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Differential protein expression patterns between planktonic and biofilm cells of *Salmonella enterica* serovar Enteritidis PT4 on stainless steel surface

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ABSTRACT

In the present study, the proteome of a strain of *S. enterica* serovar Enteritidis PT4, grown either as biofilm on stainless steel surface or as free-floating (planktonic) in Brain Heart (BH) broth, was investigated in order to detect the strong differences in whole-cell protein expression patterns between the two growth styles. The proteins extracted from both types of cells were subjected to 2-D PAGE, followed by in-gel tryptic digestion, extraction, subsequent MALDI-TOF mass spectrometry (MS) analysis and finally database searches for protein identification. Using this approach, 30 proteins were identified as differentially expressed between the two growth modes on an "on-off" basis, that is, proteins that were detected in one case but not in the other. In particular, 20 and 10 proteins were identified in biofilm and planktonic-grown cells, respectively. The group of proteins whose expression was visible only during biofilm growth included proteins involved in global regulation and stress response (ArcA, BtuE, Dps, OsmY, SspA, TrxA, YbbN and YhbO), nutrient transport (Crr, DppA, Fur and SufC), degradation and energy metabolism (GcvT, GpmA, RibB), detoxification (SseA and YibF), DNA metabolism (SSB), curli production (CsgF), and murein synthesis (MipA). To summarize, this study demonstrates that biofilm growth of *S.* Enteritidis causes distinct changes in protein expression and offers valuable new data regarding some of the proteins presumably involved in this process. The putative role of these proteins in the maintenance of a biofilm community in *Salmonella* and other bacteria is discussed.

1. Introduction

Salmonellae represent a group of Gram-negative bacteria that are recognized worldwide as major zoonotic pathogens for both humans and animals. In the EU, salmonellosis was the second most commonly reported zoonotic infection in 2009, with 108,614 human cases confirmed and a case fatality rate of 0.08%, which approximately corresponds to 90 human deaths (EFSA-ECDC, 2011). The two most common *Salmonella* serotypes, implicated in the majority of outbreaks, are Typhimurium and Enteritidis (52.3% and 23.3%, respectively, of all known serovars in human cases). The native habitat of salmonellae is considered to be the intestinal tract of taxonomically diverse group of vertebrates, from which salmonellae can spread to other environments through faeces.

Interestingly, salmonellae have been shown to survive for extended periods of time in non-enteric habitats, including biofilms on abiotic surfaces (Giaouris et al., 2012; White et al., 2006). A biofilm can be broadly defined as a microbially derived sessile community characterized by cells that are attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002). It has been observed that the antimicrobial resistance of biofilm cells is significantly increased compared to planktonic cells (Mah and O'Toole, 2001; Wong et al., 2010). The general features of biofilms also apply for *Salmonella*, which is able to form biofilms on both biotic and abiotic surfaces (for an overview on *Salmonella* biofilms, see Steenackers et al., 2011). Thus, biofilm formation enhances the capacity of pathogenic *Salmonella* bacteria to survive stresses that are commonly encountered within food processing and/or during host infection.

In the food industry, biofilms may create a persistent source of product contamination, leading to serious hygienic problems and also economic losses due to food spoilage (Brooks and Flint, 2008). Improperly cleaned surfaces promote soil build-up and, in the presence of water, contribute to the development of bacterial biofilms which may contain pathogenic microorganisms, such as *Salmonella*. Cross contamination occurs when cells detach from biofilm structure once food passes over contaminated surfaces or through aerosols originating from contaminated equipment (Kusumaningrum et al.,

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2003). Till now, there is only limited information on the presence of *Salmonella* in biofilms in real food processing environments (Rasschaert et al., 2007). However, numerous studies have shown that *Salmonella* can easily attach and form biofilms on various food-contact surfaces, such as stainless steel, plastic and rubber (for a review see Giaouris et al., 2012). Taking into account that all these surfaces are commonly encountered in farms, slaughter houses, industrial food processing facilities, and kitchens, it is obvious that the risk for public health is serious.

Scientific interest in the process of bacterial biofilm formation has erupted in recent years and studies on the molecular biology and physiology of biofilm cells have begun to shed light on the driving forces behind the transition to the biofilm mode of existence (Fux et al., 2005; Ghigo, 2003; Irie and Parsek, 2008; Sauer, 2003; Smith et al., 2009). Regarding *S. enterica*, there are a number of interesting studies occupied with its cellular physiology inside a biofilm (Bhowmick et al., 2011; Brown et al., 2001; Kim and Wei, 2009; Malcova et al., 2008; Römling, 2005; White et al., 2010; Zakikhany et al., 2010). In this bacterium, the expression of the main extracellular matrix components stabilizing the biofilm structure (curli fimbriae and cellulose) is dependent on the transcriptional regulator CsgD, whose transcription seems to be influenced by a variety of regulatory stimuli (Gerstel and Römling, 2003). Interestingly, White et al. (2010) showed by comparing extracellular matrix-embedded, wild-type *S.* Typhimurium and the matrix-deficient *csgD* mutant (using a combined metabolomics and transcriptomics approach) that the two populations present distinct metabolite and gene expression patterns, with wild-type cells expressing genes mainly involved in gluconeogenesis and stress-resistance pathways.

Several other studies with various food-related bacteria have also shown that biofilm cells differ physiologically from their planktonic counterparts, presenting a modified and distinct protein expression (Dykes et al., 2003; Hefford et al., 2005; Kalmokoff et al., 2006; Oosthuizen et al., 2002; Planchon et al., 2009; Sampathkumar et al., 2006; Trémoulet et al., 2002). Regarding *Salmonella*, Hamilton et al. (2009) discovered, when determined the transcriptomic and proteomic profiles of biofilms of *S.* Typhimurium, that 124 detectable proteins were differentially expressed in the biofilm compared to the planktonic cells, and that 10% of the *S.* Typhimurium genome (433 genes) showed a 2-fold or more change in the biofilm compared with the planktonic cells. The genes that were significantly up-regulated implicated certain cellular processes in biofilm development, including amino acid metabolism, cell motility, global regulation and tolerance to stress. Two other studies have used a proteomic approach to identify *S.* Enteritidis proteins that are differentially regulated during biofilm growth on glass coverslips in response to disinfectant and different flowing rates (Mangalappalli-Illathu et al., 2008a,b). In addition, proteomic approaches have also been used to investigate the response of *Salmonellae* to food related antimicrobials (Di Pasqua et al., 2010) and other stressful conditions (Encheva et al., 2009).

An improved understanding of the physiological responses taking place inside a *Salmonella* biofilm, can be of value to work out the relative roles of benefits and forces that drive the switch to this sessile mode of growth. In order to contribute to the current knowledge of molecular changes occuring in *Salmonella* biofilms under environmental conditions relevant to food processing, mature biofilms of *S.* Enteritiditis PT4 formed on stainless steel and planktonic cultures of the same age were comparatively investigated in the present study by a proteomic analysis.

2. Materials and methods

2.1. Bacterial strain and preparation of inoculum

Salmonella enterica subsp. *enterica* serovar Enteritidis phage type (PT) 4 strain P167807, kindly provided by the School of Biomedical and Molecular Sciences of the Surrey University (Surrey, UK), was used in this study. This strain was selected due to its prevalence in cases of human salmonellosis, mainly associated with the consumption of raw shell egg products (Cowden et al., 1989). Before utilization, the microorganism was stored frozen (at −80 °C) in bead vials (Protect; Technical Service Consultants Ltd, Heywood, Lancashire, UK) and was then resuscitated by adding one bead to 100 ml of Brain Heart Infusion broth (BHI; LAB M; International Diagnostics Group Plc, Bury, Lancashire, UK) in a conical flask and incubating at 37 °C for 18 h, at which time late exponential phase was attained (preculture). Working culture was prepared by adding a 100-μl aliquot of the preculture to 100 ml of BHI broth and incubating at 37 °C for 18 h. Cells from the final working culture were harvested by centrifugation (5000 ×*g*, 10 min, at 4 °C), washed twice with 1/4 Ringer solution (Ringer's tablets; Merck, Darmstadt, Germany) and finally resuspended in 1/4 Ringer solution (*ca.* 10^8 CFU/ml), in order to be used as inoculum for the biofilm development assay.

2.2. Abiotic surface and biofilm development

Stainless steel (SS) coupons (50×20×1 mm, type AISI-304; Halyvourgiki Inc., Athens, Greece) were the abiotic substrates used for biofilm development. The coupons were initially soaked in acetone (overnight), to remove any manufacturing process debris and grease. Coupons were then washed in commercial detergent solution, rinsed thoroughly with tap water followed by distilled water and air-dried.

To produce biofilms, cleaned SS coupons were individually placed in 50 ml polypropylene centrifuge tubes (length, 114.4 mm; outside diameter, 29.1 mm; Corning Inc., Amsterdam, the Netherlands) each containing 25 ml of growth medium, in such a way that the upper part of each metallic surface (*ca.* 2 mm) was exposed to the air-liquid interface, since this interface provides a selectively advantageous niche for *Salmonella* biofilm formation (Giaouris and Nychas, 2006). Growth medium used to support biofilm development was BHI broth (pH 7.4; 0.5% w/v NaCl). Centrifuge tubes with SS coupons were autoclaved at 121 °C for 15 min. After sterilization and cooling, growth media were inoculated with *Salmonella* to yield initial bacterial populations of *ca.* 10^3 CFU/ml. Inoculated tubes were subsequently incubated at 20 °C for 6 days (144 h) under static conditions, without any nutrient refreshment, to allow biofilm development on the coupons. Incubation conditions applied here were the ones found to provide the maximum number of biofilm cells according to previous results (Giaouris et al., 2005).

2.3. Recovery of planktonic cells

On the 6th day of incubation, 1 ml of planktonic culture in plastic centrifuge tubes containing the SS coupons was collected. The advanced stationary phase cells were subsequently harvested by centrifugation (5000 ×*g*, 10 min, at 4 °C) and used directly for protein extraction.

2.4. Recovery of biofilm cells from the coupons

On the 6th day of incubation, SS coupons – carrying *S.* Enteritidis biofilm cells on them – were carefully removed from plastic centrifuge tubes using sterile forceps and were thereafter rinsed two times by pipetting 10 ml of 1/4 Ringer solution (each time onto each coupon), in order to remove the loosely attached cells. After this rinsing procedure, coupons were individually introduced in new sterile plastic centrifuge tube containing 40 ml of 1/4 Ringer solution and 30 sterile glass beads (diameter, 3 mm). The plastic tube was then vortexed for 2 min, at maximum speed, to detach biofilm cells from the coupons. Detached cells were subsequently collected by centrifugation (5000 ×*g*, 10 min, at 4 °C) and used directly for

protein extraction. In addition, the number of viable biofilm cells was estimated by serial decimal dilutions, plating onto Tryptone Soy Agar (TSA; LAB M) and counting colonies after 24 h at 37 °C.

2.5. Protein extraction

The total protein extraction procedure was performed twice, both for planktonic- and biofilm-derived samples. Each sample replicate was derived from independent bacterial cultures. All chemicals used in proteomic analysis were purchased from Bio-Rad (Bio-Rad Laboratories Inc., Athens, Greece), unless otherwise stated. Initially, collected pellets of both planktonic and sessile bacteria were washed two times with 1/4 Ringer solution by centrifugation (5000 ×*g*, 10 min, at 4 °C) and resuspended at a concentration of *ca.* 10^8 cells/ml in 1 ml of lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris, 2 mM TBP and 0.2% Bio-Lyte 3/10 ampholyte). Total cellular proteins were extracted by sonicating cells resuspended in lysis buffer thrice for 15 s each at 40 Ω amplitude on ice with 1 min interval between pulses. The suspension was centrifuged (16,000 ×*g*, 10 min; Eppendorf benchtop centrifuge) to remove unbroken cells and cell debris. The supernatant was then treated with 0.1 volume of buffer containing 50 mM $MgCl_2$, 1 mg/ml DNase I (Invitrogen) and 0.25 mg/ml RNase A (Invitrogen) for 15 min in ice. The reaction was stopped by mixing it with 3 volumes of ice-cold acetone. Proteins were then precipitated for 2 h at −20 °C. The precipitate was collected by centrifugation (16,000 ×*g*, 10 min) and dried to remove residual acetone. The protein pellet was resuspended in isoelectric focusing (IEF) buffer containing 8 M urea, 2% CHAPS, 50 mM DTT, and 0.2% Bio-Lyte 3/10 ampholyte. Protein concentration was determined according to dye-binding assay of Bradford (1976). Protein samples were stored at −20 °C until further analysis.

2.6. Two dimensional (2-D) gel electrophoresis

2-D electrophoresis was performed as described by O'Farrell (1975), with some modifications described thereafter (Görg et al., 2009). Three gels were run per sample replicate resulting in total of 6 gels per growth condition (biofilm *versus* planktonic).

2.6.1. Isoelectric focusing (IEF)

Eighteen-centimeter ReadyStrip™ immobilized pH gradient 4–7 (IPG) strips (Bio-rad) were rehydrated with 315 μl of IEF buffer (composition as above plus 0.001% bromophenol blue) containing 400 μg of total protein for 16 h at room temperature. Isoelectric focusing was performed using a PROTEAN® IEF Cell system (Bio-Rad) with the following steps: (i) 250 V with rapid increase for 30 mins, (ii) 5000 V with linear increase for 12 h, and a final focusing step (iii) at 5000 V with rapid increase for 16 h (total ~80–90 kVh). Following isoelectric focusing, the IPG strips were equilibrated twice for 20 min each in 6 ml of equilibration buffer (50 mM Tris/HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and traces of bromophenol blue), in the presence of 10 mg/ml DTT for the first equilibration, and in the presence of 25 mg/ml iodoacetamide for the second equilibration.

2.6.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The second dimensional separation was a vertical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and was performed in a PROTEAN® Plus Dodeca Cell system (Bio-Rad). Equilibrated isoelectric focusing strips were placed on top of a uniform 12% SDS-polyacrylamide gel and sealed with 0.5% low melting point agarose, according to manufacturer instructions. Second-dimension separation was carried out at 8–10 mA per gel constant current at room temperature for 12 h. Following electrophoresis, proteins in each gel were fixed in fixing solution (45% methanol and 1% glacial acetic acid) for at least 4 h and stained with home-made colloidal Coomassie [34% methanol, 0.5% glacial acetic acid, 17% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) Coomassie G250] overnight. The 2-DE protein patterns were recorded as digitalized images using a scanner.

2.7. In-gel tryptic digestion and protein identification by MALDI-TOF mass spectrometry (MS)

Protein differences between the two growth conditions (biofilm versus planktonic) on 2-D gels were manually detected and subsequently protein spots of interest were manually excised. Spots were initially treated with destaining solution [50 mM ammonium bicarbonate, 30% acetonitrile (ACN)]; rinsed with d.d. H_2O, dehydrated using a speed-vacuum instrument, and incubated overnight with 3–5 μl/spot trypsin (Roche; proteomics grade, 10 μg/ml in 10 mM ammonium bicarbonate) at room temperature. Peptides were extracted with 10 μl/spot of extraction solution [50% ACN, 0.1% trifluoroacetic acid (TFA)]. Peptide-containing solutions were applied on a 384 steel MALDI target (Bruker) followed by 1 μl of standards containing Matrix [50% ACN, 0.1% TFA, 0.3% (w/v) cyano-4-hydroxycinnamic acid, 10 pmol/ml bradykinin fragment 1–8 (m/z 904.4861) and 20 pmol/ml adrenocorticotropic hormone fragment 18–39 (m/z 2465.1983)]. MALDI ULTRAFLEX (Bruker Daltonics) was used for spectra acquisition using the software controller Bruker Daltonics FlexControl Version 2.2. The instrument, operated in Reflector Mode, was calibrated using 400 laser shots accumulated from external standards. Spectra were acquired using a laser power range of 45% – 65% and a detection range of 900 – 3500 m/z. A total of 8 × 50-laser shots were accumulated for each spot. The spectra accumulation was done after their automatic evaluation. All spectra analyzed had a resolution higher than 6500 in the range 1200–2700 m/z. For Spectra Processing Bruker Daltonics FlexAnalysis 8 Version 2.2 was used with SNAP algorithm for detection, Centroid algorithm for editing and Savitzky Golay algorithm for smoothing. The S/N ratio in the spectra analyzed was at least 2.5, and a quality factor threshold of 50 was selected. Background Peak removal was performed based on the contaminant peak list provided by Bruker Daltonics containing Tryptic auto-digest peaks and common keratin fragment peaks. A local Mascot Server Version 2.0 was used for protein identification. Several identification cycles were performed and the most stringent parameters used were the following: Swissprot/TrEMBL and NCBI databases, proteobacteria and mammalian, trypsin with zero and one missed cleavage, carbamidomethylation and methionine oxidation as fixed and variable modifications respectively, and 25 ppm error tolerance. Identified proteins had at least 4 peptides below 10 ppm. Gene ontology (GO) annotations were automatically acquired and manually processed from European Bioinformatics Institut (EBI; http://www.ebi.ac.uk/EGO).

3. Results and discussion

Till now, numerous studies with various microorganisms have demonstrated that biofilm formation triggers the expression of specific sets of proteins, compared to planktonic cells (Dykes et al., 2003; Hefford et al., 2005; Kalmokoff et al., 2006; Oosthuizen et al., 2002; Planchon et al., 2009; Sauer and Camper, 2001; Silva et al., 2011; Trémoulet et al., 2002). However, nothing is yet known about the proteomic profile inside a *Salmonella* biofilm formed on stainless steel (SS), an abiotic substratum commonly used in food processing equipment. In order to better understand the cellular mechanisms sustaining a surface-associated lifestyle of *S.* Enteritidis in food related environments, the differential protein patterns of this bacterium cultivated as biofilm on SS *versus* planktonic mode were comparatively studied in the present work.

Initially, biofilm, as well as planktonic cultures were set up. The environmental conditions used here to support biofilm development (batch system, 6 days, 20 °C, coupons partially submerged in broth, static incubation) have already been shown to promote *Salmonella* surface-associated growth (Giaouris et al., 2005). In order to compare

protein expression between biofilm and planktonic bacteria, the latter were also grown under exactly the same conditions. Under such conditions *Salmonella* reached a sessile population of *ca.* 10^6 CFU/cm^2, and a planktonic population of *ca.* 10^9 CFU/ml. These high levels of populations permitted the easy isolation of sufficient quantities of cells for the proteomic analysis. It is worth noting that, by the end of incubation period, dense pellicle formation was observed at the air-liquid interface in the incubation tubes containing the SS coupons. This pellicle, mainly composed of cellulose, is strongly correlated with the biofilm forming capacity of *Salmonella* and some other bacteria as well (Scher et al., 2005; Spiers et al., 2003).

Total protein extracts were successfully isolated from both types of cells (biofilm and planktonic), and reproducible protein patterns were obtained for each case. Subsequently, strong differences between the two protein profiles were detected manually on an "on-off" basis, that is proteins detected in one case but not in the other. Sixty-one protein spots, that were visible solely to the one of the two growth modes, were selected and analyzed by MALDI-TOF MS. These spots were specifically chosen as they were abundant and clearly separated from other spots, in order to facilitate unambiguous identification. In particular, in samples derived from planktonic cultures there was an attempt to identify a total number of 20 protein spots. The identification of 10 different proteins above confidence levels (ID score > 50) was achieved for the planktonic cell extracts (Table 1). In biofilm derived samples, 20 different proteins were identified with certainty (ID score > 50) out of a total number of 41 protein spots tested (Table 2). All these 30 identified proteins were analyzed by BLASTP (http://blast.ncbi.nlm.nih.gov/; Altschul et al., 1997), in order to compare them to protein sequence databases and find regions of similarity and therefore reveal their possible function and evaluate their presumptive importance in biofilm formation.

The 20 proteins whose expression was visible only in the biofilm-derived samples could be classified into the following five categories, based on their function: (a) proteins involved in global regulation and stress response (ArcA, BtuE, Dps, OsmY, SspA, TrxA, YbbN and YhbO); (b) proteins involved in nutrient transport (Crr, DppA, Fur and SufC); (c) proteins involved in degradation and energy metabolism (GcvT, GpmA and RibB); (d) proteins involved in detoxification (SseA and YibF) and (e) proteins involved in various processes, such as DNA metabolism (SSB), curli production (CsgF) and murein synthesis (MipA). In a similar proteomic study with *S.* Typhimurium SL1344 (grown either as biofilm on silicone rubber tubing in a modified batch system at 25 °C for 72 h, or as planktonic cells), Hamilton et al. (2009) discovered that the expression of 124 proteins was altered (> 2-fold), with the expression of 59 proteins increasing and 65 proteins decreasing during suface-associated growth compared to planktonic growth. In that study, 24 proteins up-regulated in the biofilm were finally identified, in which they were included proteins involved in cell motility, amino acid and carbohydrate metabolism, as well as proteins of unassigned function. In another comparative study with *S.* Enteritidis ATCC4931 (cultivated for 168 h in flow cells using glass coverslips as substrata for attached growth), Mangalappalli-Illathu et al. (2008a) identified 32 differentially-expressed proteins between biofilm and planktonic cells (of which 14 proteins were up-regulated and 18 were down-regulated in biofilms). Major up-regulated proteins included those involved in degradation and energy metabolism, protein translation and modification, RNA synthesis and modification, DNA transcription and adaptation. In the related species *Escherichia coli*, Trémoulet et al. (2002) found that 14 proteins were up- and 3 down-regulated in cells grown as biofilm for 7 days at 20 °C on glass fiber filter disks compared to planktonic cells of the same age. For a better overview and comparison of the results derived from proteomic studies on *Salmonella* biofilm formation reader is reffered to Table S1 (supplementary file). Suprisingly, from the 20 "biofilm proteins" identified here, only 3 (Crr, DppA and GpmA) had also been previously found to be up-regulated in *Salmonella* biofilm cells compared to planktonic cells. However, it should be noted that a direct comparison of the results between all these studies may be not appropriate, since the dynamic and environmental conditions for biofilm formation used in each study (bacterial serotype and strain, support material, biofilm incubation temperature and growth conditions) are quite different. Such biological and technical differences should greatly impact upon protein expression, and therefore the identification of biofilm related proteins, complicating the comparison of proteomic data between the different studies. Despite this, it is still interesting that half (10 out of 20) of the "biofilm proteins", which were identified here, have also been found to be implicated in biofilm formation and/or other related events in other bacteria (ArcA, Dps, TrxA, Crr, DppA, GpmA, RibB, SseA, Ssb and MipA; see Table S2, supplementary file).

In *S.* Typhimurium transcriptome analyses have recently shown that the global response regulator ArcA (aerobic respiratory control) directly or indirectly, regulates 392 genes (8.5% of the genome), while it mainly serves as a regulator/modulator of genes involved in aerobic/anaerobic energy metabolism and motility (Evans et al., 2011). An *arcA* mutant was non-motile, lacked flagella and was as virulent in mice as the wild-type strain. In *E. coli*, ArcA was also found to be important for competitiveness in biofilms (Junker et al., 2006). Interestingly, it has also been reported that, although *arcA* mutants of *Shewanella oneidensis* are capable of forming wild-type biofilms, they are compromised in their ability to undergo oxygen-depletion-induced detachment (Thormann et al., 2005). In the facultative anaerobic pathogen of the porcine respiratory tract *Actinobacillus pleuropneumoniae*, an *arcA* deletion mutant was attenuated and deficient in autoaggregation and biofilm formation under oxygen-deprived growth conditions (Buettner et al., 2008).

Table 1
List of 10 proteins identified by MALDI-TOF MS whose expression was visible only during planktonic growth.

s/n	ExPASy access. no.	Gene name	Gene locus[a]	Protein name[b]	Protein class/function[c]	Peptide coverage	Theoretical[d] pI/ molec. mass (Da)
1	B5QTN0	*nmpC*	SEN1483	Outer membrane porin protein (OmpD)	Transport (porin)	18	4.66/39695.5
2	B5QXR6	*tufA*	SEN3273 SEN3930	Elongation factor (EF-Tu)	Protein biosynthesis (elongation factor)	54	5.30/43283.5
3	B5R492	*fabI*	SEN1332	Enoyl-[acyl-carrier-protein] reductase [NADH]	Fatty acid biosynthesis (oxidoreductase)	46	5.57/27774.8
4	B5QW86	*pepQ*	SEN3778	Xaa-Pro dipeptidase	Dipeptidase (prolidase)	27	5.78/50170.2
5	B5R2Q2	*lpdA*	SEN0158	Dihydrolipoyl dehydrogenase	Oxidoreductase	44	5.87/50639.4
6	B5R2U1	*manX*	SEN1207	Mannose-specific IIAB component	Sugar transporter (PEP phosphotransferase system)	41	5.82/35017.5
7	B5QZX1	*basR*	SEN4063	Two-component response regulator	DNA-binding transcriptional regulator	68	5.84/25053.5
8	B5QX91	*pagC*	SEN1803	Outer membrane invasion protein (PagC)	Virulence (invasion)	52	6.83/20231.6
9	B5R505	*ynaF*	SEN1400	Putative uncharacterized protein YnaF	Putative universal stress protein	58	5.93/15714.2
10	B5QUS3	*atpC*	SEN3678	ATP synthase epsilon chain	ATP synthesis	62	5.84/15064.3

[a] Corresponds to NCBI reference sequence NC_011294.1 of *S. enterica* serovar Enteritidis str. P125109 complete genome (Thomson et al., 2008).
[b] Corresponds to the name submitted to protein knowledgebase UniProtKB (http://www.uniprot.org/).
[c] According to database and literature searches.
[d] Theoretical values obtained from ExPASy bioinformatics tool "Compute pI/Mw" (http://web.expasy.org/compute_pi/).

Table 2
List of 20 proteins identified by MALDI-TOF MS whose expression was visible only during biofilm growth.

s/n	ExPASy access. no.	Gene name	Gene locus[a]	Protein name[b]	Protein class/function[c]	Peptide coverage	Theoretical[d] pI/ molec. mass (Da)
Globar regulation and stress response							
1	B5R3D0	*arcA*	SEN4354	Global response regulator	Two-component response regulator	49	5.29/27262.9
2	B5QVW0	*btuE*	SEN1703	Glutathione peroxidase	Response to oxidative stress	77	5.08/20453.4
3	B5QXT6	*dps*	SEN0776	DNA protection during starvation protein	DNA starvation/stationary phase protection	68	5.71/18717.2
4	B5R2J1	*osmY*	SEN4323	Putative periplasmic protein	Osmotically inducible gene	48	5.78/21449.1
5	B5R0L5	*sspA*	SEN3175	Stringent starvation protein A	Transcriptional activator (induced by starvation)	31	5.22/24248.8
6	B5QVH3	*trxA*	SEN3721	Thioredoxin 1	Chaperone, oxidoreductase, oxidoprotectant, virulence	47	4.67/11806.6
7	B5QU93	*ybbN*	SEN0485	Thioredoxin-like protein	Chaperone, oxidoreductase	41	4.63/31768.0
8	B5QZU1	*yhbO*	SEN3104	Putative uncharacterized protein YhbO	Stress response (multiple stresses)	46	5.02/18871.4
Nutrient transport							
9	B5R4E2	*crr*	SEN2414	Pts system, glucose-specific IIA component	Sugar transporter (PEP phosphotransferase system)	55	4.73/18247.0
10	B5R4L8	*dppA*	SEN3454	Periplasmic dipeptide transport protein	Dipeptide ABC transporter	45	5.74/58491.3
11	B5QWD7	*fur*	SEN0657	Ferric uptake regulation protein	Ferric uptake regulator, pathogenicity	66	5.56/17039.1
12	B5QVT1	*sufC*	SEN1674	Putative ABC transport ATP-binding subunit	Cysteine desulferase ATPase, Fe-S biogenesis	55	4.81/27715.6
Degradation and energy metabolism							
13	B5QXI2	*gcvT*	SEN2898	Aminomethyltransferase	Degradation of glycine	40	5.27/40259.8
14	B5QX43	*gpmA*	SEN0717	Phosphoglyceromutase	Carbohydrate degradation (glycolysis)	44	5.78/28493.3
15	B5QZ31	*ribB*	SEN3037	3,4-dihydroxy-2-butanone 4-phosphate synthase	Riboflavin biosynthesis	39	4.89/23310.4
Detoxification							
16	B5R592	*sseA*	SEN2513	Putative thiosulfate sulfurtransferase	3-mercaptopyruvate sulfurtransferase	52	4.75/30831.8
17	B5R5B7	*yibF*	SEN3506	Putative glutathione transferase	Putative Se metabolism and detoxification	46	5.38/22544.1
Various processes							
18	B5QZ80	*ssb*	SEN4025	Single-stranded DNA-binding protein	DNA replication, repair, recombination	31	5.46/19074.1
19	B5QY25	*csgF*	SEN1908	Assembly/transport component in curli production	Curli production (assembly)	47	5.64/15158.9
20	B5QWL5	*mipA*	SEN1766	Putative outer membrane protein	Synthesis of murein-sacculus	64	5.51/27992.0

[a] Corresponds to NCBI reference sequence NC_011294.1 of *S. enterica* serovar Enteritidis str. P125109 complete genome (Thomson et al., 2008).
[b] Corresponds to the name submitted to protein knowledgebase UniProtKB (http://www.uniprot.org/).
[c] According to database and literature searches.
[d] Theoretical values obtained from ExPASy bioinformatics tool "Compute pI/Mw" (http://web.expasy.org/compute_pi/).

Dps is a ferritin-like protein with DNA-binding properties that is capable of offering protection during oxidative stress and during times of nutritional deprivation. Using microarray and qRT-PCR analyses, Bhomkar et al. (2010) demonstrated that FimH-mediated adhesion of *E. coli* to biocompatible substrates led to the induction of *dps* among bound cells relative to unbound cells. Strong induction of this gene during biofilm growth of *E. coli* in urine has also been shown (Hancock and Klemm, 2007). Interestingly, exposure of *E. coli* to environmental bacteriophages resulted in rapid selection for phage-tolerant subpopulations displaying increased biofilm formation and producing large amounts of the Dps protein in the outer membrane (Lacqua et al., 2006). In the common airway commensal and opportunistic pathogen *Haemophilus influenzae*, Dps was found to promote survival within biofilm communities, as well as resistance to host clearance *in vivo* (Pang et al., 2012). The importance of this protein both for biofilm formation and poultry colonization by *Campylobacter jejuni* has also been shown (Theoret et al., 2012). In another study with *E. coli* strains, Dps was found to have a putative role in attachment in a strain- and substrate-dependent manner (Goulter-Thorsen et al., 2011).

In *S.* Typhimurium, *trxA* encodes thioredoxin 1 which (together with thioredoxin 2) is involved as antioxidant in defense against oxidative stresses, such as exposure to hydrogen peroxide and hydroxyl radicals. Additionally, Negrea et al. (2009) have reported that Trx1 is required for the proper activity of *Salmonella* pathogenicity island (SPI2) type III secretion system, while a *trxA* mutant of *S.* Typhimurium was attenuated for virulence in mice (Peters et al., 2010). Interestingly, Trx1 was also included in the list of proteins up-regulated in *S.* Enteritidis planktonic and biofilm cells exposed to benzalkonium chloride (Mangalappalli-Illathu and Korber, 2006; Mangalappalli-Illathu et al., 2008b).

Crr is the glucose-specific component of phosphoenolpyruvate (PEP) phosphotransferase (PTS) system. An *S.* Typhimurium mutant unable to catabolize glucose due to deletion of *crr* showed reduced replication within murine macrophages (Bowden et al., 2009). GpmA is a phospoglyceromutase which catalyzes the interconversion of 2-phosphoglycerate to 3-phosphoglycerate during glycolysis. In agreement with current results, these two proteins (Crr, GpmA) also became up-regulated in 168 h-old *S.* Enteritidis biofilms relative to a planktonic cell control (Mangalappalli-Illathu et al., 2008a).

The dipeptide permease (Dpp) transports dipeptides across the *E. coli* cytoplasmic membrane with high affinity, allowing a wide variety of dipeptides to enter. The periplasmic dipeptide-binding protein DppA acts as the initial receptor for dipeptides during their uptake through this system and also plays a role in chemotaxis toward dipeptides (Dunten and Mowbray, 1995). Interestingly, DppA was one of the 12 proteins up-regulated during short-term adaptation of *E. coli* to glucose-limitation (Wick et al., 2001) and was also included in the list of proteins up-regulated (10-fold) during biofilm growth of *S.* Typhimurium on silicone rubber tubing (Hamilton et al., 2009).

Another up-regulated protein during biofilm growth was RibB, a key enzyme of the riboflavin biosynthesis pathway (Kumar et al., 2010). In agreement with this observation, RibB protein was among the proteins showing significant upregulation in *Shewanella oneidensis* biofilms when compared to planktonic cells (De Vriendt et al., 2005).

One other protein expressed during biofilm growth was SseA which is a 3-mercaptopyruvate sulfurtransferase. A role of *sseA* gene in biotic surface colonization has previously been found in *S.* Enteritidis, since a *sseA* mutant was unable to colonise liver and spleen of mice 3 weeks post-infection (Karasova et al., 2009). In *S.* Typhimurium, SseA was found necessary for the intracellular infection of both macrophages and epithelial cells and was required for virulence in mice (Coombes et al., 2003).

The single-stranded DNA-binding protein (SSB) plays an important role in DNA metabolism, such as DNA replication, repair and recombination (Huang et al., 2011). Strangely, this protein, although

essential for cell survival, was found here to be expressed solely during biofilm growth. It is likely that the low level of SSB in planktonic cells was not detectable because of the manual method used for comparison of the protein levels in the two growth conditions. It's worth noting that SSB was one of the 22 proteins found to be highly overexpressed by cold shocked immomilized *E. coli* cells entrapped in 2% w/v solution of agar (Perrot et al., 2001).

Ths MltA-interacting protein MipA of *E. coli* is known to be involved in the metabolism of the murein sacculus (Vollmer et al., 1999). Interestingly and in agreement with current results, MipA had higher expression in sessile culture of *E. coli* O157 compared to planktonic culture (Rivas et al., 2008).

Regarding the other 10 "biofilm proteins" found here (BtuE, OsmY, SspA, YbbN, YhbO, Fur, SufC, GcvT, YibF and CsgF; Table 2), the first 5 (BtuE, OsmY, SspA, YbbN, YhbO) are known to play an essential role in cellular stress response mechanisms. Some representative studies related to the specific role of each one of these 10 proteins (mainly in *E. coli* strains) are presented in Table S3 (suppementory file). Substantial protection against the elevated oxidative stress that *Salmonella* biofilms may face could be provided by BtuE protein identified in the present study. BtuE from *E. coli in vitro* catalyzes the decomposition of a variety of peroxides, mainly lipid peroxides, using thioredoxins A or C as the reducing agents, while it was also shown that *btuE*, like other *E. coli* antioxidant genes, is induced under oxidative stress conditions (Arenas et al., 2010). The RpoS-regulated *osmY* gene encodes a periplasmic protein of unknown function that has been found to be induced by both osmotic and growth phase signals (Yim and Villarejo, 1992). The stringent starvation protein A (SspA) is a RNA polymerase-associated protein and in *E. coli* has been found important for the stress response during stationary phase and under nutrient-limited conditions (Williams et al., 1994). In the same bacterium, SspA is also essential for cell survival during acid-induced stress (Hansen et al., 2005). It is suggested that thioredoxin-like protein YbbN functions *in vivo* in *E. coli* as a chaperone rather than as an oxidoreductase and cooperates with DnaK for the optimal expression of several cytoplasmic proteins (Kthiri et al., 2008). YhbO protects *E. coli* cells against many environmental stresses (Abdallah et al., 2007). In accordance with its role in stress management, YhbO is overexpressed severalfold in stationary phase and during hyperosmotic and acid stresses (Weber et al., 2006).

The Fur (ferric uptake regulator) protein is the main regulator of iron homeostasis in many bacteria. In the Fe^{2+}-bound form, *E. coli* Fur represses genes involved in respiration, flagellar chemotaxis, the TCA cycle, glycolysis, methionine biosynthesis, phage DNA packaging, DNA synthesis, purine metabolism, and redox stress resistance. As a major regulator of gene expression, it also ultimately coordinates intracellular iron levels with many other cellular processes, including the expression of *S. enterica* pathogenicity island 1 (SPI1) and the control of nitrate/nitrite respiration by sensing cellular redox status (Teixidó et al., 2010, 2011). SufC is an ATPase component of the SUF (mobilization of sulfur) machinery, which is involved in the biosynthesis of Fe–S clusters, required as cofactors in a wide range of critical cellular pathways (Kitaoka et al., 2006). The *sufABCDSE* operon is required for de novo Fe-S cluster biogenesis under iron starvation and oxidative stress conditions in *E. coli* (Nachin et al., 2003; Outten et al., 2004).

In *E. coli*, the *gcvTHP* operon encodes the glycine cleavage system, which breaks down glycine to produce compounds involved in variety of pathways, including methionine and purine biosynthesis (Stauffer and Stauffer, 2005). YibF is a putative glutathione (GSH) transferase, believed to be involved in Se metabolism and detoxification (Rife et al., 2003). Finally, CsgF, which was found here to be expressed only under biofilm conditions, is known to be involved in assemply of curli fimbriae (Nenninger et al., 2009), which form, together with cellulose, the two main matrix components of *Salmonella* biofilms (Gerstel and Römling, 2003).

Strangely, among the 10 proteins whose expression was visible here only in the planktonic-derived samples (NmpC, TufA, FabI, PepQ, LpdA, ManX, BasR, PagC, YnaF and AtpC), two proteins were included (TufA and YnaF) that, contrary to our results, were overexpressed in 168 h-old *S.* Enteritidis biofilms relative to a planktonic cell control (Mangalappalli-Illathu et al., 2008a) (Table 3). This clearly indicates that biofilm communities seem to be very diverse and unique, not just to the microorganism, but to the particular environment in which they are being formed. With regard to the other "planktonic proteins", there is only one protein (AtpC) with possible involvement in biofilm events, besides its central role to ATP synthesis. Thus, in a study comparing biofilm formation between *E. coli* wild type and *rpoS* mutant strain, *atpC* gene was found to present increased expression in the mutant strain biofilm, but not in the WT strain biofilm (Ito et al., 2008). To the best of our knowledge, the other 7 proteins, whose expression was found here to be visible only during planktonic growth, have never been reported before in any other biofilm related study.

To sum, present results clearly show that under surface-associated growth *Salmonella* over-produces proteins mainly related to stress

Table 3
List of 3 proteins (out of 10) whose expression was visible only during planktonic growth which, however, are also known to be implicated in biofilm formation and/or other related events in other bacteria.

Protein	Protein function	Other published studies related to the putative role of gene/protein of interest in biofilm formation and/or other related events in *Salmonella* and other microbial species			
		Strain(s)	Experimental setup	Key conclusions (related to gene/protein of interest)	Reference
TufA	Protein biosynthesis (elongation factor)	*Salm. enterica* Enteritidis ATCC4931	The influence of hydrodynamic conditions on the formation and maintenance of biofilms formed by *S.* Enteritidis (on glass coverslips in flow cells) was studied	→ TufA became up-regulated in 168 h-old *S.* Enteritidis biofilms relative to a planktonic cell control	Mangalappalli-Illathu et al. (2008a)
YnaF	Putatitve universal stress protein	*Salm. enterica* Enteritidis ATCC4931	The influence of hydrodynamic conditions on the formation and maintenance of biofilms formed by *S.* Enteritidis (on glass coverslips in flow cells) was studied	→ YnaF became up-regulated in 168 h-old *S.* Enteritidis biofilms relative to a planktonic cell control	Mangalappalli-Illathu et al. (2008a)
		Salm. enterica Enteritidis ATCC4931	The development of adaptive resistance of *S.* Enteritidis biofilms following exposure to benzalkonium chloride (BC) either continuously (1 μg ml^{-1}) or intermittently (10 μg ml^{-1} for 10 min daily) was examined	→ YnaF was included in the list of proteins up-regulated in *S.* Enteritidis biofilm cells exposed to BC	Mangalappalli-Illathu and Korber (2006)
AtpC	ATP synthesis	*Escherichia coli* MG1655	*E. coli* wild type (WT) and *rpoS* mutant strains were used to compare biofilm formation capacity (on glass surfaces in flow cells) and global gene expression	→ *atpC* gene showed increased expression in the mutant strain biofilm, but not in the WT strain biofilm	Ito et al. (2008)

management, something rather expected, supporting the well established view that biofilms are examples of multicellular behavior which enhance the capacity of microorganisms to survive multiple stresses (Anderson and O'Toole, 2008; Coenye, 2010; Fux et al., 2005; Mah and O'Toole, 2001; Seneviratne et al., 2012). Thus, almost half (8 out of 20) of the "biofilm proteins" identified here are known to be implicated in stress response pathways (ArcA, BtuE, Dps, OsmY, SspA, TrxA, YbbN and YhbO). Proteins involved in nutrient transport (Crr, DppA, Fur and SufC), together with ones involved in the complementary processes of degradation and energy metabolism (GcvT, GpmA and RibB) form the other important group of proteins found here to be induced under biofilm conditions. Three of these last proteins (Crr, DppA and GpmA) had also been previously found to be up-regulated in *Salmonella* biofilms (Hamilton et al., 2009; Mangalappalli-Illathu et al., 2008a;Table S1). Crr and DppA are involved in sugar and dipeptide membrane transport, respectively, while GpmA is a key enzyme (phospoglyceromutase) of glycolysis pathway. Undoubtedly, this small amount of proteins overlapping between the different *Salmonella* biofilm studies significantly complicates our efforts to unravel the cellular physiology of this pathogenic bacterium under biofilm conditions. Obviously, our research efforts to identify the core genes and/or proteins that are required for survival of this pathogen on different surfaces should continue preferably by using in combination approaches based on transcriptomics, mutagenesis, metabolomics and proteomics to study *Salmonella* biofilms grown in dissimilar model systems.

Overall, the results obtained here and in combination with other previously published results (Hamilton et al., 2009; Mangalappalli-Illathu et al., 2008a,b; White et al., 2010), extend our knowledge on the physiology of *Salmonella* once being enclosed in a biofilm structure formed on a typical food contact surface. However, although differential protein expression was found between planktonic and sessile cells, further investigations are undoubtedly required to determine the specific role of each one of these identified proteins during biofilm growth of this pathogen. In addition, we should always keep in mind that biofilms are microbial communities known to present great genetic and physiological hererogeneity, even those formed by the same microorganism under different environmental conditions (Stewart and Franklin, 2008). Unambiguously, the ability to recognize "how and why" *Salmonella* attach to food-contact surfaces and form biofilms on them is an important area of focus, since a better understanding of this ability may provide valuable ways toward the elimination of this pathogenic bacterium from food processing environments and eventually lead to reduced *Salmonella*-associated human illness.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2012.12.023.

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