid pLEX11.

Compound	115WT	115SLR	115SLR/pLEX11	
			bound	released
Glucose	2.34	2.34	2.34	2.34
Galactose	1.06	1.01	1.03	1.07
Pyruvate	0.84	0.78	0.83	0.81
Acetate	0.93	1.07	1.07	1.01

Conditions. Aminex HPX-87H column (organic acid analysis column, 300×7 8 mm); mobile phase, 5 mM sulfuric acid, temperature, 40°C; flow rate, 0.6 ml/min, sample injection volume, 30 ml

capsular polysaccharides of gram-negative bacteria have a lipoidal substance linked to the reducing end of the carbohydrate. The presence of this hydrophobic end causes the polysaccharide chains to aggregate in a micellar form. Therefore, the determination of the fatty acids from the EPS of *Z. ramigera* 115 and its mutant strains was carried out using the same method as Gotschlich *et al.* [6]. No fatty acid compound was identified (unpublished results). In previous analyses of other polysaccharides, no significant chemical differences have been reported between capsular and slime polysaccharides obtained from wild-type strains and their slime-forming mutants [23]. Similarly, the compositional analysis of the EPS isolated from *Z. ramigera* 115 and its mutant strains indicated that monosaccharides and organic acids composing the EPS were not related with the morphological changes of *Z. ramigera* 115. Accordingly, the factors involved in the morphological changes are still not clearly understood. However, it is known that, in some bacteria, the capsule is very firmly attached to the cell surface [1].

Nucleotide Sequencing and Analysis

The nucleotide sequence of the 3.3 kb *Hin*dIII fragment of pLEX11 was determined and is shown in Fig. 5. The 3.25 kb of the genomic insert DNA fragment was analyzed to determine the presence of open reading frames (ORFs): two ORFs encoded by the same strand were found (Fig. 5). These ORFs were designated as ORF1 and ORF2. ORF1 and ORF2 both began with a characteristic ATG start codon. The ORF1 sequence started at position 1146, contained 830 bp. and had a coding potential for a 30.0 kDa protein. The ORF2 sequence started at position

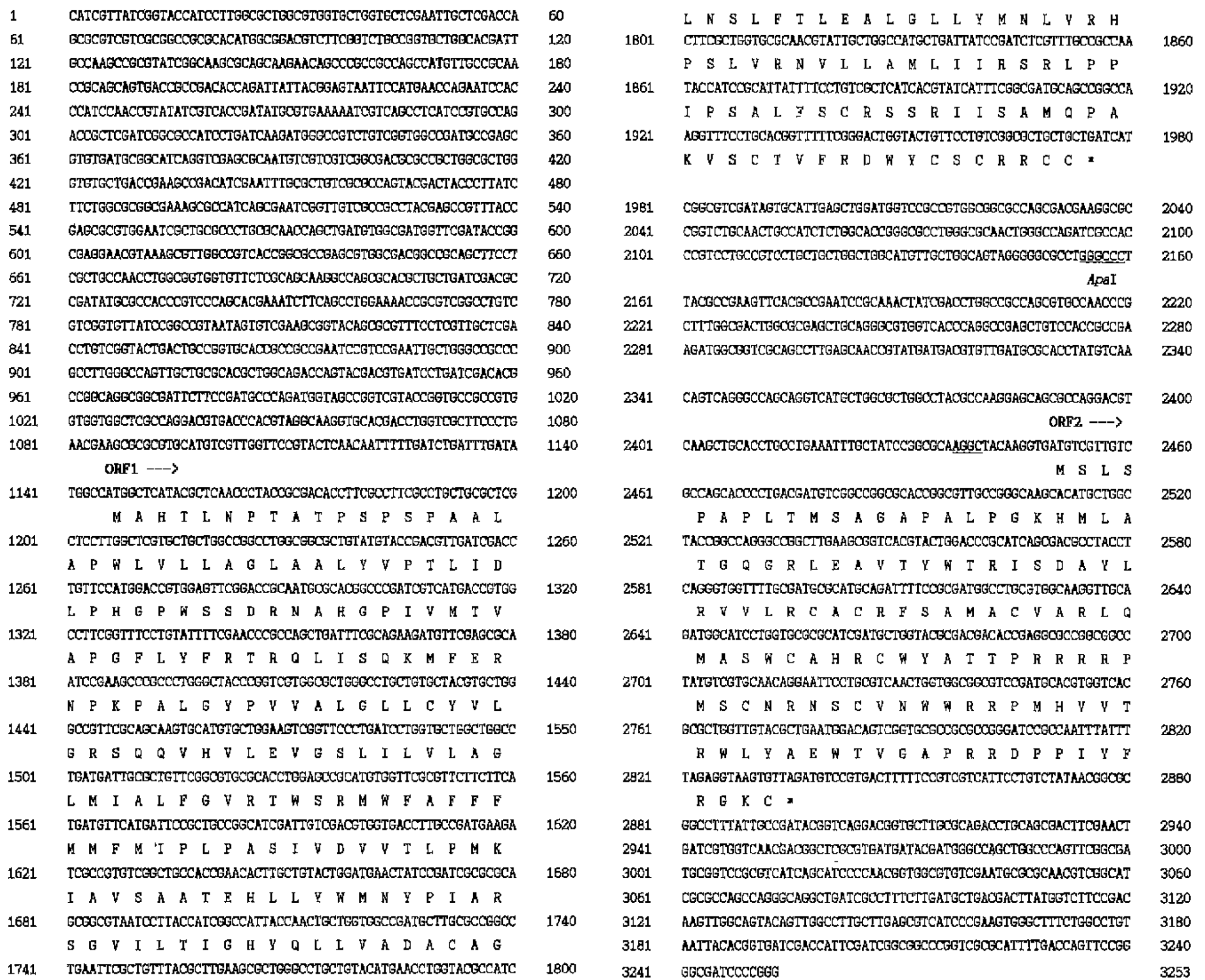

Fig. 5. Nucleotide and deduced amino acid sequences of the 3.25 kb genomic fragment of *Z. ramigera* 115SLR.
The nucleotide sequence of one strand of the DNA fragment is presented in the 5' to 3' direction. The two identified ORFs are indicated by the deduced amino acid sequence written below the nucleotide sequence. The arrows indicate the direction of the transcription The putative Shine-Dalgarno (SD) ribosomal binding sequence is underlined.

2450, contained 386 bp. and had a coding potential for a 14.3 kDa protein. ORF1 and ORF2 were not proceeded by a site similar to an *E. coli* consensus ribosomal binding (Shine-Dalgarno) sequence. The presence of two protein-coding regions potentially indicates the clustered organization of an operon. The cluster of genes involved in the EPS biosynthesis in *X. campestris* is well known [8]. Based on the restriction and complementation analysis, ORF2 was recognized as the essential DNA fragment which was able to complement the morphological mutation of *Z. ramigera* 115SLR. To identify the homology of the amino acid sequence. the amino acid sequences derived from ORF1 or ORF2 were analyzed by the BLAST search program. The similarity and identity of the amino acid sequences were compared with the amino acid sequences of other known proteins. None of the two proteins were found to share a homology with any of the other known proteins. Therefore, it was concluded that the gene products from ORF1 and ORF2 are novel proteins.

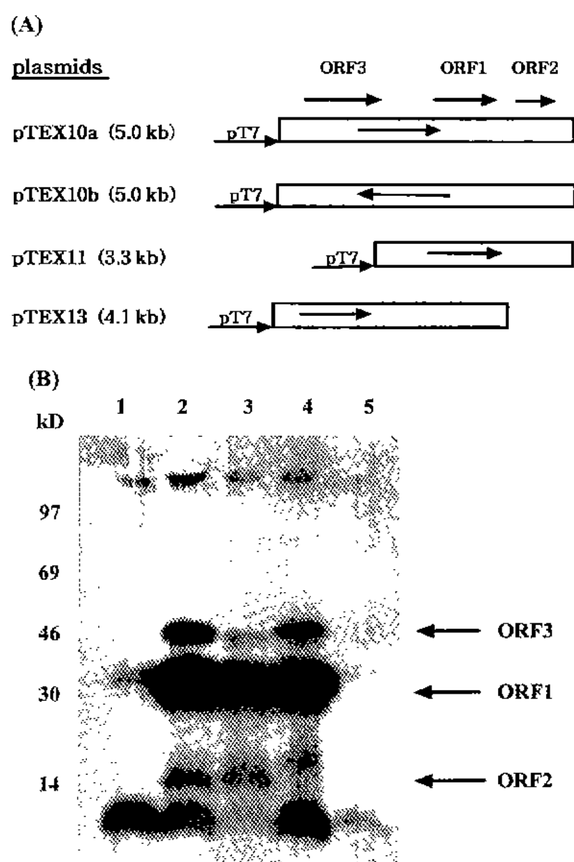

Fig. 6. Expression and autoradiograph of recombinant plasmids by T7-RNA polymerase.

(A) Schematic representation of three ORFs deduced from the nucleotide sequence analysis. including their orientation and sizes (B) Fluorography of polypeptides labeled with ^{35}S-methionine. Lane 1. plasmid pTEX10b: Lane 2, plasmid pTEX10a: Lane 3. plasmid pTEX11; Lane 4. plasmid pTEX13; Lane 5, plasmid pT7-7.

In Vivo Gene Expression

In order to confirm the DNA sequence and gene product from each ORF. an *in vivo* gene expression was performed. The recombinant plasmids, pTEX10a, pTEX10b, pTEX11, and pTEX13, were expressed by using the *in vivo* T7 RNA polymerase. Figure 6 shows the orientation of the insert DNA in each plasmid and gene expression patterns. It is evident from the fluorography that the ORF1 and ORF2 sequences present in plasmid pTEX11 express a 30 and 14 kDa protein, respectively (Fig. 6). As expected from the ORFs in the DNA sequence, proteins with the same molecular weight were expressed by the same DNA fragments. Plasmid pTEX13 with a 4.1 kb DNA in its upstream region produced two proteins of 50 kDa and 30 kDa. A 14 kDa protein was not expressed by plasmid pTEX13, in which the ORF2 sequence in the DNA fragment had been deleted. This indicates, therefore, that the 14 kDa protein is the product of ORF2. Plasmid pTEX10a with a 5.0 kb DNA fragment showed three proteins of 50 kDa. 30 kDa, and 14 kDa. Furthermore, the upstream region (1.7 kb) in plasmid pTEX10 included another open reading frame encoding a 50 kDa protein. In contrast, plasmid pTEX10b that contained the reverse orientation of the same gene did not produce any protein products as a control plasmid pT7-7. This indicates, therefore, that the gene from each ORF is expressed by the T7 RNA polymerase and its promoter present in the pT7-7 vector. In particular. the gene product of ORF1 was expressed strongly compared with the other ORFs. The extra bands observed near the 50 kDa protein band are most likely to be false initiations or termination products. Accordingly, the *in vivo* gene expression confirmed the presence of three ORFs in the 5.0 kDa DNA fragment and determined their gene products.

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