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ORIGINAL PAPER

Heterogeneity of S-layer proteins from aggregating and non-aggregating *Lactobacillus kefir* strains

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Abstract Since the presence of S-layer protein conditioned the autoaggregation capacity of some strains of *Lactobacillus kefir*, S-layer proteins from aggregating and non-aggregating *L. kefir* strains were characterized by immunochemical reactivity, MALDI-TOF spectrometry and glycosylation analysis. Two anti-S-layer monoclonal antibodies (Mab5F8 and Mab1F8) were produced; in an indirect enzyme-linked immunosorbent assay Mab1F8 recognized S-layer proteins from all *L. kefir* tested while Mab5F8 recognized only S-layer proteins from aggregating strains. Periodic Acid-Schiff staining of proteins after polyacrylamide gel electrophoresis under denaturing conditions revealed that all *L. kefir* S-layer proteins tested were glycosylated. Growth of bacteria in the presence of the N-glycosylation inhibitor tunicamycin suggested the presence of glycosydic chains O-linked to the protein backbone. MALDI-TOF peptide map fingerprint for S-layer proteins from 12 *L. kefir* strains showed very similar patterns for the aggregating strains, different from those for the non-aggregating ones. No positive match with other protein spectra in MSDB Database was found. Our results revealed a high heterogeneity among S-layer proteins from different *L. kefir* strains but also suggested a correlation between the structure of these S-layer glycoproteins and the aggregation properties of whole bacterial cells.

Keywords Glycosylation · Immunochemistry · *Lactobacillus kefir* · S-layer · Spectrometry

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Introduction

Surface layers (S-layers) are planar arrays of proteinaceus or glycoproteinaceus subunits ranging in molecular mass (Mr) from 40 to 200 kDa which can be aligned in unit cells of different symmetries on the outermost surface of many prokaryotic microorganisms. S-layers were relatively unknown three decades ago but, at this time, S-layer proteins have been identified on over 250 species from Bacteria and Archaea domains (Sára and Sleytr 2000).

S-layers have been considered to function as protective coats, cell shape determinants, molecular

sieves, ion traps, and structures involved in cell surface interactions (Schneitz et al. 1993; Antikainen et al. 2002; Horie et al. 2002; De Leeuw et al. 2006). In some pathogenic microorganisms, S-layer contributes to virulence (Blaser and Pei 1993; Sabet et al. 2003; Sakakibara et al. 2007). On the other hand, S-layer proteins have shown to be related with anti-pathogenic activity of some probiotic bacteria (Horie et al. 2002; Johnson-Henry et al. 2007; Golowczyc et al. 2007).

Presence of S-layer has been demonstrated in many species of genus *Lactobacillus*, but, to date, only 21 S-layer protein encoding genes have been cloned and sequenced, showing an overall lack of identity between different species. Although glycosylation is commonly found in S-layer proteins, most of S-layers from lactobacilli appear to be non-glycosylated (Avall-Jääskeläinen and Palva 2005), and to date a detailed glycan structure has been reported only for S-layer from *Lactobacillus buchneri* (Möschl et al. 1993).

Different strains of *Lactobacillus kefir* isolated from kefir grains have been identified and characterized using phenotypic and nucleic acid based methods (Garrote et al. 2001; Delfederico et al. 2006). These strains showed differences in surface properties and inhibitory power against intestinal pathogens in vitro (Golowczyc et al. 2007, 2009). Recently we described the presence of S-layer on the surface of these *L. kefir* strains and we investigated the relationship between the presence of S-layer proteins and surface properties (e.g., adhesion to Caco-2 cells, autoaggregation and hemagglutination) (Garrote et al. 2004). Additionally, we observed that S-layer proteins from two different strains of *L. kefir* were capable to exert a protective action against invasion of *Salmonella enterica* serovar Enteritidis to Caco-2 cells in vitro (Golowczyc et al. 2007). In the present study we performed a characterization of S-layer proteins from *L. kefir* strains with different aggregation properties in terms of their immunochemical reactivity, peptide fragmentation pattern and glycosylation.

Materials and methods

Bacterial strains and culture conditions

Lactobacillus kefir CIDCA 83111, 83113, 83115, 8321, 8325, 8335, 8344, 8345, 8347 and 8348 isolated from kefir grains (Garrote et al. 2001), *L. kefir* JCM 5818 and *Lactobacillus brevis* JCM 1059 obtained from the Japanese Collection of Micro-organisms (Reiken, Japan), and *L. kefir* ATCC 8007 and *L. brevis* ATCC 8287 obtained from American Type Culture Collection (Manassas, VA, USA) were used to obtain S-layer proteins. Bacteria were cultured in MRS broth (Biokar Diagnostics, Beauvais, France) at 30°C for 48 h, under aerobic conditions.

S-layer protein extraction

S-layer protein extraction from bacterial cells was performed using LiCl 5 M as described before (Golowczyc et al. 2007). The homogeneity of S-layer extracts was tested by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (1970). Gels were migrated on a BioRad Mini-Protean II (BioRad Laboratories, Richmond, CA, USA), with LMW-SDS Marker kit (GE Healthcare, Sweden) as a Mr reference.

Autoaggregation assays

Aggregation capacity of *L. kefir* strains before and after S-layer protein extraction was determined as previously described by Golowczyc et al. (2007). Briefly bacterial cells were resuspended in PBS (pH 7.0) to an OD 550 nm = 1.0 and optical density was measured at regular intervals without disturbing the suspension.

Monoclonal antibodies production

Monoclonal antibodies were derived by somatic cell hybridization as described by Galfré and Milstein (1981) using polyethylene glycol as fusogenic agent. Briefly, BALB/c mice were immunized by three intraperitoneal injections (separated every 15 days) with a LiCl-extract of S-layer from *L. kefir* CIDCA 8348 emulsified with incomplete Freund's adjuvant (30–40 μg of protein per mouse). NSO myeloma cells were fused with spleen cells from immunized mice and the resulting hybridomas were cloned by limiting dilution. Anti—S-layer reactivity was tested by indirect enzyme-linked immunosorbent assay (ELISA) with S-layer protein from *L. kefir* CIDCA 8348 as antigen.

Indirect ELISA

Polystyrene microtitre plates (Microlon, Greiner, Denmark) were coated with S-layer extracts from

different *L. kefir* strains diluted in carbonate buffer (pH 9.0) at 0.1 μg per well. Incubation was carried out at 37°C for 1 h. Then wells were washed three times with 200 μl of PBS plus 0.05% (v/v) Tween 20 (PBS-T) and incubated with 3% non-fat dry milk dissolved in PBS at 37°C for 2 h. The plates were washed three times with PBS-T and then 100 μl of hybridoma supernatant culture medium were applied to each well and incubated at 37°C for 2 h. Washing was repeated as described above, and then 100 μl of the peroxidase-labeled secondary antibody (horseradish peroxidase-conjugated goat anti-mouse antibody; BioRad Laboratories) diluted 1:2000 was added to each well. The plates were incubated at 37°C for 1 h. After another cycle of washing, the reaction was visualized by adding a solution containing 2 mg ml^{-1} *o*-phenylenediamine (Sigma) and 1 μl ml^{-1} 30% H_2O_2 (Merck) in 0.1 M citrate-phosphate buffer, pH 5.0. The reaction was stopped by the addition of 50 μl of 4 N sulphuric acid per well. Absorbance was determined at 492 nm in a Tecan SpectraRainbow microplate reader.

Periodic acid—schiff (PAS) reaction

Each well of a discontinuous denaturing acrylamide gel was charged with 15 μl of S-layer protein extract (ca. 3.0–4.5 μg of protein). SDS–PAGE was performed as described above and then S-layer proteins were fixed soaking gels in a mixture of 10% acetic acid and 35% methanol in water for 60 min. Then gels were treated with 0.7% $NaIO_4$ in 5% acetic acid for 30 min. After that, gels were treated with 20 ml of Schiff's reagent until magenta bands appeared and then with 0.2% $Na_2S_2O_5$ in 5% acetic acid for 60 min. S-layer proteins from *L. brevis* JCM 1059 were used as non-glycosylated control (Yasui et al. 1995).

Tunicamycin treatment

Lactobacillus kefir strains CIDCA 8348, CIDCA 8344 and JCM 5818 were subcultured twice in MRS broth with tunicamycin (0, 5, 10, 20 and 50 μg ml^{-1}) (Sigma) at 30°C for 48 h under aerobic conditions, before S-layer extraction and examination by SDS–PAGE and PAS staining were performed as described above.

Mass spectrometry

In-gel protein digestion

S-layer extracts were subjected to SDS–PAGE and then the protein bands of interest were excised using a fresh scalpel and diced into 1 mm^3 pieces. Gel pieces were further destained in 3 × 100 μl of 25 mM NH_4HCO_3, 5% Acetonitrile (ACN) (pH 9.0), then washed with 100 μl of 25 mM NH_4HCO_3, 50% ACN (pH 9.0) and finally with 100 μl of ACN. Proteins were treated in gel with 100 μl 10 mM DTT in 25 mM NH_4HCO_3, 5% ACN at room temperature for 1 h and then with 50 mM iodoacetamide in 25 mM NH_4HCO_3, 5% ACN at 37°C for 1 h to achieve reduction and alkylation of the sulphydriles from cysteines. Another washing process was performed before the enzymatic digestion of the proteins. Trypsin (Promega Trypsin Gold, TPCK treated) in 25 mM NH_4HCO_3, 5% ACN was added to the gel pieces at an approximate trypsin to protein ratio of 1:20, and the enzymatic reaction was carried out at 37°C for 3 h. Peptides were extracted from the gel pieces in three steps with 100 μl of 0.2% TFA each and finally with 15 μl of 50% ACN, 0.1% TFA. Supernatants were dried with a Speed-Vac™ and then the peptides were suspended in 5 μl of 50% ACN, 0.1% TFA. All assays were realized in ZipPlate™ micro-SPE Plate with a MultiScreen™ Vacuum Manifold 96-well (MILLIPORE, Billerica, MA).

MALDI-TOF-MS analysis

MALDI-TOF-MS analyzes were used to determine protein identities by peptide mass fingerprint (PMF). Analysis was performed using an UltraFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) at the SePBio-IBB Proteomics and Bioinformatics Service (UAB, Barcelona, Spain). Samples were spotted on a GroundSteel target (Bruker Daltonics) mixing 0.5 μl of each sample with 1 μl of freshly prepared matrix solution of 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) in a 30% ACN and 0.1% TFA aqueous solution. External calibration was performed using peptide calibrants (Bruker Daltonics). Peptide masses were acquired within a range of ca. *m/z* 800–4000.

Database search parameters

Singly charged monoisotopic peptide lists were generated and used as inputs for database searching using flex Analysis software (Bruker Daltonics), after external and internal calibration of spectra. Searches were performed against MSDB database using MASCOT PMF database search software (www.matrixscience.com). The oxidation of methionine was included as possible modification as well as the alkylation of cysteines when appropriate. Up to two missed tryptic cleavages were considered, and the mass tolerance for the monoisotopic peptide masses was set to 100 ppm. Comprehensive peak assignments were accomplished using BioTools software (Bruker Daltonics).

Results

Aggregation assays

Aggregative capacity of some *L. kefir* strains that was previously reported by Golowczyc et al. (2007) was confirmed in the present study. We found aggregation percentages in 60 min ranging from 29 to 59% for strains CIDCA 83115, CIDCA 8321, CIDCA 8325, CIDCA 8345, CIDCA 8347 and CIDCA 8348, and of less than 1% for strains CIDCA 83111, CIDCA 83113, CIDCA 8335, CIDCA 8344, JCM 5818 and ATCC 8007. Aggregating strains completely lost this capacity after extraction of S-layer proteins with 5 M LiCl, while no changes were observed in non-aggregating ones (data not shown).

Immunochemical characterization of S-layer proteins

In order to characterize the immunoreactivity of surface proteins from different *L. kefir* strains, we obtained monoclonal antibodies (Mabs) against S-layer proteins from *L. kefir* CIDCA 8348. Two hybridomas secreting specific antibodies (Mabs 1F8 and 5F8) were selected. Both Mabs showed specificity at a species level in an indirect ELISA, since they did not react with S-layer proteins from *L. brevis* JCM 1059 and ATCC 8287. Mab 1F8 showed high reactivity against all the *L. kefir* S-layer proteins analyzed while Mab 5F8 recognized only S-layer proteins from aggregating *L. kefir* strains (CIDCA 83115, 8321, 8325, 8345, 8347 and 8348) (Fig. 1).

Glycosylation analysis

PAS staining

Since glycosylation is one of the most commonly reported post-translational modifications in S-layer proteins, we performed a PAS staining of S-layer extracts previously subjected to SDS–PAGE. This assay revealed that all the S-layer proteins from *L. kefir* analyzed were glycosylated (Fig. 2). S-layer protein from *L. brevis* JCM 1059 was not stained, as

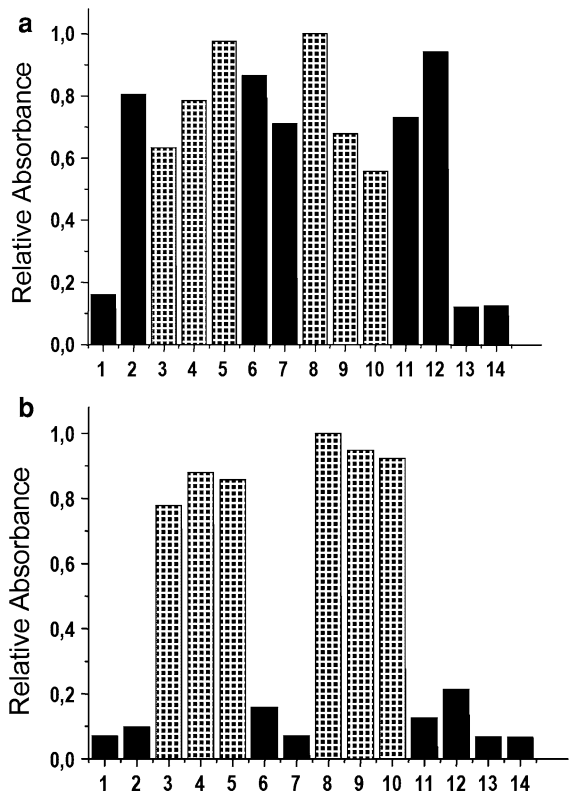

Fig. 1 Anti-S-layer reactivity of monoclonal antibodies (Mab) present in hybridoma culture supernatants determined by indirect ELISA. Reactivity of Mab 1F8 (**a**) and Mab 5F8 (**b**) against S-layer proteins from aggregating (*dotted bars*) and non-aggregating (*black bars*) strains of *L. kefir* and *L. brevis* is shown. Control without antigen (*1*), *L. kefir* CIDCA 83113 (*2*), 83115 (*3*), 8321 (*4*), 8325 (*5*), 8335 (*6*), 8344 (*7*), 8345 (*8*), 8347 (*9*), 8348 (*10*), JCM 5818 (*11*) and ATCC 8007 (*12*); *L. brevis* JCM 1059 (*13*) and ATCC 8287 (*14*)

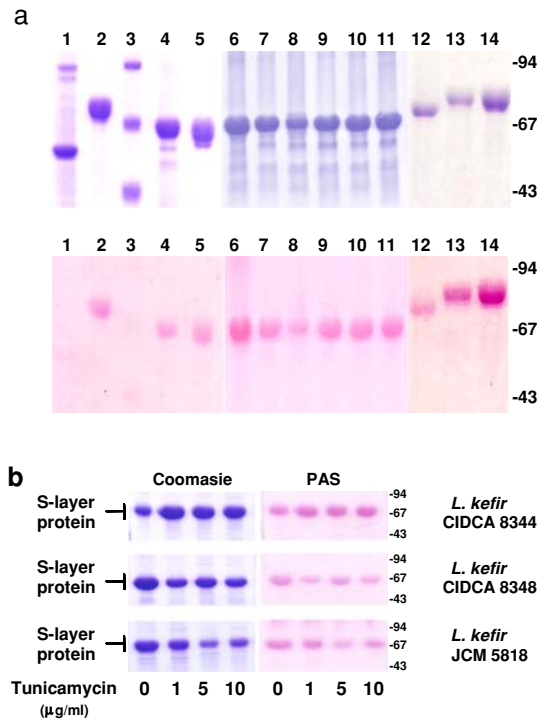

Fig. 2 Glycosylation analysis of S-layer proteins from lactobacilli in SDS–PAGE (10% gel). **a** Coomasie Blue (*upper panel*) or PAS (*lower panel*) staining of S-layer proteins from *L. brevis* JCM 1059 (*lane 1*) or *L. kefir* strains JCM 5818 (*lane 2*), CIDCA 8345 (*lane 4*), CIDCA 8335 (*lane 5*), CIDCA 83113 (*lane 6*), CIDCA 83115 (*lane 7*), CIDCA 8321 (*lane 8*), CIDCA 8325 (*lane 9*), CIDCA 8347 (*lane 10*), CIDCA 8348 (*lane 11*), ATCC 8007 (*lane 12*), CIDCA 8344 (*lane 13*) and CIDCA 83111 (*lane 14*); molecular mass standards (*lane 3*). **b** Coomasie Blue (*left panels*) or PAS (*right panels*) staining of S-layer proteins from *L. kefir* CIDCA 8344, CIDCA 8348 and JCM 5818 subcultured twice in MRS with different concentrations of tunicamycin (0, 1, 5 and 10 µg ml^{-1}) for 48 h. Molecular mass markers are shown on the *right* of each panel

was expected for a non-glycosylated protein (Yasui et al. 1995).

Tunicamycin treatment

Tunicamycin is an antibiotic that interferes with N-glycosylation pathways by inhibiting the enzyme GlcNAc phosphotransferase, without abrogate the O-linked glycan chains addition. The addition of tunicamycin to the growth medium at concentrations from 1 to 10 µg ml^{-1} had no effect on S-layer protein from *L. kefir* CIDCA 8348, CIDCA 8344 and JCM 5818, either in apparent molecular mass determined by SDS–PAGE analysis or in PAS staining (Fig. 2) showing that glycosylation was not affected. Higher concentrations of tunicamycin (20 and 50 µg ml^{-1}) completely inhibited bacterial growth.

Mass spectrometry analysis

Twelve different *L. kefir* S-layer proteins were analyzed to determine peptide-mass fingerprint by MALDI-TOF spectrometry. Mass spectrometry results showed different spectral patterns, evidencing a high heterogeneity in primary structure among these proteins. S-layer proteins from aggregating strains showed very similar MS spectra, with a large amount of coincident peptides not present in MS spectra of the other S-layer proteins analyzed (Table 1). MALDI-TOF peptide profiles of four strains are shown in Fig. 3 where the two aggregating strains (CIDCA 8321 and CIDCA 8348) have the same profile, which is different from those of the non-aggregating ones (CIDCA 83113 and JCM 5818). Moreover, no positive match with other protein spectra in MSDB Database was found. We used as an internal control the S-layer protein from *L. brevis* ATCC 8287, whose gene was already sequenced (Vidgren et al. 1992), and we were able to identify it properly using this methodology and the cited database.

Discussion

We previously reported variations in molecular mass of S-layer proteins from 20 *L. kefir* strains isolated from kefir grains, with Mr ranging from 66 to 71 kDa (Garrote et al. 2004), a common trait also found for S-layer proteins from different strains of *Lactobacillus acidophilus* (Masuda 1992), *L. brevis* (Yasui et al. 1995) and *Clostridium difficile* (McCoubrey and Poxton 2001).

Among all bacteria studied in the present work, only *L. kefir* strains CIDCA 83115, 8321, 8325, 8345, 8347 and 8348 showed aggregative capacity. Since this capacity was completely lost after treatment with 5 M LiCl, it became apparent that this surface property is mediated by the S-layer in agreement with previous reports (Kos et al. 2003, Garrote et al. 2004). However, the sole presence of this layer of proteins is not an aggregating determinant by itself, as several *L. kefir* strains bearing an S-layer showed no aggregative capacity. In this sense although all

Table 1 MALDI-TOF peptides with coincident m/z among S-layer proteins from different *L. kefir* strains

Aggregating strains						Non-aggregating strains					
CIDCA 83115	CIDCA 8321	CIDCA 8325	CIDCA 8345	CIDCA 8347	CIDCA 8348	CIDCA 83113	CIDCA 8335	CIDCA 83111	CIDCA 8344	ATCC 8007	JCM 5818
1,103.56	1,103.54	1,103.64	1,103.54		1,103.53	1,103.53		1,103.56	1,103.58	1,103.57	1,103.53
						1,351.72			1,351.66		
						1,459.69	1,459.70				
						1,510.81	1,510.80				
						1,543.76	1,543.73	1,543.74	1,543.74	1,543.73	
1,585.81	1,585.80	1,585.89	1,585.78	1,585.81	1,585.80						
								1,632.87		1,632.87	
1,633.74		1,633.84	1,633.72	1,633.75	1,633.75						
			1,638.84	1,638.90		1,638.88					
1,649.74	1,649.74	1,649.84	1,649.73	1,649.75	1,649.76						
1,680.90	1,680.88	1,680.99	1,680.88	1,680.91	1,680.90						
1,784.91	1,784.90	1,785.00	1,784.89	1,784.91	1,784.91						
	1,838.89		1,838.91						1,838.91		
1,868.99	1,868.97	1,869.07	1,868.96	1,868.98	1,868.99						
						1,870.00	1,869.99				
								1,901.94		1,901.93	
								1,918.95		1,918.96	
						1,931.96	1,931.96				
1,958.99	1,958.98	1,959.08	1,958.96	1,958.99	1,958.99						
								2,054.07		2,054.06	2,054.07
						2,265.99		2,266.02			
2,270.10			2,270.05	2,270.09							
						2,310.04	2,310.04	2,310.04	2,310.04	2,310.04	
						2,345.14	2,345.14	2,345.14		2,345.13	
								2,413.20		2,413.20	
						2,438.13	2,438.13	2,438.14		2,438.14	
2,468.16	2,468.16	2,468.28	2,468.14	2,468.15	2,468.15						
								2,518.29		2,518.29	
						2,542.39	2,542.39				
								2,617.32		2,617.31	
2,632.30	2,632.30	2,632.43	2,632.29	2,632.29	2,632.29						
2,652.23	2,652.23	2,652.37	2,652.23	2,652.24	2,652.22						
2,780.32	2,780.32	2,780.47	2,780.32	2,780.32	2,780.31						
						2,803.38	2,803.39				
2,857.50	2,857.50	2,857.65	2,857.51	2,857.50	2,857.48						
								2,909.49		2,909.49	
								2,965.51		2,965.52	
3,106.61	3,106.63	3,106.78	3,106.64	3,106.62	3,106.58						
3,127.51	3,127.48	3,127.70	3,127.55	3,127.54	3,127.47						
3,406.56		3,406.76	3,406.64	3,406.63			3,406.54				
						3,619.82	3,619.82				
								3,749.73		3,749.80	

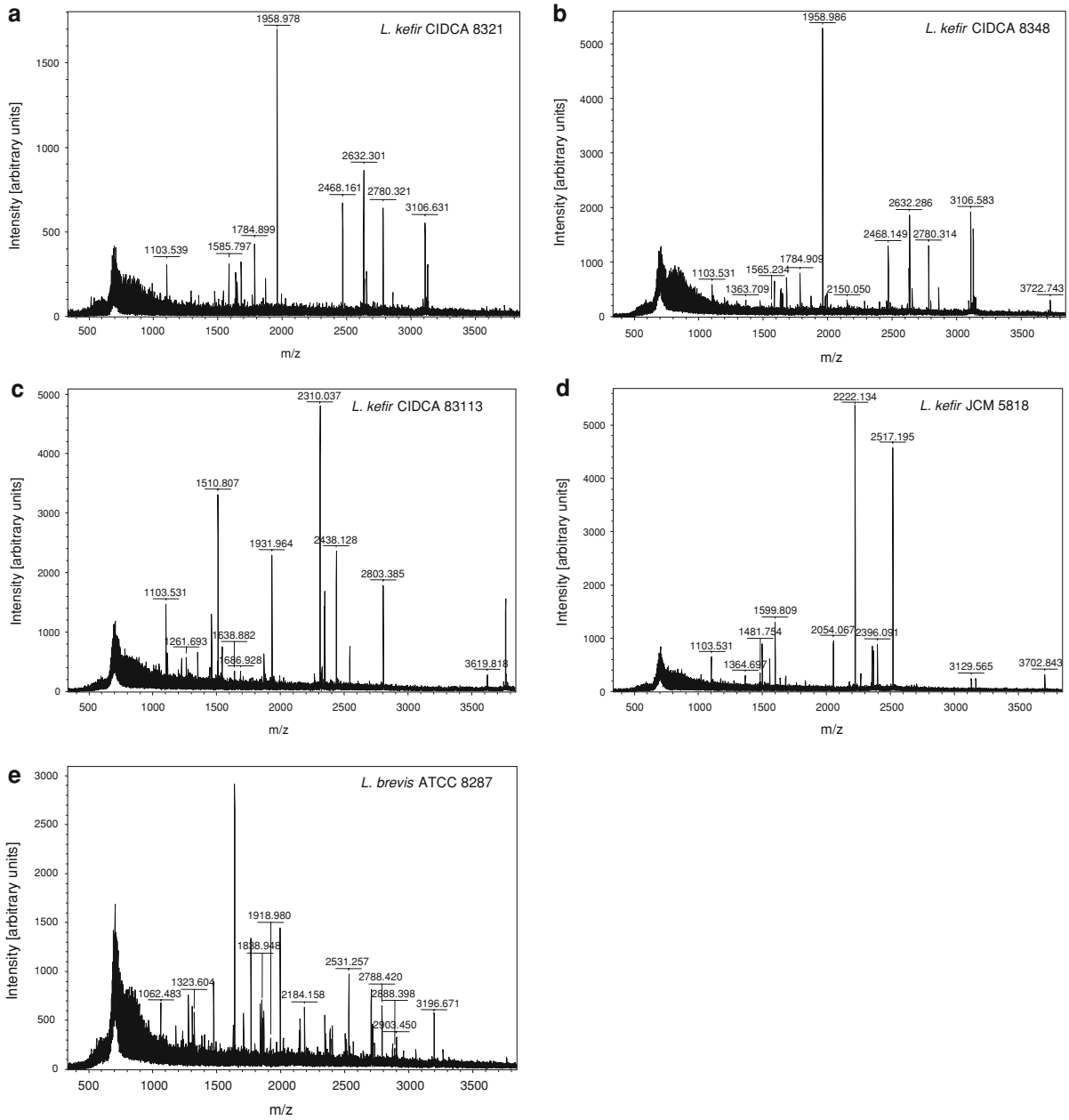

Fig. 3 MALDI-TOF spectra of S-layer proteins from different aggregating (**a**, **b**) or non-aggregating (**c**, **d**) *L. kefir* strains and from *L. brevis* ATCC 8287

aggregating strains bear S-layer proteins of Mr 66 kDa, S-layer proteins of this size are also present in non-aggregating *L. kefir* strains as CIDCA 83113 and CIDCA 8335. These observations suggest that S-layer proteins from aggregating *L. kefir* strains present some particular structural features which could contribute to confer this surface property to the whole bacterial cell.

Anti-S-layer protein Mabs 1F8 and 5F8 showed reactivity against different epitopes on S-layer proteins. The similar reactivity that Mab 1F8 showed against all of *L. kefir* S-layers tested suggests the existence of a common structure present in all S-layer proteins from this species. Nevertheless this region seems not to be an aggregation determinant by itself, because it is present not only in aggregating strains

but also in non-aggregating ones as well. On the other hand, Mab 5F8 reacted only with S-layer proteins of Mr 66 kDa from aggregating strains showing that there are structural differences among S-layer proteins from *L. kefir*, even in strains bearing S-layer proteins of the same apparent molecular mass. Moreover, these results demonstrate the existence of at least one epitope that is present only in S-layer proteins from aggregating strains.

These structural characteristics could be attributed to differences in aminoacidic sequences or to the introduction of posttranslational modifications in the proteins.

Glycosylation represents the most common post-translational modification in S-layer proteins and has been demonstrated in many archaeal and bacterial genera. In the present work the glycosylation of *L. kefir* S-layer proteins was demonstrated by PAS staining. This is a remarkable result since to present most of works reported non-glycosylated S-layers in lactobacilli (Avall-Jääskeläinen and Palva 2005) with the exception of Möschl et al. who presented the only evidence of glycosylated S-layer proteins in lactobacilli (*L. buchneri* 41021/251 and *Lactobacillus plantarum* 41021/252) in 1993. Preliminary analysis of sugar composition for S-layer glycoproteins from four *L. kefir* strains showed differences in total carbohydrate content and variety and relative amounts of constitutive monosaccharides, with glucose and mannose as main monosaccharide residues (data not shown), in concordance with data reported for other S-layer proteins (Schäffer and Messner 2004). To gain insight on the glycosylation of *L. kefir* S-layer proteins, cells were grown in presence of tunicamycin, a drug known to interfere with N-glycosylation of proteins in Archaea (Grogan 1996) and in Bacteria (Küpcü et al. 1984). The failure of tunicamycin in interfering with the protein glycosylation in *L. kefir* suggests that sugar residues are not N-linked to protein moiety, which is in concordance with data reported by Möschl et al. (1993) who described the presence of O-linked glucose residues in *L. buchneri* S-layer protein. The growth inhibition at higher concentrations of tunicamycin could be attributed to the interference with peptidoglycan synthesis as described by Bettinger and Young (1975).

Since all the S-layer proteins studied in the present work were glycosylated, it is evident that the sole presence of a carbohydrate moiety in those proteins is not related to the aggregation ability of *L. kefir* strains.

Mass spectrometry was employed by different authors to identify, characterize and compare sequences of S-layer proteins from distinct bacteria (Allmaier et al. 1995; Karjalainen et al. 2001; Schär-Zammaretti et al. 2005). The analysis of *L. kefir* S-layer proteins by MALDI-TOF MS showed that there is a high heterogeneity among these proteins, since different MS spectral patterns were obtained for S-layer proteins from different *L. kefir* strains. Although no information was available about amino acid sequences of *L. kefir* S-layer proteins, our results are in concordance with the high heterogeneity reported in S-layer proteins from other lactobacilli, i.e. the strain-specific differences in the amino acid sequences of S-proteins from *Lactobacillus gallinarum* obtained by Hagen et al. (2005) using quadrupole-time of flight (Q-TOF) analysis. Since no positive match with other protein spectra was found, S-layer proteins from the *L. kefir* strains studied in this work were different to S-layer proteins from other lactobacilli and from other bacterial genera described in MSDB Database to date. Interestingly S-layer proteins from aggregating strains presented very similar MS spectra, with many coincident peptides between them which were, however, absent in MS spectra from non-aggregating strains. These results contribute to sustain the hypothesis that S-layer protein structure could be involved, at least in part, with the aggregative properties of the whole bacterial cell. In concordance with our present results, it was recently reported that S-layer proteins from aggregating and non-aggregating *L. kefir* strains showed differences in their secondary structure composition (β-sheet 23.0–28.0% for aggregating strains and 36.0–42.1% for non-aggregating ones) as determined by FTIR spectroscopy (Mobili et al. 2008).

In conclusion, this is the first report of characterization of glycosylated S-layer proteins from different strains of a single species in genus *Lactobacillus*. The high heterogeneity observed for S-layer proteins from *L. kefir* is in concordance with data previously reported for surface proteins from other bacterial species, although the results of reactivity with Mab 5F8 and peptide-mass fingerprint for S-layer from aggregating *L. kefir* strains suggest that particular structural characteristics of these glycoproteins could have influence in determining the surface properties of whole bacterial cells.

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