The S-layer gene of *Lactobacillus helveticus* CNRZ 892: cloning, sequence and heterologous expression

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***Lactobacillus helveticus* CNRZ 892 contains a surface layer (S-layer) composed of protein monomers of 43 kDa organized in regular arrays. The gene encoding this protein (*slpH*) has been cloned in *Escherichia coli* and sequenced. *slpH* consists of 440 codons and is preceded by a ribosome-binding site (RBS) and followed by a putative ρ -independent terminator. Indeed, Northern analysis revealed that *slpH* is a monocistronic gene. The gene is preceded by a possible promoter of which the –35 and –10 hexanucleotides are separated by 17 nt. By primer extension analysis the transcription start site was mapped at 7 nt downstream of the –10 sequence while the deduced amino acid sequence of SlpH shows a leader peptide of 30 aa. The *slpH* gene has been amplified by PCR and the fragment, carrying the complete gene from the RBS to the stop codon, has been cloned in a lactococcal gene expression vector downstream of promoter P32. *Lactococcus lactis* MG1363 carrying the resulting plasmid produced and secreted an S-layer monomer with the same molecular mass as the authentic *L. helveticus* CNRZ 892 SlpH, as judged by SDS-PAGE. Immunoelectron microscopy revealed that SlpH was bound to the lactococcal cell walls in small clumps and accumulated in the growth medium as small sheets.**

Keywords: S-layer, *Lactobacillus helveticus*, cloning, heterologous expression

INTRODUCTION

Surface layers (S-layers) composed of protein monomers arranged in crystalline arrays have been described on *Lactobacillus* cell walls (Messner & Sleytr, 1992; Lortal, 1993). Monomeric subunits of *Lactobacillus* S-layers have been successfully isolated by extraction with urea (Masuda & Kawata, 1979), guanidine HCl (Masuda & Kawata, 1980), SDS (Bhowmik *et al.*, 1985) and LiCl (Callegari *et al.*, 1990; Lortal *et al.*, 1992). The amino acid composition of *Lactobacillus* S-layers has been determined by chemical methods (Masuda & Kawata, 1992) and has revealed the presence of a high proportion of hydrophobic amino acids. The molecular mass of S-layer monomers isolated from lactobacilli was found to range from 40 to 60 kDa. Hexagonal, square and oblique

symmetries have been observed in the ultrastructures of S-layers (Messner & Sleytr, 1992). Upon removal of agents used for their isolation, S-layer monomers extracted from *Lactobacillus brevis* (Masuda & Kawata, 1980) and *Lactobacillus helveticus* (Lortal, 1993) have been shown to be able to spontaneously reassemble into crystalline arrays with the same characteristics as those observed in intact cells.

The functional properties of S-layers of lactobacilli have not yet been elucidated; it appears that freeze-drying can induce the detachment of S-layer monomers from the cell wall of *Lactobacillus acidophilus* but structural damage is not related to loss of cell viability (Ray & Johnson, 1986). Resistance to lysozyme treatment has been related to the presence of an S-layer as the outermost envelope of *L. helveticus* ATCC 12046 (Lortal *et al.*, 1992). A positive relationship has also been suggested between the ability to adhere to chicken epithelia and the presence of an S-layer in an avian isolate of *L. acidophilus* (Schneitz *et al.*, 1993). We have

The EMBL/GenBank accession numbers for the nucleotide sequences reported in this paper are X91199 (ORF of *slpH*) and AJ001931 (promoter region of *slpH*).

suggested a role for the S-layer of *L. helveticus* CNRZ 892 as receptor for the virulent bacteriophage CNRZ 832-B1 (Callegari *et al.*, 1990).

The deduced amino acid sequences of S-layer monomers of *L. brevis* ATCC 8287 (Vidgren *et al.*, 1992) and *L. acidophilus* ATCC 4356 (Boot *et al.*, 1993) show both proteins to be positively charged (deduced isoelectric points of 9.37 and 9.40, respectively) with limited (35.7%) amino acid sequence similarity. From the latter strain an additional silent gene sharing extensive homology with the active gene of the same strain has been identified, cloned and sequenced (Boot *et al.*, 1995). With antisera raised against the *Lactobacillus buchneri* ATCC 4005 S-layer protein it was shown that S-layer monomers isolated from lactobacilli belonging to different species are not immunologically related (Masuda & Kawata, 1983). Nevertheless, the N-terminal amino acid sequence of the *L. helveticus* ATCC 12046 S-layer protein (Lortal, 1993) and the deduced N-terminal amino acid sequence of the *L. acidophilus* ATCC 4356 protein turned out to be nearly identical (Boot *et al.*, 1993). These two species belong to the obligatory homofermentative group of *Lactobacillus* and have been shown to be phylogenetically related by DNA-DNA hybridization data and rRNA sequences analysis (Kandler & Weiss, 1986).

Here we present the cloning and nucleotide sequence of the gene encoding the S-layer protein of *L. helveticus* CNRZ 892 and its expression in the Gram-positive heterologous host *Lactococcus lactis*.

METHODS

Bacterial strains and culture conditions. *L. helveticus* CNRZ 892 (ATCC 10386) and ATCC 12046 were propagated in MRS (Oxoid) broth or agar at 42 °C. *Escherichia coli* strains TB1 and LE 392 were cultivated at 37 °C in LB broth (Sambrook *et al.*, 1989). *Lactococcus lactis* MG1363 was grown at 30 °C in M17 (Oxoid). Ampicillin was used at 100 µg ml⁻¹ for *E. coli*. Erythromycin was used at 5 µg ml⁻¹ for *L. lactis*.

Purification of S-layer protein and preparation of specific antibodies. S-layer protein (SlpH) of *L. helveticus* CNRZ 892 and ATCC 12046 was purified following procedures based on

a 6 M LiCl extraction as described previously (Callegari *et al.*, 1990). The extraction procedure was repeated until SDS-PAGE (Laemmli, 1970) revealed a single band of SlpH monomers stainable with Coomassie brilliant blue. Purified SlpH was used to obtain a rabbit antiserum (kindly provided by Centro Zooprofilattico, Brescia, Italy).

DNA techniques, transformation and nucleotide sequence analysis. General DNA techniques were performed as described by Sambrook *et al.* (1989). All DNA modification enzymes were purchased from Boehringer Mannheim. Genomic DNA of *L. helveticus* CNRZ 892 was isolated following the procedure described by Lewington *et al.* (1987). Chromosomal DNA of *L. helveticus* CNRZ 892 was partially digested with *Sau3A*I and cloned in λGEM-11 *Xho*I half site arms (Promega). The Packagene *in vitro* Packaging System (Promega) was used for *in vitro* packaging of recombinant phages. *E. coli* and *L. lactis* were electrotransformed as described by Dower *et al.* (1988) and Holo & Nes (1989), respectively. Nucleotide sequencing was performed on the Applied Biosystems 373A automated DNA sequencer using the ABI PRISM Dye Terminator Cycle Sequencing and Dye Primer Cycle Sequencing kits (Perkin Elmer).

The Genetic Computer Group sequence analysis package (University of Wisconsin) was used to assemble and analyse the sequences. Nucleotide and predicted amino acid sequences were compared to those in the databases (GenBank, Release 50.0; SWISS-PROT, Release 33.0; Prosite, Release 13.0) using the FASTA (Pearson & Lipman, 1988) and BLAST (Altschul *et al.*, 1990) programs.

Oligonucleotides and PCR. Oligonucleotides used in this work are listed in Table 1. They were synthesized with an Applied Biosystems model 391 DNA synthesizer. Primer 1 was based on the N-terminal amino acid sequence of *L. helveticus* ATCC 12046 S-layer protein (Lortal *et al.*, 1992). Inverse PCR with primers 2 and 3 on *Asn*I-digested and self-ligated chromosomal DNA of *L. helveticus* CNRZ 892 (20 ng) was done using 25 cycles of 1 min at 94 °C for denaturation, 1 min at 50 °C for annealing and 2 min at 72 °C for extension. Prior to PCR the *Asn*I fragments of the proper size were recovered from an agarose gel using GeneClean (Bio-101). Primers 6 and 7 were used to amplify a chromosomal DNA fragment containing *slpH* devoid of its promoter by using 25 cycles of 1 min at 94 °C, 1 min at 45 °C and 2 min at 72 °C. *Taq* polymerase (Boehringer Mannheim) was used for all amplification reactions. The fragment resulting from this PCR procedure was digested with *Sph*I and *Sal*I (these two

Table 1. Oligonucleotides used as primers in PCR reactions or probes in this study

Primer	Sequence*	Position from AUG start codon
1	GCA/T ACT/A ACT/A ATT/C AAT/C GCA/T GAT/C AGT/C GCA/T ATT/C AAT/C	90 to 123
2	AAC ATT ACA TCT GTG GTA GC	662 to 681
3	CTT AAT TTG TAC ACT TGA AAG C	522 to 544
4	GGT GCA ACA GCT AAT AAA GCA GCA GCA GCA GC	27 to 59
5	GTA ATA GGC CAT AGG TTC AGC ATG AGA	-59 to -32
6	CAG ATG ATA TCG CAT GCT TAT TCA AAG TTA GCA ACC TTA AC	1297 to 1320
7	AAC GCG TCG ACA TGC ATC ATT ATA GGC TCC TTT CTC ATG	-74 to -52
8	GCC TTG CCG TTT TCG ATT AC	1070 to 1090

* The sequences in bold are not present in the *slpH* gene but they were added in designing primers to introduce useful restriction sites at both ends of the PCR products.

restriction sites were added to the 5' end of primers 6 and 7, respectively) and ligated to the expression vector pMG36e (van de Guchte *et al.*, 1989) cut with *Sall* and *SphI*. The resulting construct, pMC01, was recovered after electrotransformation of *L. lactis* MG1363.

Southern blotting and hybridization. DNA was transferred from agarose gels to a positively charged nylon membrane (Boehringer Mannheim) using the method of Southern (1975). Hybridization and probe detection were done using DIG-labelled oligonucleotides according to the instructions of the manufacturer (Boehringer Mannheim).

RNA methods. Total RNA was isolated from 200 ml of a culture of *L. helveticus* CNRZ 892 grown to exponential phase (OD_{600} 0.5). Cells were collected, washed with TE buffer (pH 8) and resuspended in 3 ml of the same buffer. The cell suspension was dispensed in six tubes (0.5 ml per tube). After addition of 170 μ l 2% macaloid, 500 μ l phenol/chloroform (24:1), 50 μ l 10% SDS and 0.8 g glass beads, cells were disrupted using a Shake-it-baby (Biospec Products). Glass beads were collected by centrifugation at 12000 *g* for 15 min. After two extractions with phenol/chloroform (24:1) RNA was precipitated by addition of 0.1 vols 2 M NaCl and 2.5 vols ethanol and collected by centrifugation at 17000 *g* for 12 min.

The pellet was dissolved in 20 mM EDTA (pH 8) and the RNA was precipitated with 2 vols 4.5 M sodium acetate (pH 7). RNA was pelleted by centrifugation (17000 *g* for 10 min), washed twice with ethanol (70%) and dissolved in distilled water. RNA gel electrophoresis, Northern blotting, labelling of primers and primer extension were performed as described by Sambrook *et al.* (1989). The lengths of the primer extension products were determined by comparison with the products of a sequencing reaction using primer 5 and an *E. coli* clone containing the inverse PCR product as template.

SDS-PAGE and Western analysis. Cells from 1 ml of an overnight culture of *L. lactis* were collected by centrifugation and resuspended in 100 μ l water and 20 μ l solubilizing solution (10% β -mercaptoethanol, 10% glycerol, 4% SDS, 500 mM Tris/HCl, pH 8, 0.02 M EDTA, pH 8, 10% bromophenol blue). The sample was boiled for 5 min and centrifuged. The supernatant was examined by SDS-PAGE according to Laemmli (1970) using a stacking gel containing 3% acrylamide and a 12% running gel. The supernatant of the bacterial culture was precipitated with TCA as described by Séchaud *et al.* (1989). The pellet obtained was resuspended in distilled water and then analysed by SDS-PAGE.

S-layer proteins extracted by the same procedure from cells and supernatants of strain CNRZ 892 and ATCC 12046 were used as positive controls. After electrophoresis proteins on the gels were electroblotted onto nitrocellulose membrane (Bio-Rad). Anti-SlpH serum was diluted 1:10000. Goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma) was used as a secondary antibody to detect the S-layer protein antigen.

Immunogold labelling and electron microscopy. Cells from 1 ml of a culture of *L. lactis* were collected by centrifugation and resuspended in 300 μ l blocking solution (1% BSA in PBS) containing 10 μ l of a 1:1000 dilution of anti-SlpH serum. After two washings in PBS, 500 μ l of a 1:100 dilution of a 5 nm gold-labelled anti-rabbit IgG (Sigma) in blocking solution were added. After 10 min of incubation, cells were washed in PBS, prefixed with 2.5% glutaraldehyde and fixed with 1% OsO_4 (Lortal *et al.*, 1991). The samples were embedded in Epon

resin (Fluka) and thin sections (50 nm) were prepared using a Reichert-Jung Ultracut microtome (Optische Werke). The sections were stained with 4% uranyl acetate and then with 0.4% lead citrate and examined with a JEOL JEE 1200 EXII electron microscope at 80 kV.

RESULTS

Purification of the S-layer protein of *L. helveticus* CNRZ 892

LiCl extraction is a generally employed method to isolate S-layer proteins from *L. helveticus* (Callegari *et al.*, 1990; Lortal *et al.*, 1992). Sequential extraction with a concentrated (6 M) LiCl solution was used to purify a single protein from *L. helveticus* CNRZ 892 as shown by SDS-PAGE and staining with Coomassie brilliant blue (Fig. 1a) as well as with silver staining (data not shown). The molecular mass of this protein, SlpH, was estimated by SDS-PAGE to be close to 43 kDa. Rabbit antiserum raised against the protein cross-reacted with the S-layer protein of *L. helveticus* ATCC 12046, suggesting that there is some homology between the two proteins (Fig. 1b).

Cloning of *slpH* gene of *L. helveticus* CNRZ 892

Oligonucleotide 1 (Table 1), based on the N-terminal amino acid sequence of the S-layer protein of *L. helveticus* ATCC 12046 (Lortal *et al.*, 1992), hybridized with a *Sau3AI* partial digest of chromosomal DNA of *L. helveticus* CNRZ 892. A genomic library of *L. helveticus* CNRZ 892 in λ GEM-11 (4×10^4 recombinant phages)

Fig. 1. (a) PAGE (14% acrylamide) of S-layer monomers purified from *L. helveticus* CNRZ 892. The gel is stained with Coomassie brilliant blue. (b) Western immunoblot analysis of S-layer proteins extracted from strains *L. helveticus* CNRZ 892 (lane A) and ATCC 12046 (lane B) using serum raised against SlpH. Bio-Rad low molecular mass markers were used as reference.

Fig. 2. (a) Northern hybridization analysis. Total *L. helveticus* CNRZ 892 RNA (20 µg) was run on a 1% agarose gel, blotted onto a positively charged nylon membrane and hybridized with ³²P-labelled primer 5 (Table 1) as probe. RNA molecular mass markers were used to determine transcript size. (b) Results of a primer extension experiment done with 15 µg total RNA isolated from *L. helveticus* CNRZ 892 using primer 5 (Table 1) as a start point for the extension. Lanes: 1, extended product; GATC, partial sequence of the putative promoter region using the same primer. The asterisk shows the start point of *slpH*.

was screened using the rabbit antiserum raised against SlpH. Four positive phages were identified and all four shared a 6.3 kb *SalI* fragment (data not shown). A DNA fragment of this size also reacted in Southern hybridizations of chromosomal DNA cut with *SalI* using oligonucleotide 1 as probe (data not shown). The common *SalI* fragment gave four fragments (3.7, 1.6, 0.5 and 0.4 kb) when digested with *HindIII*. These fragments were ligated to pUC18 cut with *HindIII* alone or in combination with *SalI*. Two different *HindIII* clones carried fragments of 526 and 435 bp which were sequenced completely. The nucleotide sequences of these two fragments showed a high degree of homology to the 3' end of the *L. acidophilus* ATCC 4356 *slpA* gene (Boot *et al.*, 1993) with the 435 bp fragment containing the 3' end of *slpH* and the other fragment carrying sequences immediately upstream thereof (see below). All attempts to clone the 1.6 kb fragment, positive with oligonucleotide 1 in Southern hybridization, failed. Also, the production of SlpH by the recombinant phages was soon lost, indicating poor stability of the *slpH* gene in this vector. All attempts to clone the 5' end directly from chromosomal DNA fragments of *L. helveticus* CNRZ 892 failed. A detailed restriction enzyme map was made of the chromosomal region around *slpH* and by deduction it was concluded that an *AsnI* fragment would contain the start of *slpH* with its promoter. Two primers (2 and 3 in Table 1) were designed to amplify this fragment by inverse PCR. The PCR products of three independent amplification reactions were cloned in pGEM-T in *E. coli* and used to complete the nucleotide sequence of *slpH*. All PCR-amplified fragments were checked by nucleotide sequence analysis and no mistakes and/or rearrangements were detected. To verify that there is no sequence gap between the two *HindIII* fragments, a PCR amplification product, obtained with primers 3 and 8, was directly sequenced using the same primers. The sequence analysis confirmed that the two fragments were contiguous and shared a *HindIII* site.

slpH sequence analysis

The ORF of *slpH* contains 1320 nt and is preceded by a putative ribosome-binding site (AGGAGG) located 9 nt upstream of the AUG start codon. It is followed by a single stop codon (UAA) and a possible *p*-independent transcription terminator. *slpH* contains 12 direct repeated sequences ranging from 7 to 16 nt. Computer analysis suggested the presence of putative -10 (TAAAT) and -35 (TTGCTA) boxes upstream of the AUG start codon. The functionality of this putative promoter was confirmed by primer extension analysis (see below).

The deduced amino acid sequence from residues 31 to 42 of SlpH of *L. helveticus* CNRZ 892 (ATTINADSAINA) is identical to the N-terminal amino acid sequence determined from the purified S-layer protein monomer of *L. helveticus* ATCC 12046 (Lortal *et al.*, 1992). This indicates that *L. helveticus* SlpH has a signal peptide of 30 aa. This 30 aa N terminus complies with the rules of

Fig. 3. Immunoblotting analysis of *Lc. lactis* MC01 proteins extracted from 3 ml of supernatant (lane 2) and from 1 ml of cells taken from an overnight culture (lane 4). As negative control, proteins recovered by TCA treatment from 3 ml of supernatant of *Lc. lactis* MG1363 were loaded in lane 1, while lane 3 contains protein extracted from 1 ml of cells of the same strain. In lane 5 proteins extracted from the positive control, *L. helveticus* CNRZ 892, are visible. Bio-Rad low molecular mass markers were used as reference.

von Heijne (1986) for genuine prokaryotic signal sequences. The predicted cleavage site is located between alanine residues 30 and 31. The deduced molecular mass of mature SlpH is 43.1 kDa, which is in accordance with the molecular mass of 43 kDa as determined by SDS-PAGE. The deduced isoelectric point is 10.19.

Analysis of mRNA of *slpH*

Primer 5 is located 31 bp upstream of the AUG start codon of *slpH* and was used as probe in Northern hybridization. A 1.5 kb transcript was detected, indicating that *slpH* is a monocistronic gene (Fig. 2a). Primer extension using primer 5 revealed a major transcription start point (Fig. 2b) located at a G residue 190 nt upstream of the AUG start codon. The coding region is preceded, at 198 nt from the AUG start codon and at 7 nt from the transcription start point, by a possible -10 sequence (TAAAAT). A -35 sequence (TTGCTA) is located 17 nt upstream of the -10 sequence. A similar promoter structure has been found upstream of *slpA* of *L. acidophilus* ATCC 4356 (Boot *et al.*, 1993).

Cloning and expression of the S-layer protein in *Lc. lactis* MG1363

As the entire *slpH* gene was unstable in *E. coli*, we decided to clone the gene behind another promoter in *Lc. lactis*. To do this a fragment carrying the complete gene from putative RBS up to the stop codon was amplified by PCR using primers 7 and 6 (from position -74 to -52 and from 1297 to 1320, respectively). The resulting 1390 bp fragment was digested with *SalI* and *SphI* and ligated into the lactococcal expression vector

pMG36e (van de Guchte *et al.*, 1989) cut with the same enzymes. Thus, *slpH* was placed under control of the strong lactococcal promoter P32 present in the vector. The ligation mixture was used to electrotransform *Lc. lactis* MG1363. Transformants were selected for erythromycin resistance and on the basis of a positive reaction in Western blotting with anti-SlpH antiserum. One transformant, carrying the expected pMC01, produced SlpH with a molecular size by SDS-PAGE similar to that of SlpH isolated from *L. helveticus* CNRZ 892 and was selected for further characterization (Fig. 3). The S-layer protein could be precipitated from the culture supernatant of *Lc. lactis*(pMC01) with TCA and was present in boiled samples of these cells. Electron microscopy revealed that the protein is shed from the surface of the cells and accumulates in the growth medium (Fig. 4a). Immunogold labelling indicated the presence of few sites on the lactococcal cell walls where small clumps of SlpH are visible (Fig. 4b). A large proportion of the lactococcal cells are bound to each other by what seem to be bridges of SlpH protein.

DISCUSSION

Our results indicate that the amino acid sequence of SlpH is highly similar to that determined for the S-layer protein SlpA of *L. acidophilus* ATCC 4356 (Boot *et al.*, 1993). Similarity to *L. acidophilus* SlpA is particularly evident in the C-terminal region (from aa 193 to 438) while the middle region shows a lower degree of similarity with the corresponding region of SlpA.

Our failure to clone the entire gene of SlpH in *E. coli* resembles the problems observed in cloning of other S-layer protein genes (Boot *et al.*, 1993, 1995; Vidgren *et al.*, 1992; Yamagata *et al.*, 1987). Instability of *L. acidophilus* *slpA* was postulated to be related to the presence of several direct repeats (Boot *et al.*, 1995). Similar observations have already been described in *Bacillus stearothermophilus* and *Aeromonas salmonicida* (Kuen *et al.*, 1994; Chu *et al.*, 1991). In contrast we faced no problem in cloning in *E. coli* the promoter and the 5' end of *slpH* and the entire coding region from the RBS to the stop codon.

The *slpH* gene was stably maintained in the Gram-positive host *Lc. lactis* MG1363. The recombinant clone (MC01) carrying *slpH* under control of P32 had a reduced growth rate in M17 compared to the wild-type strain. Moreover, small 'sand-like' particles were formed by aggregation of cells at the end of the exponential growth phase, that precipitated and left the supernatant completely clear. MC01 produced a protein of the expected size as observed by immunological detection using SlpH antiserum. *Lc. lactis* MG1363 did not give a signal in immunoblotting even when the amount of examined protein was very high. Immunoelectron microscopy revealed that gold-labelled, SlpH-specific antiserum recognized regularly ordered protein aggregates that were not bound to the lactococcal cell surfaces. Proteins reacting with the SlpH antiserum were not present on the *Lc. lactis* MC01 surface except for

Fig. 4. (a), (b) and (d) Transmission electron microscopy observations of *Lc. lactis* MC01 thin sections. Cells were treated with anti-SlpH serum and then with 5 nm gold-labelled anti-rabbit IgG. Thin sections were stained with 4% uranyl acetate and with 0.4% lead citrate. (c) *Lc. lactis* MG1363 cells were used as control. Bars, 100 nm.

clumps localized in small spots on each cell (Fig. 4b). The real significance of these aggregation sites has to be investigated further. Moreover, cells were attached to each other by protrusions containing SlpH antigens (Fig. 4d). This observation could explain the sand-like aggregations observed during growth of *Lc. lactis* MC01 in broth medium.

A different composition of lactococcal cell walls could explain the observed inability of the cloned S-layer protein to form crystalline arrays on the surface of the new bacterial host: cloning and expression studies of the same construction in a range of lactic acid bacteria, including more closely related lactobacilli, are planned to study reattachment of the protein to heterologous hosts.

The results presented here allow us to investigate more deeply the role played by SlpH in the binding process of the lytic phage CNRZ 832-B1 (Callegari *et al.*, 1990). Spontaneously arising phage-resistant mutants, unable to bind phage particles, are already available (Callegari *et al.*, 1990) and the nucleotide sequences of their *slpH* genes will be checked to identify mutations possibly involved in the blocking of phage absorption.

ACKNOWLEDGEMENTS

This work was supported by grants from the European Union BIOTECH (contracts BIO2-CT94-3055 and BIO4-CT96-0402 STARLAB).

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Received 28 July 1997; revised 13 October 1997; accepted 20 October 1997.