Molecular characterization of the levansucrase gene from the endophytic sugarcane bacterium *Acetobacter diazotrophicus* SRT4

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The *Acetobacter diazotrophicus* SRT4 gene encoding levansucrase (EC 2.4.1.10) (*lsdA*) was isolated from a genomic library. The nucleotide sequence of a 2.3 kb DNA fragment sufficient for complementation of a levansucrase-deficient mutant (obtained by EMS treatment) was determined. The *lsdA* gene (1751 bp) coded for a polypeptide of molecular mass 64.9 kDa with an isoelectric point of 5.2. The N-terminal amino acid sequence of the extracellular levansucrase indicated the presence of a precursor protein with a putative signal sequence of 51 residues which is possibly cleaved in two successive steps. Expression of the *lsdA* gene from the *lac* promoter in *Escherichia coli* resulted in the production of a protein with levansucrase activity. The deduced amino acid sequence of the *lsdA* gene was 48% and 46% identical with the levansucrases from the Gram-negative bacteria *Zymomonas mobilis* and *Erwinia amylovora*, respectively, but only 28–31% identical with levansucrases from Gram-positive bacteria. Multiple alignments of published levansucrase sequences from Gram-negative and Gram-positive bacteria revealed eight conserved motifs. A comparison of the catalytic properties and the sequence of the *A. diazotrophicus* levansucrase with those of the *Bacillus subtilis* levansucrase suggested that one of these motifs may be involved in the specificity of the synthesized product. Disruption of the *lsdA* gene in the genome of *A. diazotrophicus* resulted in a mutant lacking both levansucrase activity and the ability to utilize sucrose as a carbon source, suggesting that levansucrase is the key enzyme in sucrose metabolism of *A. diazotrophicus*.

Keywords: *Acetobacter diazotrophicus*, levansucrase, *lsdA* gene

INTRODUCTION

Acetobacter diazotrophicus is the only nitrogen-fixing species identified in the genus *Acetobacter* (Gillis *et al.*, 1989). It is a Gram-negative bacterium and has been isolated from sucrose-rich plants including sugarcane, sweet potato, sweet sorghum and Cameroon-grass (Paula *et al.*, 1991). *A. diazotrophicus* infects sugarcane roots, stems and leaves, and is mostly found in the apoplastic fluid of stem parenchyma where the sucrose content is about 12% (0.34 M) and the pH 5.5 (Dong *et al.*, 1994).

Under similar conditions in the free-living state, the bacterium synthesizes an extracellular levansucrase (Hernández *et al.*, 1995). This enzyme releases fructo-oligosaccharides and levan, a high-molecular-mass fructosyl polymer, from sucrose. These exopolysaccharides may be a recognition signal in the micro-organism-plant interaction (James *et al.*, 1994).

Many micro-organisms belonging to Gram-negative and Gram-positive species produce levansucrase. Comparison of the levansucrase structural genes sequenced to date shows that several short domains of the deduced polypeptide sequences are conserved (Song *et al.*, 1993). It has not been possible to correlate the small differences observed in these motifs with differences in their catalytic

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specificities. One reason for this is that the catalytic properties of most of these enzymes are poorly characterized.

The catalytic mechanism of *A. diazotrophicus* levansucrase was recently studied in detail (Hernández *et al.*, 1995) and compared with that of the well-characterized levansucrase produced by *Bacillus subtilis*. Both enzymes form a fructosyl enzyme intermediate as the first step of transfructosylation with similar kinetic parameters, but the patterns of the products released are different. Levansucrase of *A. diazotrophicus*, unlike the enzyme of *B. subtilis*, causes accumulation of large quantities of tri- and tetrasaccharides but small quantities of high-molecular-mass levan. Differences in the primary structures of the two enzymes may be correlated with their different product profiles.

All known levansucrases are secreted proteins. Characterization and comparison of their intrinsic secretion signals may help elucidate differences in the mechanisms of secretion from Gram-negative and Gram-positive bacteria.

Here, we report the cloning of the *A. diazotrophicus* levansucrase structural gene and the determination of its sequence.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in Luria broth, supplemented with 50 µg ampicillin ml⁻¹ and 12.5 µg tetracycline ml⁻¹ as appropriate. *A. diazotrophicus* strains were grown either in LGIE medium [LGI medium salts (Cavalcante & Döbereiner, 1988): 1 g tryptone l⁻¹ and 0.2 g yeast extract l⁻¹ supplemented with 5% (w/v) sucrose and 1% (v/v) glycerol], SB liquid medium (Coego *et al.*, 1992) or GYC agar medium (De Ley *et al.*, 1984): 1% (w/v) yeast extract, 3% (w/v) D-glucose, 3% (w/v) CaCO₃ and 2.5% (w/v) agar.

Isolation of *A. diazotrophicus* mutants deficient in levan synthesis by EMS mutagenesis. The method described by Miller (1972) was used for EMS mutagenesis, with some modifications. *A. diazotrophicus* strain SRT4 was grown aerobically in SB medium at 30 °C for 48 h. Bacteria (1.5 ml culture in an Eppendorf tube) were harvested by centrifugation, washed with 1.5 ml LGI salts, resuspended in 0.5 ml 2% (w/v) EMS in 0.2 M Tris/HCl, pH 7.4, and incubated at 30 °C for 90 min. The treated cells were collected by centrifugation, washed with 1 ml 5% (w/v) Na₂S₂O₃ in 0.2 M Tris/HCl, pH 7.4, resuspended in 3 ml SB medium supplemented with 1% glycerol and grown with aeration for 16 h at 30 °C. The bacterial culture was plated onto LGIE medium and incubated at 30 °C. Colonies with a non-mucous phenotype were picked, grown in liquid LGIE medium and assayed for levansucrase activity. The mortality resulting from EMS treatment was 80%.

Cosmid library construction. Total DNA from *A. diazotrophicus* strain SRT4 was partially digested with the restriction endonuclease *Sau*3AI. Fragments of 15–30 kb were isolated from a low-melting-temperature agarose gel and ligated to *Bam*HI-cleaved and dephosphorylated vector pPW12 DNA. The library was packaged into lambda phage particles using Gigapack Plus

mixtures. The packaged cosmids were transferred by infection to *E. coli* strain S17-1 which was then plated onto LB medium supplemented with tetracycline (12.5 µg ml⁻¹). The gene bank (approximately 7000 clones) was collected in 4.8 ml LB medium, and kept frozen at -70 °C in 20% glycerol.

Complementation experiments. The cosmid bank in *E. coli* S17-1 was mobilized into the *A. diazotrophicus* Lev⁻ mutant M21 by conjugal mating. The donor and recipient bacteria were mixed on GYC agar plates and incubated for 24 h at 30 °C. The mating mixture was collected and plated onto LGIE agar medium with 5% sucrose supplemented with 20 µg tetracycline ml⁻¹ for transconjugant selection and 25 µg ampicillin ml⁻¹ to counterselect *E. coli* donors. Single colonies which had recovered the mucous phenotype were picked. Plasmid DNA from four colonies was purified, used to transform *E. coli* strain S17-1 and again transferred by conjugation to the *A. diazotrophicus* mutant M21 to confirm that plasmids complemented the Lev⁻ phenotype. Subfragments of the 7.8 kb fragment common to the recombinant cosmids p21R1 and p21R2 (Table 1) were subcloned into pUC18 and the mobilizable broad-host-range vector pRK293.

Electroporation of *A. diazotrophicus* cells. Bacteria were grown in LGIE medium (200 ml) with vigorous shaking at 30 °C to early exponential phase (A_{600} of 0.3–0.4). The culture was then chilled on ice for 15 min. Cells were harvested by centrifugation, washed twice with 100 ml ice-cold 10% glycerol and resuspended in 2 ml 10% glycerol. Aliquots of 0.15 ml were frozen in liquid nitrogen and stored at -70 °C. A mixture of 0.3 µg plasmid DNA and 0.04 ml cells previously thawed on ice was transferred to a chilled 2 mm electroporation cuvette. Electroporation was performed with a Gene Pulser Apparatus (Bio-Rad) at 9000 V cm⁻¹, 200 Ω, 25 µF. The resulting time constant was 4.0–4.8 ms. Immediately after electroporation, 1 ml cold LGIE medium was added to the cuvette, cells were resuspended gently, transferred to polypropylene tubes and incubated at 30 °C for 3 h with slow shaking. Bacterial dilutions were plated on LGIE medium supplemented with kanamycin (120 µg ml⁻¹) and incubated at 30 °C.

Purification of levansucrase from *A. diazotrophicus* culture supernatants. *A. diazotrophicus* SRT4 was grown to stationary phase in LGIE medium containing 2% glycerol as carbon source at 30 °C. Under these conditions, levansucrase represents more than 70% of total extracellular protein. Bacteria were removed by centrifugation. The culture supernatant was collected and concentrated fivefold using a rotatory evaporator. High-molecular-mass exopolysaccharides were precipitated by ethanol [60% (v/v) final concentration] and centrifugation. Levansucrase remained in the supernatant fraction from which ethanol was removed using a rotatory evaporator. The enzyme solution was dialysed against 20 mM Tris/HCl, pH 7.5, and passed through a DEAE-Sepharose CL-6B (fast flow) column (2.5 × 13 cm) equilibrated with 20 mM Tris/HCl, pH 7.5. The proteins adsorbed on the column were selectively eluted with a linear concentration gradient from 100 to 500 mM NaCl. The peak fractions with levansucrase activity were pooled, dialysed against a solution of 1% (w/v) NH₄HCO₃, pH 8.0, and lyophilised. The purified levansucrase gave a single band of approximately 60000 Da following SDS-PAGE.

N-terminal sequencing of the extracellular *A. diazotrophicus* levansucrase. The N-terminal sequence analysis of the purified levansucrase was performed by the Edman degradation procedure (Bauw *et al.*, 1989) using a gas-phase sequencer (model 470A, Applied Biosystems) equipped with an on-line phenylthiohydantoin amino acid derivative analyser (model 120A).

Table 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Genotype or description	Reference or source
<i>E. coli</i>		
XL1-Blue	<i>thi recA1 endA1 gyrA96 (Nal^r) hsdR17(r_k⁻m_k⁺) supE44 relA1 Δ(lac-proAB) [F'proAB lacI^qZ DM15 Tn10]</i>	Stratagene
S17-1	<i>thi pro hsdR recA</i> [RP4-2(Tc::Mu) (Km::Tn7)]	Simon <i>et al.</i> (1983)
<i>A. diazotrophicus</i>		
SRT4	Wild-type, Lev ⁺	Coego <i>et al.</i> (1992)
M21	EMS mutant, Lev ⁻	This study
AD1; AD2	SRT4 <i>lsdA::nptII-ble</i> cassette, Lev ⁻	This study
AD3; AD4	SRT4 <i>lsdA::pALS40</i> , Lev ⁺	This study
Plasmids		
pPW12	Derivative of cosmid pLAFR1 with <i>EcoRI</i> - <i>Bam</i> HI- <i>EcoRI</i> linker, Tc ^r	*
pRK293	IncP-mobilizable cloning vector, Tc ^r Km ^r	Ditta <i>et al.</i> (1985)
pUC4-KIXX	pUC4K-based plasmid carrying the Tn5 Km ^r and Bl ^r resistance genes, Ap ^r Km ^r Bl ^r	Pharmacia
pUC18; pUC19	Cloning vector, Ap ^r	Pharmacia
p21R1	pPW12 with a 20 kb insert of <i>A. diazotrophicus</i> DNA containing the <i>lsdA</i> gene, Tc ^r	This study
p21R2	pPW12 with a 24 kb insert of <i>A. diazotrophicus</i> DNA containing the <i>lsdA</i> gene, Tc ^r	This study
pALS5	5.2 kb <i>SmaI</i> - <i>EcoRI</i> fragment from p21R1 inserted into pUC19, Ap ^r	This study
pALS10	2.3 kb <i>Bgl</i> II fragment from p21R2 inserted in the <i>Bam</i> HI site of pUC18, Ap ^r	This study
pALS11	pUC18 carrying a 2.8 kb <i>SmaI</i> fragment from p21R2, <i>plac-lsdA</i> fusion, Ap ^r	This study
pALS15	pALS10 <i>Hind</i> III-linearized and inserted in the <i>Hind</i> III site of pRK293, Ap ^r Tc ^r	This study
pALS40	pALS5 with the <i>nptII-ble</i> cassette from pUC4-KIXX inserted in the <i>Bam</i> HI site of <i>lsdA</i> , Ap ^r Km ^r Bl ^r	This study

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The initial sequencing yield was 1.4%, indicating strong blocking of the N-terminus.

DNA sequencing. Sequencing was performed using pALS10 (Table 1) as the template by the dideoxy chain-termination method (Sanger *et al.*, 1977) with a Sequenase sequencing kit. The whole sequence of the levansucrase gene was determined on each strand using direct and reverse primers and the primer walking procedure with 16-mer oligonucleotides as primers. The nucleotide sequences were compiled and analysed by the BioSOS program package (Bringas *et al.*, 1992). FASTA (Pearson & Lipman, 1988) and BLAST (Altschul *et al.*, 1990) programs were used for similarity searches in the SwissProt and EMBL databases, and the Waling option in the molecular modelling program WHAT IF (Vriend, 1990) used to obtain multiple sequence alignments. Protein secondary structure in levansucrases were predicted by the PHD program (Rost *et al.*, 1995).

Expression of the *A. diazotrophicus* levansucrase gene in *E. coli*. A 2.8 kb *SmaI* DNA fragment (from nucleotide 369, corresponding to the position five amino acids downstream from the cleavage site of the processed levansucrase, Fig. 2) was ligated into the *SmaI* site (in-frame in the 5' region of *lacZ'*) of pUC18 under the control of the *lac* promoter. The resulting

plasmid, named pALS11, was used to transform *E. coli* strain XL1-Blue. After induction by IPTG (0.1 mM), total proteins were assayed for levansucrase activity and analysed by Western blotting.

Other techniques

DNA manipulations. Standard methods were used for recombinant DNA procedures (Sambrook *et al.*, 1989).

Levansucrase assays. Levansucrase enzyme assays both in solution and on SDS-polyacrylamide gels were as reported previously (Hernández *et al.*, 1995).

Antibody preparation. Rabbit polyclonal antibodies were raised against extracellular levansucrase purified by preparative SDS-PAGE from culture supernatants of *A. diazotrophicus* SRT4.

Western blotting. Protein electrophoresis was carried out on a SDS-polyacrylamide gel (12%, w/v, acrylamide). After electrophoresis, proteins were transferred onto a nitrocellulose membrane, probed with polyclonal antibodies against the *A. diazotrophicus* levansucrase, and binding detected using ¹²⁵I-labelled Protein A.

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase and oligonucleotides were purchased from Heber Biotec and

radiochemicals from Amersham. EMS was from Koch-Light, sequencing kits from US Biochemicals and Gigapack Plus packaging mixtures from Stratagene.

RESULTS

Isolation and characterization of *A. diazotrophicus* EMS-induced mutants deficient in levan synthesis

A. diazotrophicus strain SRT4 forms mucous colonies surrounded by levan when grown on sucrose-containing media due to the action of an extracellular levansucrase. Nine non-mucous colonies were isolated after EMS treatment at a frequency of 2.5×10^{-4} . There were two classes of mutants according to their phenotype. Class I mutants (two colonies) did not express detectable levansucrase activity either in culture supernatants or in sonicated cell pellets. These mutants were totally complemented by the introduction of the wild-type levansucrase gene (*lsdA*) indicating that mutations in the structural gene for the enzyme were likely. Class II mutants (seven colonies) were defective in levansucrase secretion but retained the intracellular activity of the wild-type strain. These mutants remained unable to secrete levansucrase even after introducing the structural *lsdA* gene.

A class I mutant, named M21, was used as host for the genetic complementation experiments to isolate the levansucrase gene from an *A. diazotrophicus* SRT4 genomic library.

Cloning of an *A. diazotrophicus* genomic fragment encoding levansucrase

A genomic library of *A. diazotrophicus* was constructed in the broad-host-range cosmid pPW12 (*mob*⁺) in *E. coli* S17-1 (Tra⁺). The library was transferred by conjugal mating into the levan-deficient mutant M21. Two recombinant cosmids, p21R1 and p21R2, sharing a common 7.8 kb region, were found to restore the Lev⁺ phenotype to the mutant M21. The restriction map of the common 7.8 kb region was determined (Fig. 1a). In subcloning experiments, a 2.3 kb *Bgl*II fragment was obtained in pALS15, sufficient for complementation of the mutant M21.

The presence of a single *lsdA* locus on the *A. diazotrophicus* genome was deduced from Southern hybridization analysis (Fig. 1b).

DNA sequence analysis of the complementing region

The sequence of the 2.3 kb fragment was determined on both strands (Fig. 2). It contained two ORFs in opposite orientations. ORFI has two putative translation initiation codons preceded by potential ribosome-binding sites: ATG (nt 201–203) and ATG (nt 325–327). It ends at two adjacent stop codons (nt 1954–1959). There is a potential rho-independent transcription terminator [$\Delta G = -24.1$ kcal mol⁻¹ (-100.8 kJ mol⁻¹)] 9 nt downstream from the last stop codon. The hairpin comprises a stem of twelve base pairs and a loop of four unpaired bases, the stem-loop

Fig. 1. (a) Restriction map of the 7.8 kb fragment common to p21R1 and p21R2 cosmids. E, S, Sp and B represent *Eco*RI, *Sal*I, *Sph*I and *Bam*HI restriction sites, respectively. The *lsdA* ORF is represented by the arrow. The insertion of the *nptII-ble* cassette in the *Bam*HI site of the *lsdA* gene is shown. (b) Southern hybridization analysis of *A. diazotrophicus* SRT4. Total DNA was digested with *Eco*RI (lane 1), *Sph*I (lane 2) or *Sal*I (lane 3) and probed with a ³²P-labelled 2 kb *Sph*I fragment containing the *lsdA* gene from *A. diazotrophicus*. Hybridization was done in 0.5 M sodium phosphate buffer, pH 7.2, containing 7% (w/v) SDS, 1% (w/v) BSA at 65 °C. The nitrocellulose sheet was then washed in 0.1 × SSC containing 0.1% SDS at 65 °C.

structure is followed by four consecutive U residues. ORFII overlaps completely with ORFI in the opposite direction, extending beyond the end of the *Bgl*II fragment in pALS10.

Identification of ORFI as the *A. diazotrophicus* levansucrase gene

The expression of ORFI in *E. coli* under the *lac* promoter of pUC18 resulted in a polypeptide with levansucrase activity detected by the enzymic synthesis of levan after fractionation of a cell extract on a SDS-polyacrylamide gel. The protein exhibited the expected molecular mass (60 kDa) and was recognized by polyclonal antibodies raised against the extracellular levansucrase (Fig. 3). The polypeptide encoded by ORFI consisted of 584 amino acids from the first ATG codon. Residues 52–61 of the predicted amino acid sequence were identical to the N-terminal sequence G-G-P-L-F-P-G-R-S-L of the secreted levansucrase determined by Edman degradation. The putative processed protein had a calculated molecular mass of 58.1 kDa and an isoelectric point of 5.2, which is

GATCTGGTATTGTGCGGAGCCGCCATGATTCCGCTTAAATCTTTTATTAAATAAATTGACATCCGTAAATCCCCCGTGATCTTCTGC 90
 GCGCGCGCTCAGCGGAAAAACCGGGGAAAGTCCCTTATAAATCACTGAATACAGTGACGCGCGGTCGTGGTGTCTGCAATCTGAAG 180
 AGTTTCCTTACAGGAGTGGAAATGGCGCATGTACGCCGAAAGTAGCCACGCTGAATATGGCGTGGCGGGTCCCTGCTCATGGTGTCTG 270
 MetAlaHisValArgArgLysValAlaThrLeuAsnMetAlaLeuAlaGlySerLeuLeuMetValLeu
 GCGCGCAAGTGGCTGGCGCAAGGAATTTACGCCGCGAGGAAGCCGCGCATGGCGCACCGTCCGGTGTGTATGGCTCGTGGCGGC 360
 GlyAlaGlnSerAlaLeuAlaGlnGlyAsnPheSerArgGlnGluAlaAlaArgMetAlaHisArgProGlyValMetProArgGlyGly
 CCGCTCTTCCCGGGCGGTGCTGGCGGGGTGCGGGCTTCCCGCTGCCGAGCATTATACGCGAGCGGTATGACCGCGAGTCGGAC 450
 ProLeuPheProGlyArgSerLeuAlaGlyValProGlyPheProLeuProSerIleHisThrGlnGlnAlaTyrAspProGlnSerAsp
 TTTACGCCGCTGGACACGTGCGCAGCATTTGAGATCAAGGCGCATTGGATGCGACGGTCCGCGCGGCGAGAATTCCCTGCGCGCG 540
 PheThrAlaArgTrpThrArgAlaAspAlaLeuGlnIleLysAlaHisSerAspAlaThrValAlaAlaGlyGlnAsnSerLeuProAla
 CAACTGACCATGCCAACATCCCGCGGACTTCCCGGTGATCAATCCGGATGTCTGGGTCTGGGATACCTGGACCTGATCGACAAGCAC 630
 GlnLeuThrMetProAsnIleProAlaAspPheProValIleAsnProAspValTrpValTrpAspThrTrpThrLeuIleAspLysHis
 GCCGATCAGTTTACGTATAACGGCTGGGAAGTCAATTTTCTGCTGACGGCGCACCCCAATGCCGGATACGGTTTCGACGACCGCCAGTG 720
 AlaAspGlnPheSerTyrAsnGlyTrpGluValIlePheCysLeuThrAlaAspProAsnAlaGlyTyrGlyPheAspAspArgHisVal
 CATGCCGCTCCCGGGCTTCTTCTATCGTCCGCGGGTATTCCCGCCAGCGCGCGCGGTGAATGGCGGTGGACCTATGGCGGCATCTC 810
 HisAlaArgIleGlyPhePheTyrArgArgAlaGlyIleProAlaSerArgArgProValAsnGlyGlyTrpThrTyrGlyGlyHisLeu
 TTCCACGACGCGGCGGCGGAGGTCTACGCGCGGAGCTACACGAACAGCGGGAATGGTCCGGTCTCGTGGCTGTATCGACGATG 900
 PheProAspGlyAlaSerAlaGlnValTyrAlaGlyGlnThrTyrThrAsnGlnAlaGluTrpSerGlySerSerArgLeuMetGlnIle
 CATGGCAATACCGTATCGGTCTTCTATACCGAGTGGCGTCAACCGTGACGCCAACGCCAACATCACCCCGCGCGAGCCATCATC 990
 HisGlyAsnThrValSerValPheTyrThrAspValAlaPheAsnArgAspAlaAsnAlaAsnAsnIleThrProProGlnAlaIleIle
 ACCACGACCGTGGCGGATCCAGCGGACTTCAACCATGCTCTGGTTCACGGGCTTACCGCCACACGCGCTGCTGCGACCGCGCGC 1080
 ThrGlnThrLeuGlyArgIleHisAlaAspPheAsnHisValTrpPheThrGlyPheThrAlaHisThrProLeuLeuGlnProAspGly
 GTGCTGTATCAGAACGTCGCGAGAACGAATTTCAATTTCCGCGATCCGTTCACCTTCGAGGACCCGAGCATCCGCGGTGAACATC 1170
 ValLeuTyrGlnAsnGlyAlaGlnAsnGluPhePheAsnPheArgAspProPheThrPheGluAspProLysHisProGlyValAsnTyr
 ATGGTGTTCAGGGAATACCGCGGCGGACGTCGCGTCCCACTGCACCGAGGCGGATCTGGGCTTCCGCGGACGATCCCAATGCG 1260
 MetValPheGlnSerThrAlaGlyGlnArgGlyValAlaAsnCysThrGluAlaAspLeuGlyPheArgProAsnAspProAsnAla
 GAAACCTCGAGGAAGTCTGGATAGCGGGCTATTACAGAACGCAATATCGGCTGGCCATCGCCACGGATTTCGACCTGTGCGAAA 1350
 GluThrLeuGlnGluValLeuAspSerGlyAlaTyrTyrGlnLysAlaAsnIleGlyLeuAlaIleAlaThrAspSerThrLeuSerLys
 TGGAGTTCTCTGCGCGCTGATTTCGGCCAACTGCGTCAATGACAGACCGAAGCGCGCAGGTGTACCTCCATAACGGAATACTACT 1440
 TrpLysPheLeuSerProLeuIleSerAlaAsnCysValAsnAspGlnThrGluArgProGlnValTyrLeuHisAsnGlyLysTyrTyr
 ATCTTCACCATCAGCCACCGCAGCACTTCCGCGCGGTGTCGATGGACCGGACGGCTCTACGGCTTCTGGGTGACGGCATCCCGAGT 1530
 IlePheThrIleSerHisArgThrThrPheAlaAlaGlyValAspGlyProAspGlyValTyrGlyPheValGlyAspGlyIleArgSer
 GACTTCCAGCCGATGAATATGGCAGCGGCTGACGATGGGCAATCCGACCGACCTCAACACGCGCGGAGGACCGATTTCGATCCAGC 1620
 AspPheGlnProMetAsnTyrGlySerGlyLeuThrMetGlyAsnProThrAspLeuAsnThrAlaAlaGlyThrAspPheAspProSer
 CCGGACAGAACCCGCGGGCTTCCAGTCTTATCGCACTACGTCATGCCGGGGGACGTTGGTTGAATCGTTTCATCGACCGGTGGAAAAC 1710
 ProAspGlnAsnProArgAlaPheGlnSerTyrSerHisTyrValMetProGlyGlyLeuValGluSerPheIleAspThrValGluAsn
 CGTCGCGGGGTACCTTGGCGCCACGGTCCGGTGGCATCGCCCAAGACCGTCCGCGGTGACCTGCGGTACGGCAATGGCGGCTG 1800
 ArgArgGlyGlyThrLeuAlaProThrValArgValArgIleAlaGlnAsnAlaSerAlaValAspLeuArgTyrGlyAsnGlyGlyLeu
 GGCGGTATGGCGATATTCGGCCAAACCGCGGACGTGAACATCGCCGGCTTATCCAGGATCTGTTGGCCAGCCCACTGCGGGCTG 1890
 GlyGlyTyrGlyAspIleProAlaAsnArgAlaAspValAsnIleAlaGlyPheIleGlnAspLeuPheGlyGlnProThrSerGlyLeu
 GCGCGCAGGCGTCCACCAACAATGCCAGGTGCTGGCGCAGGTTCGCCAATTCCTGAACAGTAATGACGGGGTTCGACAGCCCGCCC 1980
 AlaAlaGlnAlaSerThrAsnAsnAlaGlnValLeuAlaGlnValArgGlnPheLeuAsnGln * *
 TCCGCGGGCTGGTCTTTTCTGATATTGAT

Fig. 2. Nucleotide and deduced amino acid sequences of the *A. diazotrophicus* *lsdA* gene. The potential ribosome-binding site (SD) is in italics. The N-terminal amino acid sequence of the extracellular levansucrase determined by Edman degradation is underlined. The inverted repeat sequence downstream of the two stop codons which may serve as transcriptional terminator is indicated by arrows.

Fig. 3. Western blot analysis of *lsdA* gene expression in *E. coli*. Lanes: 1, extracellular levansucrase purified from *A. diazotrophicus* SRT4; 2, *E. coli* XL1-Blue(pUC18); 3, *E. coli* XL1-Blue(pUC18) (IPTG-induced); 4, *E. coli* XL1-Blue(pUCL528); 5, *E. coli* XL1-Blue(pUCL528) (IPTG-induced).

in good agreement with data obtained from purified extracellular levansucrase (Hernández *et al.*, 1995). Similarity searches in the SwissProt and EMBL databases revealed the highest scores for bacterial levansucrases, particularly from Gram-negative bacteria. Thus, we conclude that ORF1 (hereafter referred to as the *lsdA* gene) encodes the *A. diazotrophicus* levansucrase.

Codon usage of the *lsdA* gene

The overall G + C content of the *lsdA* gene was 63 mol %, in agreement with the high G + C percentage of *A. diazotrophicus* genomic DNA (De Ley *et al.*, 1984). The frequency of adenine and thymine in the third positions of the codons was only 18 %. Eleven codons were not found in the *A. diazotrophicus* *lsdA* gene, eight of these codons

Fig. 4. Multiple sequence alignment of levansucrases from Gram-negative bacteria: *Lsc*, *Erwinia amylovora*; *LevU*, *Zymomonas mobilis*; *LsdA*, *Acetobacter diazotrophicus*. Asterisks indicate identity and dots indicate conservative changes (S-T-A, L-V-I-M, K-R, D-E, Q-N, F-Y-W). Regions that are conserved in the three levansucrases are overlined and numbered from 1 to 10. Regions conserved in all levansucrases, including those from Gram-positive bacteria, are boxed and numbered from 1 to VII. A vertical arrow indicates the His residue in the position corresponding to the Arg-331 of *B. subtilis* levansucrase (see Discussion). Numbering refers to the processed form of the *A. diazotrophicus* levansucrase.

(TTA, CTA, TCT, TCA, AGA, AGG, ACT and CCA) are also rare in sequenced genes from various other *Acetobacter* species (Fukaya *et al.*, 1990; Saxena *et al.*, 1990; Tamaki *et al.*, 1991).

Similarity with other bacterial levansucrases

An alignment of the deduced amino acid sequence of *A. diazotrophicus* levansucrase with those published from other bacterial levansucrases showed a high degree of identity with enzymes produced by the Gram-negative bacteria *Zymomonas mobilis* (48%) (Song *et al.*, 1993) and *Erwinia amylovora* (46%) (Geier & Geider, 1993). The most similar regions were detected predominantly in the C-terminal part of the proteins (Fig. 4).

The sequence is only 28–31% identical to levansucrases from the Gram-positive bacteria *Streptococcus mutans* (31%) (Shiroza & Kuramitsu, 1988), *B. subtilis* (28%) (Steinmetz *et al.*, 1985) and *Bacillus amyloliquefaciens* (28%) (Tang *et al.*, 1990).

In the *B. subtilis* enzyme, substitution of Arg-331 in the conserved motif VII by His or Lys substantially affected the pattern of the products synthesized (Chambert & Petit-Glatron, 1991). There is a His residue at the corresponding position in the *A. diazotrophicus* levansucrase (arrow in Fig. 4). Furthermore, the *B. subtilis* levansucrase variant Arg-331 to His has catalytic properties similar to those of the *A. diazotrophicus* enzyme. Both enzymes synthesize small amounts of high-molecular-mass levan but accumulate oligofructosides (Chambert & Petit-Glatron, 1991; Hernández *et al.*, 1995). This is further evidence for an involvement of this region in levansucrase specificity.

Insertional inactivation of the *lsdA* gene in *A. diazotrophicus*

To investigate the role of *LsdA* in sucrose metabolism, the *lsdA* gene in the bacterial genome was inactivated by disruption.

Fig. 5. Southern blot analysis of insertionally disrupted *lsdA* mutants. Genomic DNA was cleaved with *SphI*. Hybridization was done, under the same conditions as in Fig. 1(b), with a 32 P-labelled 1.1 kb *NaeI* fragment downstream of the *lsdA* gene from *A. diazotrophicus*. Lanes: 1, SRT4; 2, AD3 (or AD4); 3, AD1 (or AD2).

The kanamycin-bleomycin resistance cassette (*nptII-ble*, 1.6 kb) from pUC4-KIXX was inserted into pALS5 at the *Bam*HI site of the *lsdA* coding region (Fig. 1a) to create plasmid pALS40. This plasmid was introduced by electroporation into *A. diazotrophicus* SRT4. Transformants were selected on LGIE plates containing kanamycin (120 μ g ml $^{-1}$) and were obtained at a frequency of 5×10^{-4} .

No transformants were obtained after electroporation with pUC4-KIXX, suggesting that the kanamycin-resistant transformants were the result of homologous recombination of pALS40 into the SRT4 genome. About two-thirds of the transformants had the non-mucous

colony morphology characteristic of levan-defective mutants, whereas one-third of the colonies displayed the mucous phenotype of the wild-type strain SRT4. Two colonies from each phenotype were chosen for further examination. The integration of the *nptII-ble* cassette in the transformants was confirmed by Southern hybridization. In the non-mucous mutants, designated AD1 and AD2, the *lsdA* gene was disrupted, indicating double crossover events (Fig. 5, lane 3). The mucous transformants, named AD3 and AD4, arose from single-crossover events: both the pALS40 sequence and an unaltered copy of the *lsdA* gene were present in the bacterial genome (Fig. 5, lane 2).

The *lsdA::nptII-ble* *A. diazotrophicus* non-mucous mutants AD1 and AD2 were tested for levansucrase activity and sucrose utilization in LGIE liquid medium containing sucrose as the sole carbon source. The mutants did not display levansucrase activity, and were unable to hydrolyse sucrose and consequently to use it as a carbon source for growth. These results suggest that LsdA may be responsible for all sucrose utilization by *A. diazotrophicus*.

DISCUSSION

To understand the genetic mechanism of host specificity in the symbiotic association between *A. diazotrophicus* and sucrose-rich plants, it would be useful to identify the bacterial genes involved in sucrose metabolism. Recently, we reported the isolation and enzymic properties of the *A. diazotrophicus* extracellular levansucrase. Here, we describe cloning the structural gene for the enzyme by complementation assays. To our knowledge, the levansucrase gene (*lsdA*) is the first sequenced gene encoding an *A. diazotrophicus* enzyme.

Translation of the *lsdA* gene may start either at position 201 or 325. However, only the N-terminal sequence of the deduced protein starting at the ATG at position 201 shows the characteristic features of a signal peptide (von Heijne, 1986). Assuming that the upstream ATG is the initiation codon, the *lsdA* gene encodes a precursor protein of 584 amino acids. According to von Heijne (1986), the putative signal peptidase cleavage site may be located between Ala-30 and Gln-31. However, protein sequencing of the native extracellular levansucrase suggests that the N-terminal 51 amino acids are removed. Transmembrane and periplasmic proteins in other species of the genus *Acetobacter* have long signal peptides (Inoue *et al.*, 1989; Tamaki *et al.*, 1991). However, the 51 residue sequence is longer than most signal peptides from other Gram-negative bacteria including *Acetobacter*. It is thus possible that there are two successive proteolytic cleavages during the secretion process of the enzyme, the first cleavage releasing a proprotein-like enzyme.

A long ORF completely overlaps the *lsdA* gene in the opposite orientation. Similarly, a long ORF has been found on the antisense strand of the gene encoding the 72 kDa subunit of the alcohol dehydrogenase from *Acetobacter aceti* (Inoue *et al.*, 1989). An involvement of

the antisense-strand ORF in the levansucrase gene expression of *A. diazotrophicus* cannot be ruled out.

There is a strong preference for cytosine and guanine in the third position of the *lsdA* gene codons, consistent with the high G + C content of *Acetobacter* genomic DNA (De Ley *et al.*, 1984). The codon usage in the *A. diazotrophicus lsdA* gene is similar to that of other non-regulatory genes from other species of the genus *Acetobacter* (Fukaya *et al.*, 1990; Saxena *et al.*, 1990).

No sequences similar to *E. coli* promoter consensus sequences were identified upstream from the *lsdA* gene, suggesting that the *lsdA* gene was not expressed from its own promoter in *E. coli*. Similarly, the promoters of genes from other *Acetobacter* species do not function in *E. coli* (Fukaya *et al.*, 1990; Saxena *et al.*, 1990).

Extensive sequence similarities were found among levansucrases from Gram-negative bacteria. There were conserved regions throughout the sequences although the greatest identity was in the C-terminal part of the proteins. The N-terminal conserved region W(T/S)RADA(L/M) was very close to the initiation codon of the *Z. mobilis* and *E. amylovora* levansucrases. This supports the assumption that the two enzymes are not cleaved during the transport process and are subject to a signal-peptide-independent export mechanism (Song *et al.*, 1993; Geier & Geider, 1993). This conserved region, which is absent from Gram-positive levansucrases, was also found in the N-terminal amino acid sequence of levansucrase from the Gram-negative bacterium *Pseudomonas syringae* pv. *phaseolicola* (Hettwer *et al.*, 1995). It may thus be characteristic of Gram-negative levansucrases.

In the regions of greatest similarity (Fig. 4), there are two particular differences between the *A. diazotrophicus* levansucrase and those of other Gram-negative bacteria: the substitution of Gly by Cys interrupting conserved region 5, and the presence of a 16 amino acid segment between domains 8 and 9, resulting in a longer predicted loop in the protein secondary structure.

Bacterial levansucrases catalyse transfructosylation from sucrose to water (hydrolase activity) or to levan (polymerase activity). Each type of activity may be separately modulated in the *B. subtilis* levansucrase by the single replacement of the Arg-331 (Chambert & Petit-Glatron, 1991). The substitution of this Arg with a His residue caused a reduction in the ratio of polymerase to hydrolase activity in the *B. subtilis* enzyme. Its capacity to polymerize fructose is apparently blocked at the first step, i.e. the formation of the trisaccharide kestose, whereas sucrose hydrolysis activity remains unchanged. In the Gram-negative levansucrases, a His occupies the position corresponding to Arg-331. This amino acid substitution may be a simple explanation for the relatively high levels of 1-kestose and low levels of high-molecular-mass levan synthesized by the natural levansucrase of *A. diazotrophicus* (Hernández *et al.*, 1995) and *Z. mobilis* (Crittendon & Doelle, 1993). The role of this residue could be further studied by site-directed mutagenesis. However, other domains highly conserved in the C-terminal part of the

Gram-negative enzymes may contribute to the production and accumulation of fructo-oligosaccharides.

In this study, gene disruption was found to be a feasible method for selectively mutating defined loci in *A. diazotrophicus* for functional analysis of the corresponding gene product. We constructed *A. diazotrophicus* mutants in which the *lsdA* gene was specifically disrupted. These mutants totally lost their levansucrase activity and their potential to utilize sucrose as a carbon source (Alvarez & Martinez-Drets, 1995). At the usual concentration of sucrose used in the culture medium (Hernández *et al.*, 1995), levansucrase acts more as a sucrose hydrolase than as a fructan polymerase. Therefore, we conclude that LsdA is the key enzyme in the sucrose metabolism of *A. diazotrophicus* SRT4 and is possibly involved in bacterial colonization of sucrose-rich plants.

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