Characterization of surface proteins of *Cronobacter muytjensii* using monoclonal antibodies and MALDI-TOF Mass spectrometry

Ziad W Jaradat1*, Abrar M Rashdan1, Qotaiba O Ababneh1,2, Saied A Jaradat1,3 and Arun K Bhunia4

**Abstract**

**Background:** *Cronobacter* spp. is a newly emerging pathogen that causes meningitis in infants and other diseases in elderly and immunocompromised individuals. This study was undertaken to investigate surface antigenic determinants in *Cronobacter* spp. using monoclonal antibodies (MAbs) and MALDI-TOF Mass spectrometry.

**Results:** Spleenocytes from mice that were immunized with heat-killed (20 min, 80°C) *Cronobacter* cells were fused with SP2 myeloma cells. Five desirable MAbs (A1, B5, 2C2, C5 and A4) were selected. MAbs A1, B5, 2C2 and C5 were of IgG2a isotype while A4 was an IgM. Specificity of the MAbs was determined by using immunoblotting with outer membrane protein preparations (OMPs) extracted from 12 *Cronobacter* and 6 non-*Cronobacter* bacteria. All MAbs recognized proteins with molecular weight ranging between 36 and 49 kDa except for one isolate (44) in which no OMPs were detected. In addition, MAbs recognized two bands (38-41 kDa) in four of the non-*Cronobacter* bacteria. Most of the proteins recognized by the MAbs were identified by MALDI-TOF peptide sequencing and appeared to be heterogeneous with the identities of some of them are still unknown. All MAbs recognized the same epitope as determined by an additive Index ELISA with their epitopes appeared to be conformational rather than sequential. Further, none of the MAbs recognized purified LPS from *Cronobacter* spp. Specificity of the MAbs toward OMPs was further confirmed by transmission electron microscopy.

**Conclusions:** Results obtained in this study highlight the immunological cross-reactivity among *Cronobacter* OMPs and their *Enterobacteriaceae* counterparts. Nevertheless, the identity of the identified proteins appeared to be different as inferred from the MALDI-TOF sequencing and identification.

**Background**

*Cronobacter* spp. (formerly *Enterobacter sakazakii*) is a non-spore forming, motile, facultative anaerobic Gram-negative bacillus and belongs to family *Enterobacteriaceae* [1,2]. Initially isolates of *Cronobacter* spp. (*Cronobacter*) were identified as yellow pigment producing *Enterobacter cloacae*. Later, Farmer et al. [3] reclassified them as a new species and were given the name *sakazakii* based on DNA-DNA homology, antibiotic susceptibility patterns and certain unique biochemical characteristics such as catalase production, the absence of oxidase and the production of yellow pigment in all tested strains. More recent studies utilizing full length 16S rRNA gene sequencing, ribotyping, fluorescent-amplified fragment length polymorphism and DNA-DNA hybridization have demonstrated that *Cronobacter* is a heterogenic genus exhibiting a high degree of genetic and phenotypic diversity among species and comprises six species: *C. muytjensii*, *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. dublinensis* and *C. genomospecies I* [4-7]. *Cronobacter* is considered an emerging pathogen; though, little is known about its virulence properties and antigenic determinants [8]. Recently, several studies have reported the involvement of an outer membrane protein (OMP), OmpA, in pathogenesis of *C. sakazakii*; however, nothing is known about its antigenicity. Besides, little is known about OMPs from other *Cronobacter* species [8-10]. In contrast, the virulence and antigenic properties of OMPs of closely related *Enterobacter*...
species including *E. aerogenes* [11] and *E. cloacae* [12,13] were studied well.

Prematurely born infants with low birth weights and infants in neonatal intensive care units are highly susceptible to *Cronobacter* infections with the pathogen being transmitted primarily from contaminated environments to the infant formula during the preparation [14-20]. In rare cases, nosocomial infections can happen in adults especially in immunocompromised ones [21]. In 2004, a joint FDA/WHO workshop raised an alert concerning the presence of *Cronobacter* in powdered infant formula (PIF) and recommended applying higher microbiological standards during its manufacturing [22]. This warning culminated into increased research efforts to study *Cronobacter* including the development of improved isolation and identification methods, and understanding of the growth and survival characteristics.

Antibodies are the most frequently used tools to study bacterial antigenic determinants; however, little is known about the production of monoclonal antibodies that recognize *Cronobacter* antigenic determinants. In this paper we describe the production and characterization of 5 MAbs that recognize outer membrane proteins of *Cronobacter*. In addition, antigenic properties, identification, distribution and cell surface localization of the MAbs-recognized OMPs were examined using electron microscopy and MALDI-TOF spectrometry. To our knowledge, this is the first report on using monoclonal antibodies to study the surface antigens of this pathogen.

**Methods**

**Materials**

Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin, complete Freund’s adjuvant, incomplete Freund’s adjuvant, sarkosyl, DMSO, pancreatic RNase and DNase and a mouse subsotyping kit were from Sigma-Aldrich, USA. Gold-conjugated (18 nm) anti-mouse IgG was obtained from Jackson Immunochimicals, USA. Polyethylene glycol 4000 was from Fluka, USA. Micro test plates, tissue culture plates and flasks were from Griener, Germany. Coomassie Brilliant blue G-250 was from BDH chemicals, Ireland and BSA was from Biobasic, Canada; Proteinase K was from Promega, GMBH, Austria. Recovery cell culture freezing media was from Griener, Germany. Coomassie Brilliant blue R-250 was mixed (1:1) with 30% (w/v) polyacrylamide solution; ammonium persulfate (50 μl) and TEMED (10 μl) were added to the mixture to obtain a 15% polyacrylamide gel (w/v) [24]. The gel-containing LPS was frozen in liquid nitrogen and ground with a pestle and mortar into a fine powder. The powder was dissolved in 10 ml PBS (0.1 M, pH 7.0) and immediately used for immunization [25].

**Lipopolysaccharide (LPS) extraction and antigen preparation**

LPS was prepared following the method described by Jaradat and Zawistowski [23], with minor modifications. Briefly, *C. muytjensii* ATCC 51329 cells were harvested from an overnight culture by centrifugation (5,000 × g, 10 min) and resuspended in 50 ml of 50 mM sodium phosphate buffer, pH 7.0. The cells were sonicated 5 times for 45 s intervals at 300 Watts (Branson Sonifier). The sonicated suspension was incubated with pancreatic RNase and DNase (0.1 μg ml⁻¹) in 20 mM MgCl₂ at 37°C for 10 min, followed by 10 min at 60°C and then mixed with an equal volume of preheated 90% phenol. Following incubation (70°C for 15 min) with occasional mixing, the mixture was centrifuged (18,000 × g, 1 h) and the resulting aqueous layer was collected and dialyzed using dialysis tubing of 6,000-8,000 MW cutoff at room temperature against several changes of distilled water until no detectable phenol odor remained. The dialysate was treated with 20 μg/ml of Proteinase K in 0.1 M Tris-HCl (pH 8.0) at 60°C for 1 h followed by overnight incubation at 37°C. The samples were then lyophilized and stored at -20°C until used. For antigen preparation, the extracted LPS from *Cronobacter* was mixed (1:1) with 30% (w/v) polyacrylamide solution; ammonium persulfate (50 μl) and TEMED (10 μl) were added to the mixture to obtain a 15% polyacrylamide gel (w/v) [24]. The gel-containing LPS was frozen in liquid nitrogen and ground with a pestle and mortar into a fine powder. The powder was dissolved in 10 ml PBS (0.1 M, pH 7.0) and immediately used for immunization [25].

**Outer membrane protein extraction**

OMPs were extracted using the sarkosyl-based method described by Davies et al. [26]. Briefly, *Cronobacter* cells were harvested from overnight cultures by centrifugation, and then treated with 0.1 μg of bovine RNase and DNase.
in 20 mM MgCl₂ for 10 min at 37°C. Next, the cells were sonicated for 10 min in 45 sec intervals at 300 watts on crushed ice and were centrifuged (5,000 × g for 30 min at 4°C). The supernatant was collected and re-centrifuged (29,000 × g for 2 h at 4°C). The resulting pellet was treated with 10 ml of 2% (w/v) sarkosyl for 30 min at room temperature. The mixture was centrifuged (29,000 × g for 2 h at 4°C). The final pellet, which contained OMPs, was resuspended in distilled water, aliquoted and stored at -20°C for further use.

Production of monoclonal antibodies against Cronobacter spp

Female Balb/c mice (6 to 8 weeks old) were initially immunized intraperitoneally with 200 µl (10⁸ CFU ml⁻¹) of heat-killed bacterial suspension (C. muytjensii ATCC 51329) mixed with complete Freund adjuvant at a 1:1 ratio. Subsequently, 4 booster doses were administrated at weekly intervals using the same amount of immunogen but prepared with incomplete Freund adjuvant. Simultaneously, female Balb/c mice (6 to 8 weeks old) were immunized intraperitoneally with 200 µl of polyacrylamide-LPS preparation in PBS for at least 8 wks at weekly intervals. Myeloma SP2 cells were maintained in RPMI media supplemented with 10% Fetal Calf Serum (FCS), 20 U of penicillin, 20 U streptomycin and 2.5 µg ml⁻¹ amphotercin B. At the day of fusion, the actively grown myeloma culture was washed twice using serum-free media (SFM) and adjusted to the desired concentration. The fusion was performed according to the method described by Liddell and Cryer [27] using 40% (w/v) polyethylene glycol 4000 as the fusing agent in sterile SFM adjusted to pH 7.4. Spleen cells harvested from immunized mice and myeloma cells were fused at a ratio of 8:1. Two weeks later, hybridomas were screened for the production of MAbs by ELISA using heat killed Cronobacter and non-Cronobacter cells. Hybridomas reacting specifically with Cronobacter were expanded and cloned at least three times by limiting dilution. Positive clones were frozen in recovery cell culture freezing media® or FCS supplemented with 4% (v/v) DMSO and stored at -80°C overnight before being transferred to liquid nitrogen. The positive clones were propagated either in tissue culture or by ascitic fluid using the procedure of Harlow and Lane [28]. Isotypes of purified monoclonal antibodies from ascites or spent medium were determined using the mouse type subisotyping kit according to the manufacturer’s instructions.

Table 1 Cronobacter and Non-Cronobacter strains used in this study

| Isolate # | Isolate identity | Source | Isolate ID | GenBank ID based on 16S rRNA sequence |
|-----------|-----------------|--------|------------|---------------------------------------|
| -         | C. muytjensii   | -      | ATCC 51329 | -                                     |
| C4        | C. sakazakii    | Clinical |            | -                                     |
| C6        | C. sakazakii    | Clinical | CDC 407-77 | -                                     |
| C13       | C. sakazakii    | Clinical | ATCC 29004 | -                                     |
| Jor* 44   | C. sakazakii    | Food   | EMCC 1904  | FJ906902                              |
| Jor* 93   | C. sakazakii    | Food   | EMCC1905   | FJ906906                              |
| Jor* 112  | C. muytjensii   | Food   | EMCC1906   | FJ906909                              |
| Jor* 146a | C. sakazakii    | Food   | EMCC1907   | FJ906897                              |
| Jor* 146b | C. sakazakii    | Food   | EMCC1908   | FJ906910                              |
| Jor* 149  | C. muytjensii   | Food   | EMCC1909   | FJ906912                              |
| Jor* 160a | C. sakazakii    | Environment | EMCC1910 | FJ906914                              |
| Jor* 170  | C. turicensis    | Food   | EMCC1912   | FJ906916                              |

None -Cronobacter

- C. freundii | - | ATCC 43864 | - |
- E. coli | - | ATCC 35218 | - |
- L. ivanovii | - | ATCC 19119 | - |
- P aeruginosa | - | ATCC 27833 | - |
- S. enterica Choleraesuis | - | CIP 104220 | - |
- S. sonnei | - | ATCC 9290 | - |

Jor*: Strains were isolated from food and environmental samples collected in Jordan and were deposited in the Egyptian Microbial Culture Collection (EMCC; Ain Shams University, Cairo, Egypt) and their 16S rRNA sequences were deposited in the GenBank. C: clinical samples isolated from patients obtained from CDC (Atlanta, GA, USA) and were a gift from Dr. Ben Davies Tall from U.S. FDA. All the other isolates were obtained from the American Type Culture Collection (ATCC) except for Salmonella which obtained from the Collection of Institute Pasteur (CIP) and S. sonnei which was a local strain.
**Immunochemical Methods**

**Elisa**

Screening of antisera spent medium and ascites for the presence of antibodies against *Cronobacter* was performed by an indirect non-competitive ELISA. Flat-bottom 96 well plates were coated with 0.1 ml of (10^8 heat-killed cells ml^-1) of whole cell antigen diluted in 0.05 M carbonate buffer (pH 9.6) overnight at 4°C. Alkaline phosphatase-conjugate goat anti-mouse immunoglobulin and p-nitrophenyl phosphate were used as secondary antibodies and substrate, respectively.

**Additive index elisa**

Additive index ELISA was performed on paired MAbs as described by Friguet et al., [29]. An additive index for each pair of MAbs was calculated according to the formula \([2A_{12}/(A_1 + A_2)] - 1\) \times 100, where \(A_1\), \(A_2\), and \(A_1 + A_2\) are absorbance values with antibody 1 alone, antibody 2 alone, and the two antibodies together, respectively.

**Gel electrophoresis**

Profiles of *Cronobacter* OMPs were examined using SDS-PAGE following the method described by Laemmli [30]. The runs were performed in 4% stacking and 12.5% separating gels. Equal concentrations of *Cronobacter* OMPs (20 μg well^-1) were mixed with sample buffer at a ratio of 1:5, boiled for 5 min and loaded (approx. 20 μl/lane). Gels were either stained with 1% (w/v) Coomassie Brilliant Blue G-250 or used for immunoblotting. Likewise, LPS preparations from *Cronobacter* were examined using Deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE) following the method described by Reuhs et al., [31]. Briefly, the runs were performed using 4% (v/v) stacking and 12.5% (v/v) separating gels. Equal concentrations of *Cronobacter* LPS (5 μg well^-1) were mixed with sample buffer [2 ml of 22.7% (w/v) Tris-base solution; 1 ml of 50% (v/v) glycerol; 1 ml of 1% (w/v) bromophenol blue and 6 ml distilled water] at a ratio of 1:5. The gels were pre-run in DOC-electrophoresis buffer (Tris-base, 4.5 g; glycine, 21.7 g; 2.5 g sodium deoxycholate, pH adjusted to 8.3 and volume adjusted to 1 liter) for 10 min at 80 volts before loading the samples. Samples were run in the same buffer at 80 and 120 volts for the stacking and separating gels, respectively. Upon completion, gels were either stained using the PageSilver™ silver staining kit (Fermentas) or were used for immunoblotting. Band sizes for both gels and blots were estimated using the BioRad Quantity One 1-D Analysis Software.

**Immunoblotting**

Immediately after completing the electrophoresis run, OMPs and LPSs were transferred onto NC using a mini-transblot cell (Bio-Rad). The membranes were blocked with 3% (w/v) BSA in Tris Buffered Saline (TBS) containing Tween 20 (0.05% v/v). NC membranes were then incubated with affinity purified MAbs (2 μg ml^-1) diluted in 0.15 M TBS buffer containing 1% (w/v) BSA with gentle shaking for 1 h. Membranes were then developed with goat anti-mouse-HRP in 0.15 M TBS buffer containing 1% (w/v) BSA and a diaminobenzidine (DAB) substrate solution. Color development was stopped by rinsing the membranes with distilled water.

**Protein sequencing and identification**

Extracted OMPs were separated on SDS-PAGE gels and probed with anti-OMP monoclonal antibodies. Immunoblot-positive bands were cut with sterile sharp scalpels and immersed in 1% acetic acid solution. Protein sequencing was performed using the MALDI-TOF technology at the Proteomics and Mass Spectrometry Facility at Purdue University (West Lafayette, Indiana, USA).

**Dot blot assay**

Dot blotting was performed as described by Jaradat and Zawistowski [23]. One microliter of heat-killed *Cronobacter* whole-cell suspension (10^8 cells ml^-1) was spotted on the NC membranes, allowed to air dry for 30 min and incubated in 5% (w/v) NaOH or in 38% (v/v) HCl for 10 s or left untreated. Immunoblotting was performed as described above.

**Immunoelectron microscopy**

Immunolabeling was performed essentially as described by Jaradat and Zawistowski [23] with modifications. Briefly, 5 μl of bacterial suspension in distilled water (5 x 10^6 CFU ml^-1) were placed on formvar-coated copper grids. After air-drying for 2 h at room temperature, the grids were blocked with PBS containing 3% (w/v) BSA for 30 min at 37°C. To expose antigens on bacteria, grids were incubated with 0.1 M NaOH or 0.1 M HCl for 2 h, washed with water and incubated with purified MAb solution at 37°C. Grids were then incubated with colloidal gold (18 nm)-conjugate anti-mouse IgG diluted at 1:50 in dilution buffer (0.02 M Tris, 150 mM NaCl, 0.1% [w/v] BSA, 0.005% [v/v] Tween 20, 0.4% [w/v] gelatin [pH 9]) for 20 h at room temperature. Grids were washed 6 times with water and viewed with a Zeiss Transmission Electron Microscope at various magnifications.

**Animal use**

Animals used for immunization and production of monoclonal antibodies were cared for according to the Animal Care and Use Committee (ACUC), Jordan University of Science and Technology.

**Results**

Two approaches were attempted to produce monoclonal antibodies specific to *Cronobacter* spp.: one group of...
mice was immunized with heat-killed *C. muytjensii* ATCC 51329, while the other group was immunized with LPS purified from *C. muytjensii* ATCC 51329. Repeated immunization with the LPS produced a good antibody response as judged from both ELISA and immunoblotting results using antisera from LPS-immunized mice which revealed the characteristic ladder pattern of LPS (Figure 1). However, none of the two immunization protocols resulted in a stable hybridoma producing anti-LPS antibodies. Nevertheless, mice immunized with heat-killed cells responded well yielding a high titer after 5 injections. Consequently, mice from this group were sacrificed and two fusions were performed yielding over 500 hybridomas of which approximately 180 clones were positive upon initial screening and were cloned 3 times by limiting dilution [32-34]. Of these, only 5 stable hybridomas secreted antibodies against *Cronobacter* spp. Four of the hybridomas were of IgG type (A1, B5, 2C2 and C5), while the last hybridoma (A4) was of the IgM class. The avidity of the MAbs to their epitopes was determined by ELISA. The titration curve for all protein G-column purified MAbs, except for A4, revealed that MAb-2C2 had the highest avidity followed by C5, B5 and A1 having the lowest. MAb A4, an IgM, was not tested as it was not purified by Protein G column affinity chromatography.

**Specificity of the monoclonal antibodies**

The specificity of the MAbs was determined by non-competitive ELISA with various heat-killed bacteria belonging to *Cronobacter* and non-*Cronobacter* spp. In general, all MAbs reacted with both *Cronobacter* and non-*Cronobacter* spp. with higher titers generally obtained for *Cronobacter* spp. (Titer of 3200 *Cronobacter* versus 400 for some non-*Cronobacter*). Nevertheless, some non-*Cronobacter* spp. also gave titers comparable to those obtained for *Cronobacter* (Titer 3200). The binding affinities varied among the four MAbs with MAbs 2C2 and C5 gave titers of 3200 against almost all the heat-killed *Cronobacter* strains tested, whereas MAbs A1 and B5 had titers ranging between 800 to more than 6400.

![Figure 1](http://www.biomedcentral.com/1471-2180/11/148)
In addition to ELISA, the antigenic specificity of all purified MAbs was tested against OMPs extracted from 12 Cronobacter and 6 non-Cronobacter strains by SDS-PAGE followed by immunoblotting.

SDS-PAGE profiles of both Cronobacter and non-Cronobacter revealed the presence of several proteins with molecular weights ranging from 12 to 100 kDa (data not shown) with the majority of OMPs profiles contained 3 to 5 major proteins having molecular weights between 34 and 55 kDa. Upon immunoprobing, all MAbs produced similar reaction profile of proteins with molecular weights between 36 to 49 kDa with only one major band for all Cronobacter OMP profiles except isolate Jor44 which did not exhibit any protein (Figure 2, lane 7, C. sakazakii, lower panel). In general, the 36 kDa protein was the most common among all Cronobacter OMP profiles. It was detected by immunoblotting in 5 out of the 12 tested Cronobacter strains (Figure 2, lower panel; lanes, 5, 6, 9, 10, 11; C. sakazakii, C. turicensis, C. sakazakii, C. sakazakii, C. muytjensii, respectively), and a 42 kDa protein was detected in two C. sakazakii isolates (Figure 2, lower panel; lanes, 1 and 12), while a 41 kDa protein was detected in two isolates (C. sakazakii, and C. muytjensii, lower lanes 4 and 8 respectively). In addition, proteins of 37 and 39 kDa were detected each in one C. sakazakii isolate (Figure 2, lower panel; lanes 2 and 3, respectively). OMPs from a number of non-Cronobacter species were also tested for reactivity against the purified MAbs by immunoblotting. All MAbs (A1, B5, 2C2, C5 and A4) displayed strong and specific reaction against a 44 kDa OMP (Figure 4). Although these MAbs were produced from two different fusion experiments, they all reacted strongly and specifically against the same 44 kDa OMP. Furthermore, to compare the epitope specificity of all MAbs, additive index ELISA was conducted for each pair of the MAbs. All scores obtained were very low and fall in the range of 0 to 10 indicating that all MAbs were raised against the same epitope within the 44 kDa OMP.

Protein identification by MALDI-TOF peptide sequencing

Representatives of the immunoblot-positive proteins were subjected to peptide sequencing and identification using MALDI-TOF Mass spectrometer. Table 2 shows the identified proteins by MALDI-TOF. The 44 kDa protein that was recognized by all the monoclonal antibodies in C. sakazakii appeared to be a novel protein that did not match with any identified protein thus was termed a hypothetical protein.

In addition, the 35 kDa protein identified in the Cronobacter isolate 146A also appeared to be a novel protein termed a hypothetical protein that did not match...
with any known protein sequence deposited in the protein sequence bank (Table 2). Two *Cronobacter* isolates (160A and C13) exhibited a 42 kDa protein with identity as a flagellar hook protein FlgE and an outer membrane porin protein in the two isolates respectively. Further, a 40 kDa protein was recognized in *Cronobacter* isolate 112, and appeared to be an outer membrane protein F which is similar to an outer membrane protein F in *E. coli*. Both *E. coli* and *Salmonella* contained another similar protein with a MW of 38 kDa and was identified as an outer membrane protein A. In addition, both exhibited a 35 kDa porin protein yet appeared to be somewhat different.

**Effect of different treatments of antigens on MAbs binding affinity**

To gain insights about the nature of the binding between the MAbs and their target epitopes, ELISA and Dot-blot were carried out using different antigens (OMPs, heat killed bacterial cells, LPS) which were subjected to different treatments (acid, alkaline, denaturing agents and heat) (Figure 5). Acid and base-treatments of whole cell antigens resulted in an increase in the binding affinity between the MAbs and those antigens. These results were confirmed by immunoelectron microscopy. *Cronobacter muytjensii* ATCC 51329 cells displayed intense colloidal gold labeling after reaction with MAb 2C2 (similar results were obtained with the other MAbs) (Figure 6).

Finally, to determine whether the MAbs recognized sequential (Linear) or conformational epitopes, OMPs were either left intact or denatured by 1% (w/v) SDS and boiled for 5 min and then used as antigens for ELISA. The magnitude of binding of MAbs to antigens was higher for untreated OMPs than the denatured proteins (Table 3). This indicates that, the epitope is conformational and loses its recognition sites once denatured.


**Discussion**

Antibodies against surface antigens of pathogens aid not only in characterization but also in their classification [35]. In this study monoclonal antibodies were produced against outer membrane proteins of Cronobacter muytjensii. However, we were unable to produce antibodies against LPS. Inability to produce stable hybridomas against LPS could be attributed to the simplicity of the LPS structure which is a linear unbranched chain of repeating polysaccharide units as reported by MacLean et al., [7]. The linearity of the structure was probably responsible for the inability to elicit a significant immune response which was reflected on the inability to produce monoclonal antibodies against LPS of this strain. Luk and Lindberg [36] initially failed to produce stable antibody-producing hybridomas against LPS of Salmonella. Later, they succeeded when they used whole bacterial cells coated with LPS as immunogen. Similarly, Jongh-Leuvenink et al., [37] and Jaradat and Zawistowski [23] were able to produce monoclonal antibodies against LPS of Salmonella. This could be due to differences in the nature of the structure and composition of LPS between Salmonella and Cronobacter spp. and even among different Salmonella serovars.

In this study, the anti-OMP antibodies were characterized for specificity and all 5 monoclonal antibodies not only reacted with Cronobacter species, but also recognized other Enterobacteriaceae. The low specificity indicates that the major outer membrane proteins in the family Enterobacteriaceae are perhaps well conserved as indicated by their antigenic cross-reactivity. The specificity of the monoclonal antibodies was further tested using SDS-PAGE and immunoblotting. The SDS-PAGE protein profiles for the OMPs observed in this study were similar to those of OMPs described by other researchers for other members of the Enterobacteriaceae [38,39]. Overall, most of the isolates contained OMP proteins with MW ranging from 34-55 kDa (Figure 2 upper panel) with majority of the isolates exhibiting proteins in the range of 36-49 kDa with the 49 kDa protein appeared in all Cronobacter species (Figure 2 upper panel). In contrast, the non-Cronobacter isolates (Figure 3) showed slightly different protein profiles among the Enterobacteriaceae members and even a slight shift in the tested Gram-positive strain, L. ivanovii. The cross-reactivity observed among all Cronobacter strains used in this study indicated that some of these OMPs share common and highly antigenic epitopes. These patterns of cross-reactivity of MAbS with OMPs from bacterial strains within the same species are commonly reported especially for members of the Enterobacteriaceae [38-42]. On the other hand, fewer studies have reported the production of anti-OMP MAbS within species that were non-cross reacting and exhibiting a high degree of specificity [43,44]. The reactivity of MAbS to OMP and the lack of any reactivity against LPS indicated that Cronobacter OMPs appeared to be more antigenic than their LPS. This observation coincides with several other reports in which it was demonstrated that OMPs were stronger immunogenes than LPS, and were responsible for producing antibodies with higher affinities [45,46].

All MAbS tested by immunoblotting against OMPs extracted from C. muytjensii ATCC 51329 were able to recognize a 44 kDa protein. This protein appears to contain a highly antigenic epitope capable of eliciting strong immune response in mice against the Cronobacter strain used in the immunization procedure. The identity of this protein was determined by MALDI-TOF MS to be a hypothetical outer membrane protein ESA_03699 [Enterobacter sakazakii ATCC BAA-894]. This protein appeared to be dominant in this particular strain and protruding to the surface making it highly accessible to the host immune system. The specific function of this

**Table 2** Protein bands identified by MALDI-TOF mass spectrometer

| Band | Strain            | Predicted MW (kDa) | Protein annotation (NCBI database) | Accession No.                  | No. of peptides identified by MS/MS |
|------|-------------------|--------------------|-----------------------------------|-------------------------------|-----------------------------------|
| 1    | 160(C. sakazakii) | 42                 | Flagellar hook protein FlgE [Shigella sonnei Ss046] | gi|74311638                        | 1                                 |
| 2    | Escherichia coli  | 35                 | Outer membrane protein (porin) [Escherichia coli B171] | gi|75211632                        | 5                                 |
| 3    | Escherichia coli  | 38                 | Outer membrane protein A [Escherichia coli 536] | gi|110641146                       | 7                                 |
| 4    | Salmonella CIP    | 35                 | Outer membrane protein (porin) nmcp precursor [Escherichia coli CFT073] | gi|264247429                       | 6                                 |
| 5    | Salmonella CIP    | 38                 | Outer membrane protein A [Escherichia coli 536] | gi|110641146                       | 8                                 |
| 6    | C13(C sakazakii)  | 42                 | P COG3203: Outer membrane protein (porin)[Escherichia coli 101-1] | gi|83587007                        | 1                                 |
| 7    | 112 (C. muytjensii) | 40                 | Outer membrane protein F [Escherichia coli SMS-3-5] | gi|170682361                       | 1                                 |
| 8    | 146, (C. sakazakii) | 35                 | Hypothetical protein ESA_02413 [Enterobacter sakazakii ATCC BAA-894] | gi|156934579                       | 8                                 |
| 9    | C. muytjensii ATCC | 44                 | Hypothetical protein ESA_03699 [Enterobacter sakazakii ATCC BAA-894] | gi|156935823                       | 3                                 |
protein is unknown but it would be of significant interest in future studies since it was not detected in other strains. Other proteins from Cronobacter and non-Cronobacter (E. coli and Salmonella) recognized by the MAbs were also sequenced and aligned against known protein sequences deposited in protein sequence banks. It appeared that most of these proteins are related in terms of their structure and probably function as most of them were outer membrane proteins. However, a 42 kDa protein that was identified in two different Cronobacter spp. appeared to be different both in structure and function as one appeared to be a flagellar protein (Cronobacter 160A), while the second was identified as an outer membrane protein (Cronobacter C13). Further, as shown in Table 2 some of the proteins with the same MW (e.g. 35 kDa) were identified in three different bacteria and each appeared to have a different peptide sequence and consequently different function yet share epitope similarity as they were all recognized by the same MAb indicating a similar function too. Interestingly, similar to the 44 kDa protein, the 35 kDa protein identified in Cronobacter isolate number 146A appeared as novel protein and was termed as a hypothetical protein ESA_02413 with unknown function. Further, a protein of 40 kDa MW was identified in Cronobacter isolate number 112 as an outer membrane protein F which is similar to a protein in other E. coli as revealed from the protein bank sequence (Table 2).
Figure 6 Transmission electron micrographs of *C. muytjensii* ATCC 51329 treated with 0.1 N NaOH A, or 0.1 N HCl B and probed with MAb 2C2 followed by goat anti-mouse Ig conjugated to 18 nm gold spheres. Magnification × 50,000.
The findings in the current study provide an evidence of great similarity among Cronobacter spp. and the other members of Enterobacteriaceae. Such findings were comparable to several previous studies which reported similar cross reactivity among major OMPs in Gram negative bacteria and among members of the Enterobacteriaceae [38-42]. For example, monoclonal antibodies that recognized buried epitopes of theompC from Salmonella typhi were shown to cross react with porins extracted from 13 species of Enterobacteriaceae [41]. In addition, it appeared that OMPs extracted from Cronobacter and non-Cronobacter spp. in this study shared similar epitopes. This was evident in the multiple proteins which were recognized by the same MAbs that appeared to be specific toward the 44 kDa OMP extracted from the Cronobacter strain used forimmunization. Indeed, these results highlighted the heterogeneityof the OMPs in the Cronobacter isolates.

The effect of acid or base treatment on the reactivity of monoclonal antibodies to their antigens was investigated. Acid or base treatment increased binding affinity of the antibodies to Cronobacter cells. This might be due to an increase in the accessibility of MAbs to the surface protein antigens due to removal of some extracellular molecules and/or LPS that might have hindered the binding of MAbs to their target proteins in the case of whole bacterial cells. For example, LPS accounts for up to 70% of the outer monolayer [47]. Indeed, the masking effect of LPS against binding of antibodies to antigens has been reported and therefore it can not be under estimated [48]. These observations were further confirmed by immuno electron transmission microscopy (Figure 6). When live untreated Cronobacter cells were probed with MAb 2C2, there was no binding to the primary antibodies and hence no gold particle labeling. However, when Cronobacter cells were treated with NaOH (Figure 6A) or HCl (Figure 6B), the antibodies appeared to have gained access to their target represented by increased labeling. In addition, the MAbs were shown to be bound more strongly to conformational rather than sequential (linear) epitopes highlighting the specificity of the MAbs to their epitopes as appeared in Table 3[41].

**Conclusions**

To our knowledge, this is the first study that describes the production of monoclonal antibodies against whole cells of C. muytjensii with concomitant identification of the recognized proteins by MALDI-TOF spectrometry. All MAbs produced in this study were reactive against the whole cell antigen and Cronobacter OMPs. MAbs reacted with OMPs of molecular weight ranging between 36 and 49 kDa. However, none of the MAbs showed any reaction with LPS extracted from Cronobacter. All MAbs recognized conformational epitopes rather than sequential as it is evident from the decrease in their binding affinity to fully denatured OMP antigens. Moreover, all MAbs exhibited a high cross-reactivity against the whole cell antigen and OMPs from non-Cronobacter. As apparent from the MALDI-TOF protein identification, the overall results indicated that, the major OMPs found in the Enterobacteriaceae are sufficiently conserved thereby, promoting antigenic cross-reactivity between genera. Furthermore, the single-banding pattern and the high titers obtained in immunoblotting and ELISA for the Cronobacter strains indicated that the OMPs of closely related strains are more conserved compared with other genera evaluated. The results from this study can be of great help for possible vaccine production against this pathogen in infants and young children.

**Acknowledgements**

The authors would like to acknowledge the Deanship of Research at Jordan University of Science and Technology for funding this research project (project number 85/2008). In addition, the authors extend their deep gratitude for Professor Greg Blank, from the University of Manitoba, for his critical review of the manuscript and Hyochin Kim from Purdue University for assistance with MALDI-TOF analysis of proteins, and Muneeer Khodor, from Yarmouk University, for his assistance with Electron microscopy.

**Author details**

1Department of Biotechnology and Genetic Engineering, P. O Box 3030, Jordan University of Science and Technology, Irbid 22110, Jordan.
4. Iversen C, Waddington M, Farmer JJ, Forsythe SJ, Jaradat Z, Hartantyo SHP, Abdullah Sani N, Estuningsih S, Forsythe SJ: Cloning and sequencing of the ompA gene of Enterobacter sakazakii subsp. C. sakazakii. BMC Evolut Biol 2006, 6, 69.

5. Iversen C, Lechner A, Mullanoe N, Bidlas E, Kleenwerck I, Marugg J, Fanning S, Stephann S, Roostenn H: The taxonomy of Enterobacter sakazakii: proposal of a new genus Cronobacter gen. nov. and descriptions of Cronobacter sakazakii comb. no. Cronobacter sakazakii subsp. sakazakii, comb. nov., Cronobacter sakazakii subsp. Malonaticus subsp. Nov., Cronobacter dublinensis subsp. Nov. and Cronobacter genomospecies I. BMC Evol Biol 2007, 7, 46.

6. Iversen C, Mullanoe N, McCardell B, Tall BD, Lechner A, Fanning S, Stefann S, Roostenn H: Cronobacter gen. nov., a new genus to accommodate the biogroups of Enterobacter sakazakii, and proposal of Cronobacter sakazakii gen. nov., comb nov., C. maculans sp. nov., C. turicensis sp. nov., C. mutagens sp. nov., C. dublinensis sp. nov., Cronobacter genomospecies I, and of three subspecies. C. dublinensis sp. nov. subsp. dublinensis subsp. nov. C. dublinensis sp. nov. subsp. lasuensis subsp. nov., and C. dublinensis sp. nov. subsp. lactaridi subsp. Nov. Int J Syst Evol Microbiol 2008, 58:1442-1447.

7. MacLean LL, Pagotto F, Farber JM, Perry MB: The structure of the O-antigen in the endotoxin of the emerging food pathogen Cronobacter (Enterobacter) mundtijrensi strain 3270. J Bacteriol 2003, 185:667-671.

8. Nair MK, Venkatarayanan KS: Cloning and sequencing of the ompA gene of Enterobacter sakazakii and development of an ompA-targeted PCR for rapid detection of Enterobacter sakazakii in infant formula. Appl Environ Microbiol 2006, 72:2539-2546.

9. Nair MK, Venkatarayanan KS, Silbark LK, Kim KS: Outer Membrane Protein A (OmpA) of Cronobacter sakazakii binds fibrinogen and contributes to invasion of human brain microvascular endothelial cells. Foodborne Pathog Dis 2009, 6:495-501.

10. Singamsetty VK, Wang Y, Shimada H, Prasadnaar NV: Outer membrane protein A expression in Enterobacter sakazakii is required to induce microtubule condensation in human brain microvascular endothelial cells for invasion. Microb Pathog 2008, 45:181-191.

11. Mazi M, Saint N, Molle G, Pagès JM: The Enterobacter aerogenes outer membrane efflux proteins TolC and Efc have different channel properties. Biochim biophys Acta 2007, 1768:2559-2567.

12. de Kort G, van der Bent-Klootwijk P, van de Klundert JA: Immunodetection of the virulence determinant OmpX at the cell surface of Enterobacter cloacae. FEMS Microbiol Lett 1998, 158:115-20.

13. de Kort G, Bolton A, Martin G, Stephan J, van de Klundert JA: Invasion of rabbit ileal tissue by Enterobacter cloacae varies with the concentration of OmpX in the outer membrane. Infect Immun 1994, 62:4722-4726.

14. Agostini C, Aexission I, Goulet O, Koletko B, Michaelisen FP, Putnis WL, Rigo J, Shimar R, Szejewska H, Turck D, Vandendplas Y, Weaver LT: Preparation and Handling of Powdered Infant Formula: A Commentary by the ESPGHAN Committee on Nutrition. J Pediat Gastroenterol Nutr 2004, 39:320-322.

15. Bown AB, Braden CR: Invasive Enterobacter sakazakii Disease in Infants. Emerg infect Dis 2006, 12:1185-1189.

16. Drudy D, Mullane NR, Quinn T, Wall PG, Fanning S: Enterobacter sakazakii: An emerging pathogen in powdered infant formula. Food Safety 2006, 42:996-1002.

17. Kothary MH, McCardell BA, Frazer CD, Deer D, Tall BD: Characterization of the zinc-containing metalloprotease (zpm) and development of a species-specific detection method for Enterobacter sakazakii. Appl Environ Microbiol 2007, 73:4142-4151.

18. Chap J, Jackson P, Siqueria R, Gasper N, Quintas C, Park J, Osaili T, Shaker R, Jaradat Z, Hanttano YSH, Abdullah Sani N, Estuningsih S, Forsythe SJ: International survey of Cronobacter sakazakii and other Cronobacter spp. in follow up formulas and infant foods. Int J Food Microbiol 2009, 136:185-188.

19. Jaradat ZW, Ababneh QQ, Saadoun IM, Samara NA, Rashdan MA: Isolation of Cronobacter spp. (formerly Enterobacter sakazakii) from infant food, herbs and environmental samples and the subsequent identification and confirmation of the isolates using biochemical, chromogenic assays, PCR and 16S rDNA sequencing. BMC Microbiol 2009, 9:225.

20. Molloy M, Cagney C, O'Brien S, Iversen C, Fanning S, Duffy G: Surveillance and characterization by pulse-field gel electrophoresis of Cronobacter spp. in farming and domestic environments, food production animals and retail foods. Int J Food Microbiol 2009, 136:198-203.

21. Lai KK: Enterobacter sakazakii infections among neonates, infants, children and adults. Medicine 2001, 80:115-122.

22. Gurtler JB, Kornacki JL, Beuchat LR: Enterobacter sakazakii outer membrane lipopolysaccharide. Biochim biophys Acta 2007, 1768:2559-2567.

23. Padhye VV, Zhao T, Doyle MP: Production and characterization of Lipopolysaccharide from Porphyromonas endodontalis. J Med Microbiol 1998, 46:299-305.

24. Pupo E, Aguila A, Santana H, Nunez J, Castellanos-Serra L, Hardy E: Immune interaction with gel electrophoresis-micropurified bacterial lipopolysaccharides. Electrophoresis 1999, 20:458-461.

25. Banada PP, Shuina AK: Antibodies and immuneassays for detection of bacterial pathogens. In Principles of Bacterial Detection. Biosensor, Recognition Receptors and Microsystems. Volume Chapter 21. Edited by: Zourob M, Elwary S, Turner A. Springer, New York; 2008:567-602.

26. Davies BL, Wall RA, Borello SP: Comparison of methods for the analysis of outer membrane antigens of Neisseria meningitis by western blotting. J Immunol Methods 1990, 134:215-25.

27. Liddell JE, Ceyer A: A practical guide to monoclonal antibodies. John Wiley and Sons, Chichester, UK; 1991.

28. Hatfield ED, Lane D: Antibodies: A laboratory manual. Cold Spring Harbor, USA, 1988.

29. Fridgren B, Djavadi-Chaniance L, Golberg M: A convenient enzyme linked immunosorbent assay for testing whether monoclonal antibodies recognize the same antigenic site. Immunoenzymatic techniques Academic Press, London, 1983, 171-175.

30. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 277:680-685.

31. Rehns BL, Geller DB, Kim JS, Fox JE, Kolli VSK, Pueppke SG: Sinorhizobium fredii and Sinorhizobium melliloti produce structurally conserved lipopolysaccharides and strain-specific K antigens. Appl Environ Microbiol 1998, 64:4930-4936.

32. Padhye W, Zhao T, Doyle MP: Production and characterization of monoclonal antibodies to Verotoxins 1 and 2 from Escherichia coli of serotype O 157:H7. J Med Microbiol 1989, 30:219-226.

33. Pettersson A, Kuipers B, Pelzer M, Verhegen E, Tiesjesma RH, Tomassen J, Poolman J F: Monoclonal antibodies against the 70-kilodalton iron-regulated protein of Neisseria meningitis are bactericidal and strain specific. Infect Immun 1990, 58:3036-3041.

34. Tadine M, Mittal KR, Boudron S, Gottschalk M: Production and characterization of murine monoclonal antibodies against Haemophilus parasuis and study of their protective role in mice. Microbiology 2004, 150:3935-3945.
35. Brooks BW, Lutze-Wallace CL, Mackan LL, Vinogradov E, Perry MB. Identification and differentiation of *Taylorella equigenitalis* and *Taylorella asingeniata* by lipopolysaccharide O-antigen serology using monoclonal antibodies. *Can J Vet Res* 2010, 74:18-24.

36. Luk JM, Lindberg AA. Rapid and sensitive detection of *Salmonella* (O:6,7) by immunomagnetic monoclonal antibody-based assays. *J Immunol Methods* 1991, 137:1-8.

37. Jongs-Leuvenink J, Bouter AS, Marcelis JH, Schelleken J, Verhoef J. Cross-reactivity of monoclonal antibodies against lipopolysaccharides of gram-negative bacteria. *Euro J Clin Microbiol* 1986, 5:148-151.

38. Hofstra H, Van Tol JD, Dankert J. Cross-reactivity of major outer membrane proteins of *Enterobacteriaceae*, studied by crossed immunoelectrophoresis. *J Bacteriol* 1980, 143:328-37.

39. Jaradat ZW, Zawistowski J. Antigenically stable 35 kDa outer membrane protein of *Salmonella*. *Food Agri Immunol* 1998, 10:257-270.

40. Robertson SM, Frisch CF, Gulig PA, Kettman JR, Johnston KH, Hansen EJ. Monoclonal antibodies directed against a cell surface-exposed outer membrane protein of *Haemophilus influenzae* type b. *Infect Immun* 1982, 36:80-88.

41. Hamel J, Brodeur BR, Belmessa A, Montpiaiser S, Musser JM, Selander RK: Identification of *Haemophilus influenzae* type b by a monoclonal antibody coagglutination assay. *J Clin Microbiol* 1987, 25:2434-2436.

42. Isibasi A, Ortiz V, Vargas M, Paniagua J, González C, Moreno J, Kumate J: Protection against *Salmonella typhi* infection in mice after immunization with outer membrane proteins isolated from *Salmonella typhi* 9, 12, d, Vi. *Infect Immun* 1998, 56:2953-2959.

43. Lugtenberg B, Van Alphen L: Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. *Biochim Biophys Acta* 1983, 737:51-115.

44. Cloeckaert A, de Wergifosse P, Dubray G, Limet JN: Identification of seven surface-exposed *Brucella* outer membrane proteins by use of monoclonal antibodies: immunogold labeling for electron microscopy and enzyme-linked immunosorbent assay. *Infect Immun* 1990, 58:3980-3987.

doi:10.1186/1471-2180-11-148

Cite this article as: Jaradat et al: Characterization of surface proteins of *Cronobacter muytjensii* using monoclonal antibodies and MALDI-TOF Mass spectrometry. *BMC Microbiology* 2011 11:148.