academicJournals

Vol. 10(21), pp. 744-751, 7 June, 2016
DOI: 10.5897/AJMR2016.8004
Article Number: DA482C258763
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Hydrophobicity and specific biofilm features of *Bacillus cereus* spores subjected to pH stresses

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Received 10 March 2016, Accepted 29 April, 2016.

Bacillus cereus **is a foodborne pathogen that often persists on food processing surfaces due the formation of spores and biofilms. Spores of 12 selected *B. cereus* strains from genotypes that recurred in a pasteurized milk processing line were investigated in this study, for their surface and biofilm characteristics. The main objective was to have an insight into their persistence strategies. Spore surface hydrophobicity and acid-base properties, were assessed using the microbial adhesion to solvents (MATS) method. To determine how hydrophobicity was affected by cleaning procedures, this property was measured when spores were submitted to alkali or acidic stresses mimicking those of cleaning-in-place (CIP) procedures. Biofilms formation on stainless steel coupons by pH-treated spores was investigated in three culture media and imaged by using environmental scanning electron microscopy (ESEM). Results showed that spores were either hydrophilic or moderately hydrophobic. Alkali-stress reduced spore surface hydrophobicity, whereas acidic shock increased it. More limited hydrophobicity changes following alkaline stress suggest alkali adaptation of spores. In addition, spores submitted to pH-stresses produced specific biofilm features on stainless steel as shown by ESEM imaging. Alkali tolerance and the biofilm lifestyle are strategies that permit *B. cereus* recurrent genotypes to persist in the milk processing line. Overall, this study gives an insight into hydrophobicity and specific biofilm features of *B. cereus* spores submitted to chemical cleaning.**

Key words: *Bacillus cereus*, biofilms, spores, hydrophobicity, CIP-like stress, dairy industry, environmental scanning electron microscopy (ESEM).

INTRODUCTION

Tolerance of bacteria to low and high-pH stresses is of major concern to the food industry. As shown by several studies (Lindsay et al., 2002; Cotter and Hill, 2003; Giotis et al., 2009; Mols and Abee, 2011), the pH stresses encountered in the food processing environments may induce acidic and/or alkaline resistance of contaminating bacteria and thus contribute to their survival and persistence in the factories. Unfortunately, this adaptive

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behavior is largely reported for important foodborne pathogenic microorganisms such as *Listeria monocytogenes* and *Bacillus cereus*. As an illustration, in the dairy industry, certain *B. cereus* genotypes were shown to recur in milk processing lines for several years (Svensson et al., 2004; Shaheen et al., 2010; Malek et al., 2013). In addition, *B. cereus* forms biofilms that are responsible of spore dissemination into food environments (Wijmann et al., 2007) and resist to removal (Kumari and Sarkar, 2014).

The high adhesion potential of *B. cereus* spores is well established and related to spore surface hydrophobicity which relies on morphological structures notably exosporium and appendages (Husmark and Ronner, 1992; Faille et al., 2002; Ankolekar and Labbé, 2010). Spores of *B. cereus* have mainly been investigated for survival, adhesion and biofilm formation after CIP-like stresses (Faille et al., 2010; Salutiano et al., 2010; Shaheen et al., 2010). However, it remains unknown how the conditions encountered by spores during cleaning procedures affected spore surface hydrophobicity. CIP systems are alkaline (NaOH) and/or acidic (HNO_3) washes often performed at high temperature (70 – 80°C) (Bremer et al., 2006). Similarly, little is known about the structure of the biofilms developed by *B. cereus* on dairy processing equipment after CIP procedures. That is why this study dealt with the analysis of spore surface hydrophobicity and biofilm features following pH-stresses by using *B. cereus* dairy recurrent strains, in order to understand their persistence strategies. For this purpose, spore hydrophobicity and acid-base properties of a set of 12 *B. cereus* dairy isolates and a comparative reference strain, *B. cereus* ATCC 11778, were, first assessed. Hydrophobicity was further measured when spores were submitted to alkali and acid stresses that mimicked those of CIP systems. Finally, pH-treated spores were used to form biofilms on stainless steel coupons, under static conditions and observed in ESEM.

MATERIALS AND METHODS

Bacterial strains

B. cereus strains were previously isolated from a pasteurized milk processing line (Malek et al., 2013). These strains were fingerprinted by M13 PCR, and clustered into three distinct M13-PCR groups: one major group (genotype A), which included 17 out of 20 strains and two minor groups (genotypes B and C). Genotypes A and B which recurred in this processing line for more than four years (Table 1), were affiliated to the mesophilic *B. cereus* group III while the last genotype was affiliated with the mesophilic *B. cereus* group IV, according to the phylogenetic classification of Guinebretière et al. (2008).

Spore surface properties

Different solvents were used to evaluate the hydrophobic/hydrophilic spore surface properties of *B. cereus* and their Lewis acid–base characteristics. Both apolar solvents hexadecane and hexane were used to estimate the hydrophobicity properties of spore surfaces while the two monopolar solvents, chloroform and diethyl ether, were selected for the estimation of the Lewis acid/base (that is, electron donor/acceptor) character, according to the microbial adhesion to solvents (MATS) partitioning assay (Bellon-Fontaine et al., 1996). Hydrophobicity is expressed as the percentage (P) of adhesion to hexadecane. Spores are very hydrophilic (P < 20%), hydrophilic (20 > P < 40%), moderately hydrophobic (40 > P < 60%) and highly hydrophobic (P > 60%). The acid-base interactions can be assessed based on the comparison between the microbial cell affinity to chloroform, an acidic solvent (electron acceptor) and the apolar solvent hexadecane as well as between spore affinity to diethyl ether, a basic solvent (electron donor) and the apolar solvent, hexane (Bellon-Fontaine et al., 1996). Results are expressed as percentages of adhesion to each solvent. Spores had an electron donor character when their affinity to chloroform is higher than with hexadecane and an electron acceptor character when their affinity to diethyl ether is higher than to hexane. Spore suspensions were prepared as previously described (Simmonds et al., 2003), and prior to use, they were washed one time and suspended in saline (0,15 M NaCl) at pH 7. Hydrophobic and acid-base properties of spore surfaces were measured using the MATS method (Bellon-Fontaine et al., 1996) with modifications based on observations from other reports (Tauveron et al., 2006). In short, saline spore suspensions were adjusted to an absorbance of 0.6 to 1 at 595 nm. Spore suspension (2 mL) were added to 400 µL of the polar or apolar solvent, vortexed for 1 min and settled for 15 min. The optical density of water phase was measured using a spectrophotometer at 595 nm. As described by Bellon-Fontaine et al. (1996), the percentage of spores bound to a given solvent was expressed as $(1 - A/Ao) \times 100$, where $A0$ is the absorbance measured at 595 nm of the bacterial suspension before mixing and A is the absorbance after mixing. The mean and standard error were calculated from five measurements. Chemical products (Hexadecane, chloroform, hexane and diethyl ether) were obtained from Aldrich chemical, Co., Inc. USA.

Hydrophobicity of pH-treated spores

Spore were investigated for their surface hydrophobicity following mixing with sodium hydroxide (pH 12.7) at 80°C and into nitric acid (pH 1.2) at 70°C, to mimic CIP conditions as applied at the investigated dairy plant. Spore suspensions were pH-treated using the protocol of Faille et al. (2010), with minor modification. One volume of the stock suspensions was added to 1 volume of aqueous 2% w/v NaOH or 1% HNO_3 w/v to absorbance values between 0.8-1. Tubes containing NaOH or HNO_3 spore mixtures were respectively incubated at 80 and 70°C for 10 min. After each treatment, spores were rapidly cooled, harvested as previously described (Faille et al., 2010) and re-suspended in saline to absorbance values between 0.8-1. Hydrophobicity of the pH-treated spores was assessed as described above. Experiments were performed with repetitions. The obtained data were submitted to variance analysis and correlation tests using Matlab 7.0 France software.

Adhesion of pH-treated spores to stainless steel coupons

The pH-treated spores were used to adhere on stainless steel coupons (AISI 304 L, 10 x 10 mm), cleaned according to the protocol described by Peng et al. (2001). For the adhesion assay

Table 1. Spore surface hydrophobicity and acid-base properties of *B. cereus* isolates from a pasteurized milk processing line*.

Strains[ab]	Hexadecane (% ± SE)	Chloroform (% ± SE)	Hexane (% ± SE)	Ether (% ± SE)	Character[c]
M13PCR group A					
S78	12.5 ± 6.5	15.7 ± 6	14.9 ± 5.4	31.5 ± 7	D and A
S19	14.2 ± 3.2	17.6 ± 4.7	17.5 ± 6.2	20.1 ± 6.8	D and A
P56	18.4 ± 4.2	23.9 ± 5.3	21.1 ± 3.7	27.3 ± 3.9	D and A
P53	21.5 ± 4.5	34.4 ± 4.1	23.4 ± 7.7	27.4 ± 6.7	D and A
A9	28.4 ± 6.2	42.8 ± 7.8	34.2 ± 4.8	28.4 ± 1.9	D
P52	42.3 ± 1.6	37.3 ± 3.9	45.4 ± 7.2	19.9 ± 6.1	ND. NA
S66	43.6 ± 3.5	37.3 ± 3.2	45.2 ± 6.1	19.9 ± 5.8	ND. NA
S113	49.3 ± 5.2	21.1 ± 4.3	53.6 ± 5.6	8.4 ± 5.8	ND. NA
S79	51.6 ± 2.4	38.9 ± 2.7	53.3 ± 3.1	15.2 ± 4.2	ND. NA
M13PCR group B					
S35	23.6 ± 3.7	33.8 ± 3.6	25.8 ± 3.8	27.6 ± 3.8	D and A
A7	15.2 ± 2	29.1 ± 6.1	18.2 ± 2.8	21.1 ± 5.6	D and A
M13PCR group C					
S116	46.7 ± 2.8	nd	nd	nd	nd
Ungrouped strain					
BC ATCC 11778	71.7 ± 7.1	nd	nd	nd	nd

*Pasteurized milk was obtained from reconstituted and processed milk powder, in the investigated dairy plant. [a]*B. cereus* isolates were kindly characterized at the genotypic level at UMR 408 INRA Avignon, France in a previous work (Malek et al., 2013). [b]Strains are coded as follow: Letters indicate isolation origin and period. P: milk powder in 2010, S: milk processing equipment in 2010, A: milk processing equipment in 2006. [c] D: electron donor, A: electrons acceptor, ND. NA: non-electron donor and non-electron acceptors. Hydrophobicity is expressed as percentage of adhesion to hexadecane.

coupons were fouled with *B. cereus* spores by immersion in the wells of a 6-well polystyrene plate (Nunc multidish) for 3 h in a saline spore suspension (10^7-10^9 spores mL^{-1}) and quickly immersed in sterile water to remove weakly attached spores.

Biofilm formation by pH-treated spores

To establish laboratory conditions that mimic the industrial setting being studied, especially soiling conditions, the formation of biofilms by *B. cereus* spores was investigated in three culture media: non-diluted milk, 100 fold diluted milk and nutrient broth, at various incubation times. Milk culture medium consisted of 10% (w/v) medium heat milk powder (Germany), diluted in distilled water. The coupons with adhered spores were placed into wells containing culture media and incubated under static conditions for 24, 48 or 168 h (7 days) at 30°C. The media were refreshed every 2 days with the same fresh sterile medium and prior to that, the coupons were water rinsed to remove loosely attached cells.

Microscopy

ESEM imaging does not require any sample preparation or specific method. After the incubation time, all the above biofilm carrying stainless steel coupons were washed thrice with distilled water, air dried and exanimated in a 100 TM Hitachi environmental scanning electron microscope (Hitachi, Japan), at pressure in microscope chamber of 4 Torr. To ovoid biofilm dehydration, the samples must be rapidly observed.

RESULTS

Hydrophobicity of untreated spores

Spore surface hydrophobicity measured by MATS method was expressed as percentage of adhesion to hexadecane (Table 1). Results showed that hydrophobicity of spores varied among the analyzed *B. cereus* dairy isolates which displayed either a hydrophilic or hydrophobic character. Seven out of 12 strains were markedly (< 20%) or moderately hydrophilic (< 40%), adherence to hexadecane range between 12.5 and 28.4% respectively. Remaining strains shared moderately hydrophobic character, with hydrophobicity values spanning a narrow range from 42.1 to 51.6%. The unique highly hydrophobic strain (71.7%) was *B. cereus* ATCC 11778, included for a comparative purpose. It is interesting to note the variability in hydrophobic/hydrophilic characters

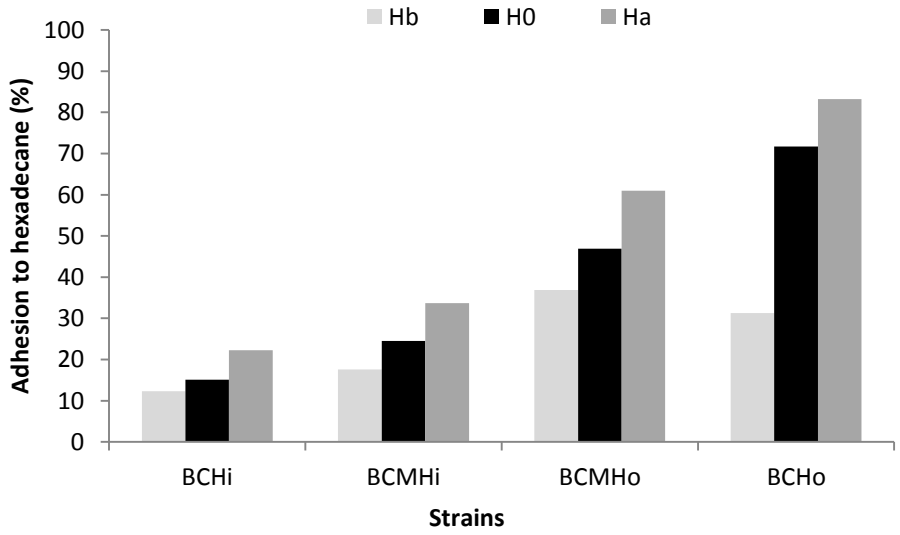

Figure 1. Variation in spore surface hydrophobicity of 13 *B. cereus* strains following alkali or acid stresses. H0: hydrophobicity of untreated spores (initial hydrophobicity), Hb: alkali-induced hydrophobicity, Ha: acid-induced hydrophobicity. H0, Hb and Ha bars represent means of spore hydrophobicity values of strains grouped based on their initial hydrophobicity. BCHi: *B. cereus* hydrophilic spores (4 strains), BCMHi: *B. cereus* moderately hydrophilic spores (3 strains), BCMHo: *B. cereus* moderately hydrophobic spores (5 strains), BCHo: *B. cereus* ATCC 11778: highly hydrophobic strain.

among closely related strains of M13 PCR genotype A, and the dominance of hydrophilic spores among *B. cereus* dairy isolates.

Lewis acid-base properties of untreated spores

The affinity of spores to the polar and apolar solvents, according to the MATS method, is presented in Table 1. The affinity of hydrophilic *B. cereus* spores of dairy origin to the polar solvents (chloroform/diethyl ether) were higher than to alkanes (hexadecane/decane), indicating their electron-donnor and electron-acceptor properties. However, their affinity for the different solvents did not exceed 40% and confirmed their hydrophilic nature. Whereas moderately hydrophobic spores sharing low affinity to both chloroform (< 40%) and diethyl ether (< 20%) did not express any acid-base characters. Conversely and regardless of the solvent used, the affinity of spores from the reference strain *B. cereus* ATCC 11778, was high (> 70%), indicating its hydrophobic nature.

The moderately hydrophilic spores produced by *B. cereus* strain A9 showed higher affinity for the electron acceptor solvent (chloroform) than hexadecane indicating an electron donor character. The electron acceptor property expressed by a higher affinity to diethyl ether (basic solvent) than to hexane was not observed for all spore surfaces.

Hydrophobicity of pH-treated spores

The variation in the hydrophobic/hydrophilic character of spores when mixed with 1% v/v sodium hydroxide (pH 12.7) at 80°C or 0.5% v/v nitric acid (pH 1.2) at 70°C is presented in Figure 1. Results showed that high alkaline stress led to a decrease in spore surface hydrophobicity while high acid stress increased it. The analysis of variance indicated that the variability in the hydrophobicity values was explained by the initial hydrophobicity and not by the strain effect. Nevertheless, in contrast to acid-induced hydrophobicity, the alkali-induced changes were significantly correlated with the initial hydrophobicity of spores (Pearson coefficient r = 0.579 [P < 0.05]). Accordingly, lower spore hydrophobicity values, resulted in lower alkali-induced hydrophobicity values indicating that hydrophilic spores were the least affected by alkaline shock.

Structures of biofilms formed by pH- treated spores

A selection of representative ESEM pictures is shown in Figures 2 to 4. ESEM images were captured at different incubation times. Biofilms incubated both in nutrient broth and non- diluted milk or diluted milk for 24 or 48 h, were little developed structures. The 48 h old biofilm shown in Figure 3a is a less elaborated monolayer structure which presents dehydration signs. In comparison, native spores

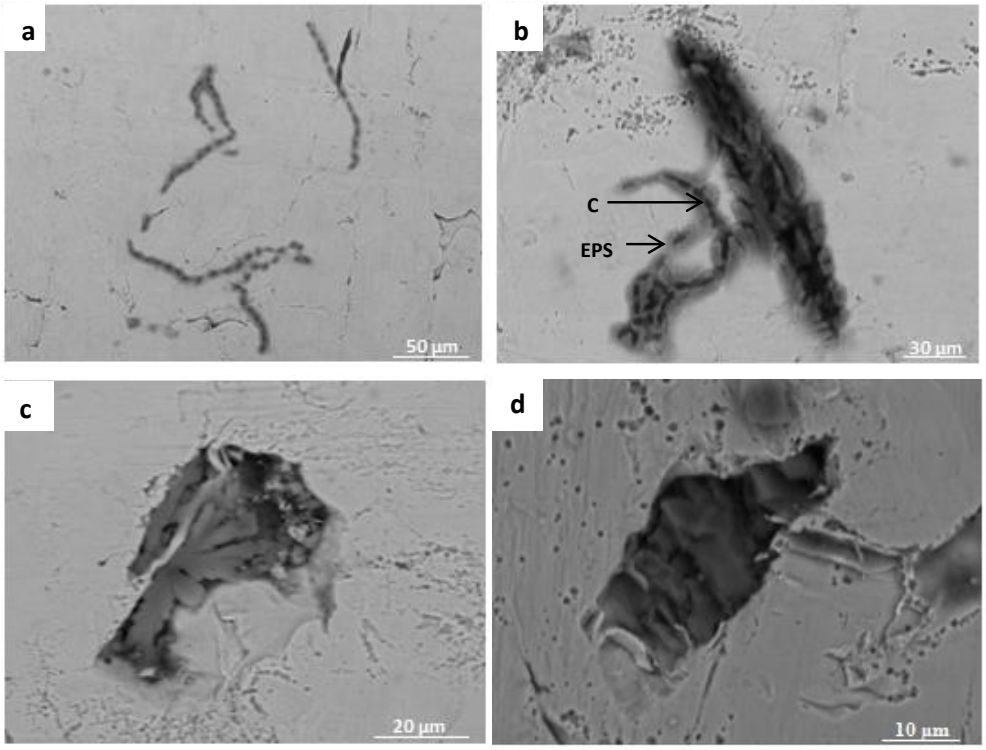

Figure 2. ESEM micrographs of 24 h old biofilms formed in diluted milk by *B. cereus* strain P53. (a) adherent cells surrounded by clear areas of EPS. (b) cells surrounded or covered by EPS material. (c and d) Young biofilms in crevices of damaged stainless steel. C Indicates cells and EPS exocellular polymeric substances.

of the same strain formed a substantial thick mature biofilm, at the detachment stage (Figure 3b). It is also interesting to note that, after 24 h of cultivation, the biofilms formed in 1/100 diluted milk were still at the adhesion stage (Figure 2a) or just starting to be covered with the EPS-matrix (Figure 2b). In contrast, in the biofilms formed in crevices (Figure 2c and d), cells are completely hidden in the extracellular matrix. It appears that in these harborages, the production of the biofilm-matrix was enhanced, enabling cells to form a compact matrix structure devoid of obvious pores or channels. Older biofilms (7 days) formed in non-diluted milk or nutrient broth, were also compact shapes characterized by smooth or wrinkled surface topography (Figure 4). However, these biofilms are most likely devoid of living cells.

DISCUSSION

In this study, the authors attempted to phenotypically characterize spores from a collection of *B. cereus* strains that recurred in a pasteurized milk processing line, in order to understand their persistence strategies. Interesting findings were the variability in spore surface hydrophobicity recorded among closely related *B. cereus* genotypes and the predominance of hydrophilic spores. Spores of *B. cereus* are generally recognized to be hydrophobic or highly hydrophobic (Simmonds et al., 2003; Tauveron et al., 2006, Ankelokar and Labbé, 2010). Hydrophilic spores have already been reported among strains of *B. cereus* isolated in the dairy environment (Bernardes et al., 2010; Salustiano et al., 2010), and strains of thermophilic bacilli isolated from milk powder (Seale et al., 2008). Accordingly, the dairy environment appeared to be a source of hydrophilic spores of both mesophilic and thermophilic bacilli.

Based on the results of the MATS method, Lewis acid-base properties exhibited by *B. cereus* spores were consistent with data of the literature. Electron donor and electron acceptor characters were found for very hydrophilic strains belonging to other bacterial species (Faille et al., 2002; Hamadi et al., 2004; Djeribi et al., 2013). Similarly, the lack of any electron donor electron acceptor properties was described in hydrophobic *B. cereus* spores (Faille et al., 2002). Both characteristics were associated with high adhesion potential to inert surfaces.

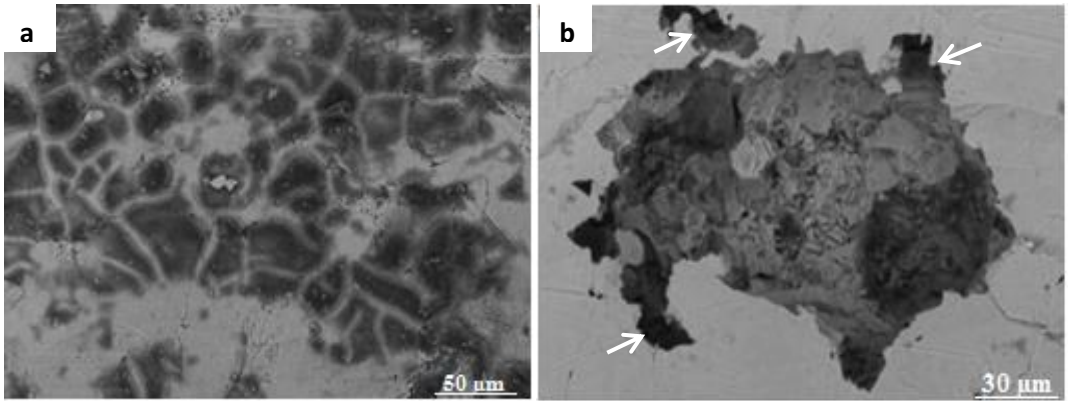

Figure 3. Comparison of 48 h old biofilms formed in nutrient broth by pH- treated (a) and untreated spores (b) of *B. cereus* strain S113. Arrows indicate detachment of small portions of the biofilm.

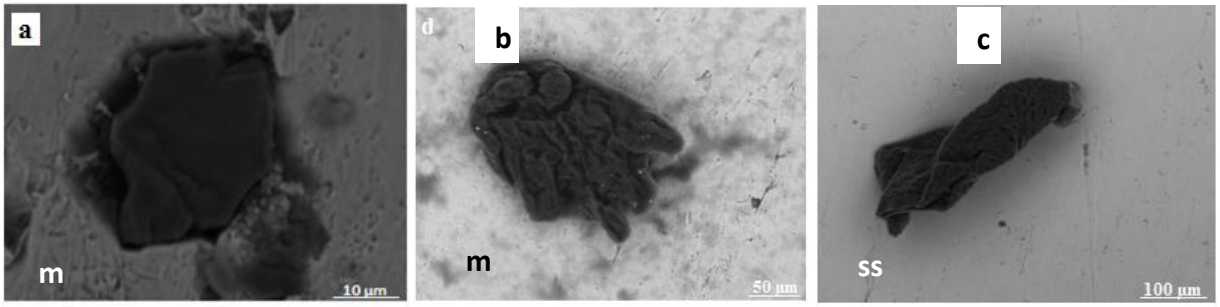

Figure 4. Smooth and wrinkled matrix surface topographies of 7 days old biofilms formed in non-diluted milk (a and b) and nutrient broth (c) by *B. cereus* strains S78 (a), A7 (b) and P56 (c). (m) Indicates milk fouling coating stainless steel surface topography and (ss) stainless steel.

The authors also attempted to explain the variability recorded in spore surface hydrophobicity among closely related *B. cereus* strains (belonging to genotype A). Hydrophobicity of *B. cereus* has been already reported to be strain-associated and not related to the ecological niche (Tauveron et al., 2006). Nevertheless, adaptation of dairy-associated *B. cereus* to alkaline pH was previously reported (Lindsay et al., 2002). Hydrophilic strains of this bacterium were isolated in the dairy industry from CIP solutions (Salustiano et al., 2010) or the filling machine (Bernardes et al., 2010). Since CIP systems, are mainly alkaline and/or acidic washes often performed at high temperature (Bremer et al., 2006), the existence of a relationship between the hydrophilic character of *B. cereus* spore surfaces and alkali adaptation is believed. To give more insight into this issue, hydrophobicity was assessed when spores were submitted to hot alkali stress or hot acidic stress mimicking those of CIP procedures. Based on the results of the percentage of adhesion to hexadecane, high-pH stress results in a decrease of hydrophobicity values while low-pH stress increases them. These results are consistent with data from previous works concerning *B. cereus* (Lindsay et al., 2000) or other bacteria (Giotis et al., 2009; Moorman et al. 2008), after exposure to mild pH-stresses. However, to the best of the authors' knowledge, this property has not been investigated when cells were submitted to more severe pH-stresses, like those encountered during cleaning procedures. Faille et al. (2010) have already shown exosporium glycoproteins to be seriously damaged by spore treatments using severe alkaline stress (2% NaOH at 80°C for 20 min) and this should result in a decrease in spore hydrophobicity as shown in the current study. On another hand, hydrophilic spores displayed more limited hydrophobicity changes as compared to highly hydrophobic spores, so that the highest percentage of hydrophobicity change (40.5%) was recorded for the most hydrophobic strain, BC ATCC 11778. More limited hydrophobicity reduction following alkali treatment has been related to cell alkali adaptation of bacteria (Giotis et al., 2009). Consequently, hydrophilic spores behave as alkali adapted cells, whilst the

reference strain should be considered as non-alkali adapted strain. This result constitutes one possible explanation for the occurrence of markedly hydrophilic strains, among these *B. cereus* dairy isolates. At the dairies, cleaning procedures may select some spores with specific surface chemistry, and it is likely that hydrophilic spores are part of such category. In good agreement with this finding, the surface chemistry of *Bacillus* spores has been described to significantly influence the efficiency of cleaning procedures (Faille et al., 2013).

The biofilms formed by pH-treated spores, in all culture media were not well developed structures, in terms of tridimensional architecture, or net-like patterns. This should be ascribed to the loss of the viability of most of the pH-treated spores or the loss of their ability to adhere due to damaged structures, as previously demonstrated (Faille et al., 2010; Shaheen et al., 2010). As an illustration, the 48 h old biofilm formed in nutrient broth was little elaborated structure which consists of a two-dimensional net-like attachment pattern, previously described for biofilms formed in poorly nutritional conditions (Marsh et al., 2003).

Likewise, in 100 fold diluted milk, the biofilm formation process is also seriously affected, since after 24 h incubation at 30°C, bacteria were still at early biofilm formation stages, namely the adhesion step. Based on the ESEM pictures, it is clear that only small numbers of spores survived the pH-stresses, and adhered to stainless steel. In good agreement, Faille et al. (2010) demonstrated that, a small percentage of adherent *B. cereus* spores were able to resist the conditions found during CIP procedures and the spores detached during the CIP procedure would re-adhere along the CIP rig. In the current study, pH-treated spores of *B. cereus* strain P53 were able to adhere on stainless steel surfaces but had lower propensity to develop a mature biofilm in 100 fold diluted milk, within 24 h. This may probably be due to the incapacity of adhered spores to rapidly germinate in diluted milk. Consistent with results of Shaheen et al. (2010), spores of some *B. cereus* strains were not capable of germinating in 10 fold diluted milk. However, ESEM pictures showed that, when protected in harborages of impaired material, spores were able to develop young biofilms, once a minimum initial bacterial load is necessary for bacteria to persist in a harborage site (Carpentier and Cerf, 2011).

Similarly, older biofilms (7 days) are associated with low numbers of living cells when they are first formed, but may be devoid of cells or contain only few spores, once the structures mature. Nevertheless, the observed old wrinkled structures were already described in *B. subtilis* biofilms and shown to be highly resistant to penetration of gas and liquid and thus to withstand biocide effects (Epstein et al., 2011). This is of crucial concern to the efficiency of cleaning procedures against biofilms exhibiting such recalcitrant structures. These results are interesting cues with regard to the persistence of foodborne pathogens in industrial settings.

Conclusion

Spores and biofilms are considered the most important reservoir of *B. cereus* in milking pipelines and on surfaces of equipment, and have to be deeply characterized. This study showed how cleaning procedure may affect spore surface hydrophobicity in *B. cereus*. Hydrophilic spores which seem to be common among dairy-associated *B. cereus* strains should be selected in the dairy environment by CIP-like procedures. Hydrophilic spores selected by cleaning systems were best able to withstand chemical cleaning, and to form specific biofilm features on stainless steel. This should constitute possible strategies whereby *B. cereus* recurrent genotypes persist in the dairy processing line. Improved knowledge on spore surface characteristics and a better understanding of *B. cereus* biofilm formation, through comparison of worldwide gathered data, may help develop efficient strategies for their control.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

ESEM imaging was performed at the University of Tlemcen, Algeria. Pr. Tabti Boufeldja is gratefully acknowledged for the facilities offered during the microscopic analysis and Cherifa Belabbassi for assistance in the ESEM observations.

REFERENCES

Ankolekar C, Labbé RG, **(**2010). Physical characteristic of spores of food-associated isolates of the *B.cereus* group. Appl. Environ. Microbiol. 76:982-984.

Bellon-Fontaine MN, Rault J, Van Oss CJ (1996). Microbial adhesion to solvents, a novel method to determine the electron–donor/electron–acceptor or Lewis acid–base properties of microbial cells. Colloid Surface B 7:47-53.

Bernardes CP, de Andrade NJ, Ferreira OS, de Sa JPN, Andrade-Araujo E, Zanom-Delatorrel DM, Pinheiro Luiz LM (2010). Assessment of hydrophobicity and roughness of stainless steel adhered by an isolate of *Bacillus cereus* from a dairy plant. Braz. J. Microbiol. 41:984-992.

Bremer PJ, Fillery S, McQuillan AJ (2006). Laboratory scale Clean-In Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. International J. Food Microbiol. 106:254-262.

Carpentier B, Cerf O **(**2011). Review-Persistence of *Listeria monocytogenes* in food industry equipment and premises. Int. J. Food Microbiol. 145:1-8.

Cotter PD, Hill C (2003). Surviving to acid test: responses of Gram-positive bacteria to low pH. Microbiology and Molecular Biology Reviews 67:429-453.

Djeribi R, Boucherit Z, Bouchloukha W, Zouaouia W, Latrache H, Hamadi F, Menaa B (2013). A study of pH effects on the bacterial surface physicochemical properties of *Acinetobacter baumannii.* Colloids and Surfaces B: Biointerfaces 102:540- 545.

Epstein AK, Pokroy B, Seimanar A, Aizenberg J (2011). Bacterial biofilm shows persistent resistance to liquid wetting and gas penetration. Current issue 108: 995-1000.

Faille C, Benezech T, Blel W, Ronse A, Ronse G, Clarisse M, Slomianny C (2013). Role of mechanical vs. chemical action in the removal of adherent Bacillus spores during CIP procedures. Food Microbiol. 33:149-157.

Faille C, Jullien C, Fontaine F, Bellon-Fontaine MN, Slomianny C, Benezech T (2002). Adhesion of *Bacillus cereus* spores and Escherichia coli cells to inert surfaces: role of surface hydrophobicity. Can. J. Microbiol. 48:728-38.

Faille C, Sylla Y, Le Gentil C, Benezech T, Slomianny C, Lequette Y **(**2010). Viability and surface properties of spores subjected to a cleaning-in-place procedure: consequences on their ability to contaminate surfaces of equipment. Food Microbiol. 27:769-776.

Giotis ES, Blair IS, McDowell, DA (2009). Effect of short-term alkaline adaptation on surfaces properties of *Listeria monocytogenes* 10403S. Open Food Sci. J. 3:62-65.

Guinebretière MH, Thompson FL, Sorokin A, Normand P, Dawyndt P, Ehling-schulz M, Svensson B, Sanchis V, Nguyen-The C, Heyndrickx M, De Vos P (2008). Ecological diversification in the *Bacillus cereus* group. Environ. Microbiol. 10:851-865.

Hamadi F, Latrache H, El Ghmari A, El Louali M, Mabrrouki, M, Kouider N (2004). Effect of pH and ionic strength on hydrophobicity and electron donor and acceptor characteristics of *Escherichia coli* and *Staphylococcus aureus.* Ann. Microbiol. 54:213-225.

Husmark U, Ronner U (1992). The influence of hydrophobic, electrostatic and morphologic properties on the adhesion of Bacillus spores. Biofouling 5:330-334.

Kumari S, Sarkar PK (2014). *In vitro* model study for biofilm formation by *Bacillus cereus* in dairy chilling tanks and optimization of clean-in-place (CIP) regimes using response surface methodology. Food Control 36:1153-158.

Lindsay D, Brozel VS, Mostert JF, von Holy A (2000). Physiology of dairy-associated Bacillus spp. over a wide pH range. J. Food Microbiol. 54:49-62.

Lindsay D, Oosthizen MC, Brozel VS, von Holy A (2002). Adaptation of a neutrophilic dairy-associated Bacillus cereus isolate to alkaline pH. J. Appl. Microbiol. 92:81-9.

Malek F, Moussa-Boudjemaa B, Aouar-Metri A, Mabrouk K **(**2013). Identification and genetic diversity of *Bacillus cereus* strains isolated from a pasteurized milk processing line in Algeria. Dairy Sci. Technol. 93:73-82.

Marsh EJ, Luo H, Wang H (2003). A three-tiered approach to differentiate *Listeria monocytogenes* biofilm-forming abilities. FEMS Microbiol. Lett. 228:203-210.

Mols M, Abee T (2011) *Bacillus cereus* response to acid stress. Environ. Microbiol. 13:2835-2843.

Moorman MA, Thelemann CA, Zhou S, Pestka JJ, Linz JE, Ryser E (2008). Altered hydrophobicity and membrane composition in stress-adapted *Listeria innocua*. J. food protection 71:182-185.

Peng JL, Tsai WC, Chou CC (2001). Surface characteristics of *Bacillus cereus* and its adhesion to stainless steel. Int. J. Food Microbiol. 65:105-111.

Salustiano VC, Andrade NJ, Ribeiro Junior JI, Fernandes PE, Lopes JP, Bernardes PC, Portugal JG (2010). Controlling *Bacillus cereus* adherence to stainless steel with different cleaning and sanitizing procedures used in dairy plants. Arch. Bras. Med. Vet.. Zootechnol. 62:1478-1483.

Seale RB, Flint SH, McQuillan AJ, Bremer PJ (2008). Recovery of spores from thermophilic dairy bacilli and effects of their surface characteristics on attachment to different surfaces. Appl. Environ. Microbiol. 74:731-737.

Shaheen R, Svensson B, Andersson MA, Christiansson A, Salkinjova-Salonen M **(**2010). Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. Food Microbiol. 27: 347-55.

Simmonds P, Mossel BL, Intaraphan T, Deeth HC **(**2003). Heat resistance of Bacillus spores when adhered to stainless steel and its relationship to spore hydrophobicity. J. Food Protection 66:2070-2075.

Svensson B, Ekelund K, Ogura H, Christiansson A (2004). Characterization of *Bacillus cereus* isolated from milk silo tanks at eight different dairy plants. Int. dairy J. 14:17-27.

Tauveron G, Slomianny C, Henry C, Faille C (2006). Variability among *Bacillus cereus* strains in spore surface properties and influence on their ability to contaminate food surface equipment. Int. J. Food Microbiol. 110: 254-262.

Wijmann JGE, de Leeuw PPLA, Moezelaar R, Zwietering MH, Abee T (2007). Air liquid interface biofilms of *Bacillus cereus*: Formation, Sporulation and Dispersion. Appl. Environ. Microbiol. 73:1481-1488.