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ORIGINAL PAPER

The role of surface charge and hydrophobicity in the attachment of *Anoxybacillus flavithermus* isolated from milk powder

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Abstract The aim of the present study was to investigate the attachment mechanisms that enable the thermophile *Anoxybacillus flavithermus* (B12) to attach to stainless-steel surfaces. Passing a B12 culture through a column of stainless-steel chips, collecting the first cells to pass through, re-culturing, and repeating the process six times, resulted in the isolation of a mutant, labeled X7, with tenfold reduced ability to attach to stainless steel as well as a reduced ability to attach to plastic. A comparison of bacterial cell-surface properties indicated that X7 was less hydrophobic than its parental strain B12. Cell-surface charge measurements also suggest that X7 had a lower net-negative surface charge. Disruption of extracellular polysaccharides and DNA appeared to have no effect on the attachment process. Removal of surface proteins caused a reduction in attachment of both B12 and X7, suggesting surface protein involvement in attachment.

Keywords *Anoxybacillus flavithermus* · Biofilm · Dairy · Surface charge · Hydrophobicity

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Introduction

Anoxybacillus flavithermus is a thermophilic, sporeforming, non-pathogenic bacillus, which is a potential contaminant of dairy products [35]. The organism is recognized as a problem in the manufacture of milk powder, as high levels of these bacteria can reduce the acceptability of the powder in local and international markets. The spores of this organism are very heat resistant, with the vegetative cells able to grow in temperatures of up to 65°C. The bacteria are normally present at low levels in raw milk but may reach 10^5CFU/g levels in the final product after 15–20 h of plant operation [19, 45]. The limited residence time of the milk in the plant during milk powder manufacture and the concentration effect of converting milk into milk powder cannot explain the number of thermophiles found in the final product. This suggests that thermophiles are attaching to the large surface area of stainless steel found within a milk evaporator and then growing and developing into biofilms, with individual cells and/or biofilm fragments sloughing off into the product line and thus contaminating the final product.

Biofilms have been defined as an aggregation of microbial cells and their associated extracellular polysaccharide (EPS) actively attached to and growing on a surface [25]. The initial adhesion event between a bacterial cell and a surface is the first essential step in the formation of any biofilm. One of the most important theories in the initial attachment of bacteria to solid surfaces was proposed by Marshall et al. [32] in which the adhesion process was divided into two distinct phases of reversible and irreversible adhesion. The reversible adhesion process was considered to involve van der Waals forces, electrostatic forces, and hydrophobic interactions. Irreversible adhesion was later described by Duanne [16] as bacteria locking onto the surface by production of

exo-polysaccharides or other cell surface structures that complex with a substratum. Perni et al. [41] concluded persistent strains of *Listeria*, i.e., strains routinely isolated over months to years from fluid dairy processing plants, were able to attach to surfaces with maximum adhesive strength following a short contact time. More recent work using atomic force microscopy (AFM) also suggests that multiple forces are involved in the process of bacterial attachment to surfaces, especially electrostatic interactions and hydrophobic interactions [1, 36, 51]. Previous work by Parkar et al. [37] suggested that surface proteins of thermophilic bacilli play a key role in the initial attachment to stainless-steel surfaces. Other groups working with *Staphylococcus* using transposon mutagenesis have also reported the important role of surface proteins in the initial attachment to a surface [11, 22, 23].

In an attempt to gain a greater understanding of how *A. flavithermus* cells attach to stainless steel, we isolated an attachment-deficient mutant of *A. flavithermus.* Biochemical characteristics of parent and mutant cells were compared to identify potential attachment mechanisms.

Materials and methods

Bacterial strains

The thermophilic strains of *Anoxybacillus* used in this study originated from milk powder. *A. flavithermus* strain B12 was obtained from a milk powder manufacturing plant and X7, an attachment-deficient mutant of B12, was isolated in our laboratory. Cultures were maintained in long-term storage by freezing at −75°C using Microbank beads (Pro-Lab Diagnostics, Austin, Texas, USA).

Isolation of spontaneous attachment-deficient mutant

Selection of a spontaneous attachment-deficient mutant of *A. flavithermus* strain B12 was carried out by passing a culture through a column of stainless-steel cuttings (1–3 mm in size) collected from an engineering lathe and soaked in three changes of 95% ethanol over 2 h. The cleaned cuttings were air dried at room temperature and placed in a 1 cm × 40 cm-long glass column. The filled glass column was autoclaved (121°C at 15 psi for 15 min) and placed in a retort stand. Then, 15 ml of a mid-log phase culture ($\approx 10^7$ cells per ml) of B12 (8 h) was added to the column and allowed to pass through. The first fraction collected after passage through the column was streaked onto Tryptic Soy Agar (TSA) and incubated at 55°C overnight. After restreaking, one isolated colony was used to inoculate 15 ml Tryptic Soy Broth (TSB), incubated for 8 h at 55°C and added to a cleaned glass column containing stainless-steel chips and the above cycle repeated. After repeating the process outlined above a total of seven times, bacterial colonies isolated from the first fraction through the column in the final run were compared to the original B12 strain in their ability to attach to stainless-steel coupons and polystyrene microtitre plates.

Random amplified polymorphic DNA (RAPD) analysis

Profiles of B12 and X7 were generated using primer OPR-13 (5′ GGACGACAAG 3′) [20, 43] under the following conditions: one cycle of 94°C for 3 min, 36°C for 15 s, and 72°C for 2 min, followed by 35 cycles of 94°C for 15 s, 36°C for 15 s, and 72°C for 2 min, followed by one cycle of 94°C for 15 s, 36°C for 15 s, and 72°C for 4 min. Amplified products were separated on 2% (w/v) agarose gels and stained in 0.005% Gelstar (Cambrex Bioscience, Rockland Inc).

API biochemical profile

The API 50 CH system (LaBalme LesGrottes, Montalieu, France) was used according to the manufacturer's instructions to provide information on any biochemical differences between strains B12 and X7.

Polystyrene microtitre plate attachment assay

The microtitre plate attachment assay was based on the methods reported by Ziebuhr et al. [54] and Loo et al. [31]. Cell cultures were centrifuged at 1,000 × *g* for 20 min and resuspended in 0.05 M phosphate buffer pH 7.0 to OD_{600nm} of 1.00. Resuspended cells (200 µl) were incubated overnight in 96-well polystyrene microtitre plates (Falcon, nontreated polystyrene plates, Bacto Laboratories, Australia) at room temperature in a closed 2-l plastic container with a moistened paper towel on the container bottom to prevent the sample from drying out. Wells were washed three times with 0.05 M phosphate buffer pH 7.0 and air dried at 55°C for 1 h. Then, 200 µl 0.1% solution of crystal violet was added and held at room temperature for 2 min and each well was washed five times with 0.05 M phosphate buffer pH 7.0. 200 µl of 95% ethanol was added to each well and OD_{490nm} measured using a MicroELISA plate reader (Dynatech, Torrance, California).

Epifluorescence microscopy of attached cells

The number of cells attached to stainless-steel surfaces was determined by epifluorescence microscopy [17, 37]. The laser-cut stainless-steel coupons (1 cm^2, 316 grade) were cleaned by heating in 50% nitric acid at 70°C for 10 min. The coupons were then washed and rinsed with distilled water and autoclaved at 121°C for 15 min. After 30 min

exposure to bacterial suspensions at room temperature, the stainless-steel samples were washed five times in sterile distilled water and then stained with 0.001% Acridine Orange (100 mM phosphate buffer, pH 7.2) for 2 min. After washing and air drying, the bacteria adhering to the stainless-steel surface were counted under an epifluorescence microscope (Leitz Ortholux II) with an H2 incident excitation filter block (Ernest Leitz Wetzlar, GmbH, Wetzlar, Germany).

Cell-surface treatments

Overnight cultures grown in TSB at 55°C were centrifuged (1,000 × *g* for 10 min) and samples resuspended to the original volume in one of the following solutions: protein removal agents, i.e., 1% trypsin at 37°C or 0.2 M glycine at pH 2.2 for 30 min; polysaccharide removing agent: 50 mM sodium metaperiodate in 0.05 M sodium acetate at pH 4.2 for 30 min; DNA disruption: 0.1 M Tris–HCl, pH 7.5 containing 2 mM $MgCl_2$ and 50 μg/ml of DNase I (Sigma) for 1 h at 25°C. After treatment, the cells were centrifuged (1,000 × *g* for 10 min) and resuspended in 0.05 M phosphate buffer pH 7.0.

Determination of cell-surface hydrophobicity

Microbial adhesion to hydrocarbon test (MATH)

In order to characterize the relative cell-surface hydrophobicity of B12 and X7 cultures, a modified version of the microorganism adhesion to hydrocarbon (MATH) test [41] was employed. Busscher et al. [8], Van der Mei [52], Doyle [14], and Ahimou et al. [2] have all suggested that electrostatic interactions can influence the MATH test because of the negative charge of hydrocarbons such as hexadecane. Doyle [14] proposed that the electrostatic interactions can be reduced by the presence of a high concentration of ions, usually NaCl. Cultures of B12 and X7 were grown to mid-log phase, centrifuged at 1,000 × *g* for 20 min and resuspended in 0.05 M phosphate buffer at pH 7.0 containing one of the following sodium chloride concentrations: 0, 1, 2, 3, or 4 M. The final OD_{600nm} was adjusted to 0.7–0.8 and 2.5 ml of the suspension was added to 2.5 ml of hexadecane, mixed for 30 s on a vortex mixer, and incubated for 20 min at 30°C. The absorbance of the aqueous phase was measured at 600 nm using a spectrophotometer (U2000 Hitachi, Japan) and the percentage hydrophobicity was determined using the formula below.

$$\% \text{ hydrophobicity} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

A_0 = OD of the bacterial suspension before mixing with hexadecane, A_1 = OD of the bacterial suspension after mixing with hexadecane.

Hydrophobic interaction chromatography (HIC)

In a further attempt to determine the cell-surface hydrophobicity of B12 and X7 cultures, a modified version of HIC as reported by Smyth et al. [46] and Peng et al. [40] was employed. Pasteur pipettes were plugged with glass wool and filled with 1 ml of suspension of hydrophobic resin (Phenyl Sepharose 6 fast flow, Amersham Biosciences). The resin was washed with 10 ml of 0.05 M phosphate buffer at pH 7.0 with the same concentration of NaCl added as the bacterial suspension to be tested, i.e., 0 M, 1 M, 2 M, 3 M, or 4 M NaCl. The bacterial cells were prepared by centrifuging a mid-log phase culture at 1,000 × *g* for 20 min and resuspending in 0.05 M phosphate buffer pH 7.0 with one of the concentrations of sodium chloride to an OD_{600nm} of 1.0. Each column was loaded with 0.3 ml of culture and eluted with 0.9 ml of 0.05 M phosphate buffer (pH 7.0) with appropriate NaCl concentration. Measuring absorbance at 600 nm, the percentage of bacteria retained in the hydrophobic column was calculated from the absorbance of a ¼ dilution of the original bacterial suspension with NaCl (OD_{600nm} 0.7–0.8) and the absorbance of the sample eluted from the column.

$$\% \text{ retained by column} = [(A_0 - A_1)/A_0] \times 100 \quad (2)$$

A_0 = OD of ¼ diluted bacterial suspension, A_1 = OD of the eluted bacterial suspension.

Determination of cell surface charge

Estimation of Zeta potential

The surface charge of both B12 & X7 was expressed as the Zeta potential in a Malvern ZetaSizer IV (Malvern Instruments Ltd, UK) [2]. Cells were grown to mid-log phase and washed twice by centrifugation at 1,000 × *g* for 20 min and resuspended in 5 mM NaCl solution. Cells were diluted to OD_{600nm} 0.5–0.6 in a solution of pH 2, 3, 4, 5, 6, 7, or 8 using phosphate-citrate buffers with ionic strength = 0.01 M.

Electrostatic interaction chromatography (EIC)

The relative surface charge of both B12 & X7 strains was assessed by separation through anionic (Dowex AG 1-8x 100-200 mesh) or cationic (Dowex AG 50W-X8 100-200 mesh) exchange resins (Bio-Rad). The method is based upon that of Pedersen [39] and Flint et al. [17]. Pasteur pipettes were plugged with glass wool and filled with 0.5 g of ion exchange resin then eluted with 5 ml of 0.01 M phosphate buffer (0.01 M KH_2P0_4 (BDH); 0.01 M Na_2HP0_4 (BDH)) at pH 7.0. Bacterial suspensions were prepared by centrifuging a mid-log phase culture (8–10 h)

at 1,000 g for 20 min and resuspending in phosphate buffer to OD_{600nm} 0.7–0.8. Each column was loaded with 1 ml of bacterial suspension and then eluted with 3 ml of phosphate buffer. The absorbance of the eluted sample was measured at 600 nm and the percentage of bacteria retained in the column was calculated from the absorbance of a ¼ dilution of the original bacterial suspension (OD_{600nm} 0.7–0.8) and the absorbance of the sample eluted from the column.

$$\% \text{ retained by Column} = [(A_0 - A_1)/A_0] \times 100$$

A_0 = OD of ¼ diluted bacterial suspension

A_1 = OD of the eluted bacterial suspension

Quantitative assay of surface polysaccharides

The amount of extracellular polysaccharide (EPS) on the surface of each strain was determined by a modified acid hydrolysis method [12]. The vegetative cells or spores were suspended in distilled water to an OD_{600nm} of 1.2, sonicated at 7-μm amplitude for 30 s, then centrifuged at 1,000 × *g* for 10 min. The polysaccharides in the supernatant were precipitated by adding 1 ml of the supernatant dropwise to 8 ml 99% alcohol and keeping at 4°C overnight. This solution was then centrifuged at 1,000 × *g* for 20 min at 4°C. The pellet was dissolved in 1 ml distilled water and this solution was dialyzed against three changes of distilled water. This treatment was not described in the original paper, but was designed to remove any cross-reacting mono- or oligosaccharides that may be released by cell lysis during ultrasonication. To each sample, 7 ml 77% sulphuric acid were added to digest the polysaccharides and the solution was cooled in an ice-bath for 10 min. Cold 1% tryptophan (1 ml) was added to each tube and mixed. All the tubes were kept in a boiling water-bath for 20 min. After cooling to room temperature, the absorbances were read at 500 nm against a distilled water blank instead of the sample. A standard curve using 0.01–0.1 mg ml^{-1} dextran (molecular weight 52,000; Sigma Chemical Co.) was prepared to permit estimation of the amount of polysaccharide.

Results

Mutant isolation and attachment screening

The cellular sieving of *A. flavithermus* (B12) vegetative cells through stainless-steel chips as outlined in Fig. 1 was conducted seven times and resulted in the successful isolation of a stable isolate labeled X7. The X7 isolate demonstrated about a 1–1.5 $\log_{10}$ per cm^{-2} decrease in attachment, with 3.69 ± 0.24 $\log_{10}$ cells per cm^{-2} on stainless-steel coupons, compared to its parental strain B12, with 5.21 ± 0.02 $\log_{10}$ cells per cm^{-2}. Errors represent the standard deviation from the mean of three replicates. Figure 2 shows that X7 also exhibited reduced attachment to polystyrene microtitre plates compared with the original B12 strain. The parental strain B12 and mutant strain X7 gave identical RAPD-PCR (Fig. 3) and API 50CHB results (data not shown), verifying that X7 was a mutant of culture B12.

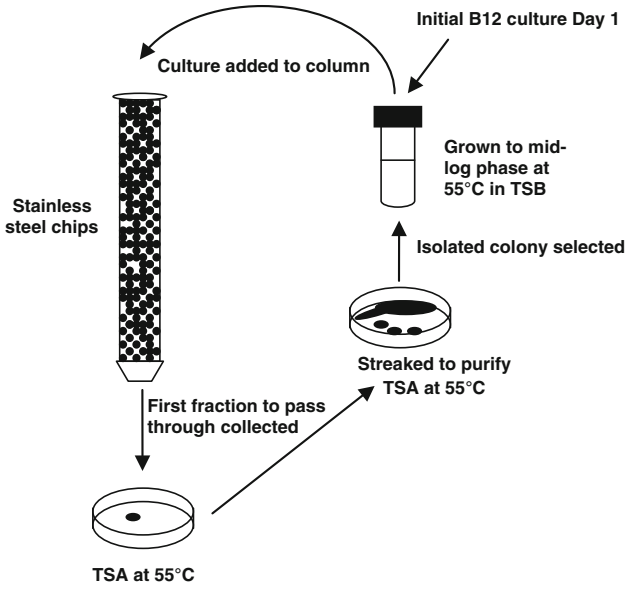

Fig. 1 Summary of strategy employed to isolate an attachment-deficient mutant from culture B12. This process was repeated six times to produce the isolate known as X7

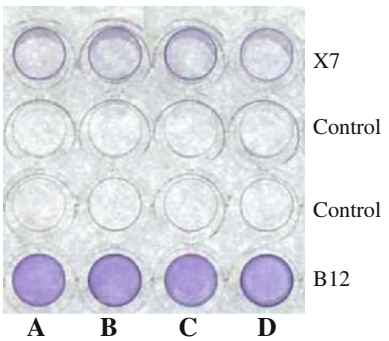

Fig. 2 Attachment of B12 and X7 cultures to polystyrene microtitre plates. All wells labeled A to D were loaded with an equal volume and density of cells. Control wells contained only water

Cell-surface charge

Both B12 and X7 were retained more on the anionic exchange resin (functional group $R\text{-}CH_2N^+(CH_3)_3$) than on the cationic column (functional group $R\text{-}SO_3^-$). Figure 4 indicates that both strains had a net-negative charge at

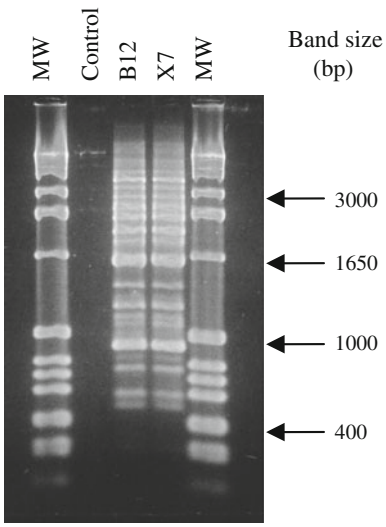

Fig. 3 RAPD profiles of B12 and X7 cells

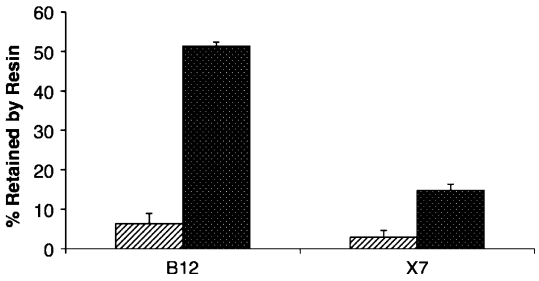

Fig. 4 Proportion of B12 and X7 cells retained on anionic (functional group R-$CH_2N^+(CH_3)_3$ *dotted bar*) and cationic (functional group R-SO_3^- *stripped bar*) at pH 7. *Error bars* represent the SD from the mean of three replicates

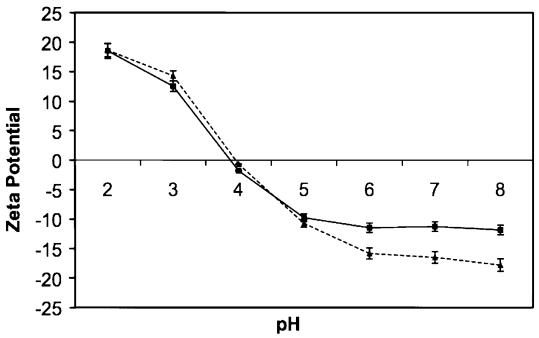

Fig. 5 Zeta potentials of strains B12 (*filled square*) and X7 (*filled triangle*) as a function of pH. *Error bars* represent the SD from the mean of three replicates

pH 7. Strain B12 demonstrated a higher retention on anionic resin at 51% compared to X7 at 14% retention, i.e., B12 had a greater negative surface charge. The electrophoretic mobility of strains B12 and X7, measured at pH values between 2 and 8 and expressed as zeta potentials, are shown in Fig. 5. Both strains showed a decrease in zeta potential with increasing pH, representative of the pH-dependent charge at the bacterial surface. Both strains had a similar isoelectric point of about pH 4, but strain X7 had a higher zeta potential (less negative charge) than B12 over the pH range 6–8.

Cell-surface hydrophobicity

In the MATH test at lower salt concentrations (0–1 M), the hydrophobicity of both B12 and X7 was very similar, but at higher salt concentrations (2, 3, and 4 M) X7 had a lower hydrophobicity than B12 (Fig. 6). In the second method (HIC), similar results were observed as in the MATH test. At low concentrations of NaCl (0–1 M) the hydrophobicity of X7 and B12 were similar, but at greater concentrations of NaCl (2, 3, and 4 M) the hydrophobicity of X7 was lower than that of B12 (Fig. 7).

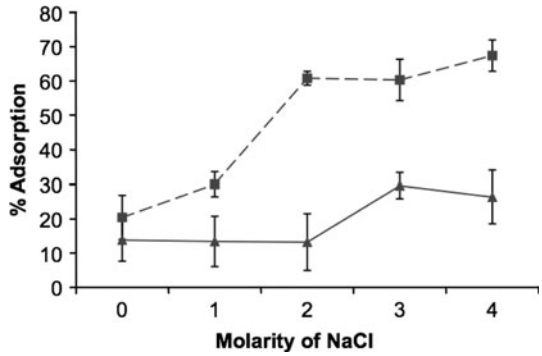

Fig. 6 Percentage adsorption of strains B12 (*filled square*) and X7 (*filled triangle*) using HIC test over increasing ionic strengths. *Error bars* represent the SD from the mean of three replicates

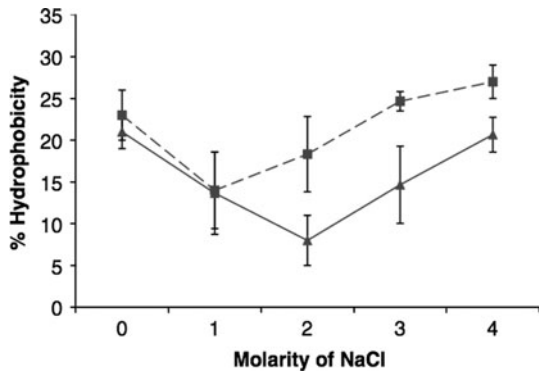

Fig. 7 Percentage hydrophobicity measurements of strains B12 (*filled square*) and X7 (*filled triangle*) using MATH test over increasing ionic strengths. *Error bars* represent the SD from the mean of three replicates

Extracellular polysaccharide

The amount of EPS recovered from standardized cell suspensions of B12 and X7 revealed little difference in the amount of EPS produced, with B12 producing 59 ± 5.8 μg dextran equivalents per 10^8 cells and X7 producing 57 ± 2.4 μg dextran equivalents per 10^8 cells.

Attachment following treatment to remove surface proteins

Treatment of B12 and X7 cells suspensions with trypsin reduced the attachment of B12 to stainless steel from 5.21 ± 0.04 $\log_{10}$ to 4.26 ± 0.03 $\log_{10}$ cells per cm^2. X7 trypsin-treated cells also demonstrated a reduction in attachment to stainless steel from 3.69 ± 0.24 $\log_{10}$ to 3.04 ± 0.14 $\log_{10}$ cells per cm^2. B12 cells treated with 0.2 M glycine pH 2.2 demonstrated reduced attachment to stainless-steel coupons from 5.21 ± 0.04 $\log_{10}$ cells per cm^2 to 4.43 ± 0.07 $\log_{10}$ cells per cm^2. Treatment of X7 cells with 0.2 M glycine pH 2.2 caused only a slight reduction in attachment 3.69 ± 0.24 $\log_{10}$ cells to 3.51 ± 0.19 $\log_{10}$ cells per cm^2 (Fig. 8).

Attachment following treatment to disrupt cell wall polysaccharides

Treatment of B12 cells with sodium metaperiodate appeared to have no effect on cell attachment to stainless steel, with attachment of B12 cells before treatment 5.21 ± 0.04 $\log_{10}$ cells per cm^{-2} compared to 5.18 ± 0.04 $\log_{10}$ cells per cm^{-2} after treatment. Treatment of X7 with sodium metaperiodate also appeared to have no effect on cell attachment to stainless steel, with X7 cells before treatment attaching at 3.69 ± 0.24 $\log_{10}$ cells per cm^{-2} compared with 3.81 ± 0.21 $\log_{10}$ cells per cm^{-2} after treatment (Fig. 8).

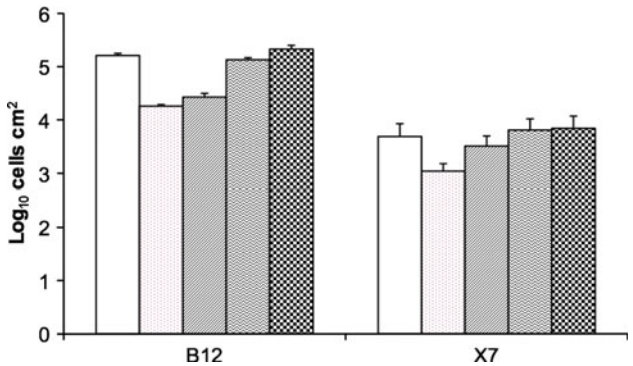

Fig. 8 Effect on the attachment of B12 and X7 cells following treatment to remove surface proteins or polysaccharides or DNA. (*empty square*) control, (*dotted bar*) trypsin, (*stripped bar*) acid-glycine, (*wavy bar*) metaperiodate and (*checked bar*) DNase I

Attachment following treatment to remove surface-associated DNA

Treatment of B12 and X7 cells with DNase I caused no apparent reduction in attachment to stainless steel with respect to control untreated cells of B12 and X7 (Fig. 8).

Discussion

Hermansson [24] considered that in the process of identifying surface characteristics that are important in bacterial attachment to surfaces, comparisons of an isogenic strain with the parental strain may result in more consistent results than different strain-to-strain comparisons. Surface heterogeneity, common when comparing different species or strains from different environments, was thus limited. In this study, a spontaneous isogenic mutant or variant strain of culture B12 with reduced ability to attach to stainless steel and polystyrene was isolated by repeatedly passing a culture through a column of stainless-steel chips. The concept of isolating by natural selection a spontaneous mutant with reduced attachment to a substrate has been reported by Mosoni et al. [33] studying *Ruminococcus albus* attachment to cellulose and by Kolenbrander [27] investigating *Actinomyces* spp. co-aggregation.

To ensure that strain the X7 was not a laboratory contaminant, random amplified polymorphic DNA (RAPD) analyses, biochemical assays, and observations of culture growth and colony morphology were used to compare B12 and X7. None of these techniques was able to distinguish between the two strains, strongly suggesting that the selection process resulted in the isolation of an isogenic mutant demonstrating a phenotype with reduced ability to attach to surfaces compared to its parental strain B12. Furthermore, the inability of standard techniques to distinguish between strains B12 and X7 shows limited heterogeneity of X7 compared to strain B12. The observed differences in their ability to attach to surfaces may be the result of differences in surface characteristics of the two strains. Epifluorescence microscopy was initially used to compare the abilities of X7 and B12 to attach to stainless-steel coupons, as previously reported by Flint et al. [18] and Parker et al. [37]. The X7 isolate consistently demonstrated approximately 1–1.5 $\log_{10}$ per cm^{-2} reduction in attachment compared to the original B12 strain. The microtitre plate method has been used by several groups [4, 31, 54] to identify isolates that demonstrate a reduced ability to attach to surfaces. In this study, the microtitre plate method also produced results supporting the epifluorescence microscopy evidence that the X7 isolate has a reduced ability to attach to surfaces compared with B12.

The basic surface properties, i.e., hydrophobicity and surface charge of B12 and X7, were compared to gain some understanding of which surface properties might play an important role in the initial attachment of *A. flavithermus* (B12) to surfaces.

Hydrophobic interactions are thought to play an important role in bacterial attachment to surfaces [21, 30, 34]. Hydrophobic interactive chromatography (HIC) and microbial adhesion to hydrocarbon (MATH) [34, 44] were employed to measure the hydrophobicity of strains B12 and X7. Overall, the two methods indicated that B12 cells are more hydrophobic than X7 cells in the presence of high concentrations of NaCl. However, one concern over the validity of the MATH and HIC tests is the possible interplay between hydrophobic and charge properties involved in the MATH and HIC tests. Ahimou et al. [2], Busscher et al. [8], and Van der Mei et al. [52] have all expressed concern over the use of the MATH test without minimizing electrostatic interactions. To overcome the potential interference of electrostatic interactions in the MATH test, Doyle [14] suggested the use of high ionic strength conditions. Additionally, Smyth et al. [46] suggested that when using HIC to measure hydrophobicity, higher ionic strengths of NaCl reduced possible electrostatic interactions. The results here indicate that strain B12 has a greater surface hydrophobicity than strain X7 in the presence of higher concentrations of NaCl. The results of the present study are similar to those of Smyth et al. [46], who demonstrated that using HIC at higher ionic strengths of NaCl to suppress electrostatic interactions, the differences in hydrophobicity between *E. coli* K88 negative and *E. coli* K88 positive strains were more pronounced.

In general, strain B12 has a greater ability to attach to plastic (hydrophobic) and stainless steel than X7. However, the hydrophobicity rating of stainless steel is not well defined: Brugnoni et al. [6] and Teixeria et al. [48] described stainless steel as hydrophobic, but Lejeune [29] and Planchon et al. [42] described it as hydrophilic. This is may be a reflection of different methods and grades of stainless steel employed to measure surface hydrophobicity. Liu et al. [30] reported that high hydrophobicity of a microbial cell surface could help attachment to both hydrophobic and hydrophilic surfaces. The observation by Liu et al. [30] could be a reflection of the relative hydrophobicity of the three phases, i.e., the aqueous phase, bacterial surface, and substratum. Consequently, if the bacterial surface and solid surface are more hydrophobic than the aqueous phase, this may cause an increase in the hydrophobic interaction between the bacteria and substrate.

Cell-surface charge is often associated with the adhesion of bacteria to surfaces [5, 50]. In this study, both Zeta potential measurements and electrostatic interaction chromatography indicated that strain X7 has a lesser net-negative charge compared to its parental strain B12 at neutral pH. Substrates such as stainless steel at neutral pH frequently possess a net-negative charge, which naturally results in the repulsion of negatively charged cells from the surface [24]. However, strain B12, has a greater ability to attach to stainless steel, despite having a stronger negative charge than X7. One explanation for this observation may lie at a more microscopic scale. Bacterial cell surfaces may possess positively charged domains which may mediate attachment, despite overall electrostatic repulsion. Cowan et al. [10] hypothesized this for the interaction between *Treponema deticola* and human erythrocytes. Busscher et al. [7] also concluded that localized charged domains on the cell surface of *Streptococcus mutans* may override the influence of global cell-surface charge on their attachment to fibronectin. Another possible explanation is that other physico-chemical properties such as hydrophobicity, van der Waals attractive forces, and steric forces override electrostatic interactions in the attachment process.

Changing the surface characteristics of cells and studying the effect on attachment may help to identify factors important in the initial attachment of *A. flavithermus* (B12) in milk powder plants. The results of this investigation suggest that cell-surface proteins play a key role in the attachment process of *A. flavithermus* to stainless steel. Removal of surface proteins by trypsin digestion or acid-glycine treatment decreased the number of cells attaching to stainless steel. Many researchers have pointed to the role played by surface proteins in the attachment of various bacterial types to surfaces [11, 16, 17, 37, 38]. Surface proteins may play a universal role in bacterial attachment. Acid-glycine surface extraction involves exposing cells to 0.2 M glycine at pH 2.2 and has been used by Calabi et al. [9] to extract surface layer proteins from *Clostridium difficile* and various *Campylobacter* species [15, 26]. Although the exact mechanism by which proteins were removed from the bacterial surface was not discussed, it is assumed that it interferes with non-covalent bonds present on a cell surface, releasing non-covalently bonded surface proteins. In this study, acid-glycine extraction of surface proteins from B12 and X7 strains resulted in a decrease in the attachment of both strains to stainless steel, but in general, trypsin treatment resulted in a greater reduction in attachment. This may be due to the limited ability of the acid–glycine treatment to remove covalently bound surface proteins compared to trypsin.

Other factors examined, i.e., production of EPS and extracellular DNA, have been implicated in the initial attachment process of bacteria to surfaces [13, 49, 53]. However, in the present study, the quantities of EPS produced by B12 and X7 were similar, suggesting that the amount of EPS produced is not responsible for the difference in the attachment of B12 and X7. Furthermore,

disruption of surface polysaccharides caused no reduction in attachment of either strain. Parkar et al. [37] also reported no correlation between EPS production and attachment of thermophilic bacilli to stainless steel and Flint et al. [17] concluded that EPS played no role in the attachment of thermophilic streptococci to stainless steel. It is likely that EPS is more important in the subsequent stages of biofilm formation [12, 28].

Several researchers have pointed to the role played by extracellular DNA in biofilm growth and development and it has even been given the acronym eDNA [3, 47]. However, the role of eDNA on initial cell attachment is not well understood. Whitchurch et al. [53] reported that DNase I added to a medium flowing over a biofilm of *Pseudomonas aeruginosa* less than 82 h old resulted in the biofilm being dissolved. Surfaces continuously exposed to a medium containing DNase I contained very few attached cells after 3 days of incubation, compared to surfaces exposed to medium without DNase I, which were extensively colonized [53]. Cells of B12 and X7 exposed to DNase I showed very similar levels of attachment compared to untreated cells, suggesting that eDNA is not involved in the initial attachment of B12 or X7 to stainless steel. However, the role of eDNA in biofilm development cannot be excluded.

In summary, the adhesion-deficient X7 mutant appears to differ from B12 mainly in that it is less hydrophobic and has a smaller negative charge. This suggests that both of these parameters may be determinants of the ability of *A. flavithermus* to adhere to stainless steel. In addition, the reduction of attachment of both X7 and B12 by treatments that remove cell-surface proteins indicates that cell-surface proteins are important in *A. flavithermus*' attachment to steel surfaces.

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