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ORIGINAL ARTICLE

Competitive exclusion as a mode of action of a novel *Bacillus cereus* aquaculture biological agent

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Abstract

Aims: To determine the contribution of potential modes of action of a *Bacillus cereus* aquaculture biological control agent in inhibition of the fish pathogen, *Aeromonas hydrophila*.

Methods and Results: When *B. cereus* was tested in plate well inhibition studies, no production of antimicrobial compounds was detected. *Bacillus cereus* had a high growth rate (0·96 h^{-1}), whereas *Aer. hydrophila* concentration decreased by *c.* 70% in co-culture experiments. In nutrient limitation studies, *B. cereus* had a significantly higher growth rate when cultured under glucose ($P < 0·05$) and iron ($P < 0·01$) limitation in comparison with *Aer. hydrophila*. *Bacillus cereus* glucose (0·30 g l^{-1} h^{-1}) and iron (0·60 mg l^{-1} h^{-1}) uptake rates were also significantly higher ($P < 0·01$) than the *Aer. hydrophila* glucose (0·14 g l^{-1} h^{-1}) and iron (0·43 mg l^{-1} h^{-1}) uptake rates. Iron uptake was facilitated by siderophore production shown in time profile studies where relative siderophore production was *c.* 60% through the late exponential and sporulation phases.

Conclusions: Competitive exclusion by higher growth rate, competition for organic carbon and iron, facilitated by siderophore production, could be identified as mechanisms of pathogen growth inhibition by *B. cereus*.

Significance and Impact of the Study: This study is the first elucidation of the mechanism of action of our novel *B. cereus* biological agent in growth attenuation of pathogenic *Aer. hydrophila*. This study enhances the application knowledge and attractiveness for adoption of *B. cereus* NRRL 100132 for exploitation in aquaculture.

Introduction

Global aquaculture is challenged by poor water quality and the outbreak of diseases (Jeney and Jeney 1995; Moriarty 1999). The use of conventional chemotherapies has resulted in the increased virulence of pathogenic strains, negative environmental impact and is often met with consumer resistance (Verschuere *et al.* 2000). Exploitation of beneficial bacteria as biological agents has potential advantages to address aquaculture challenges by improving water quality and reducing disease propensity caused by pathogenic bacteria (Fast and Menasveta 2000; Gomez-Gill *et al.* 2000; Jana and Jana 2003; Hong *et al.* 2005). Water quality and infection by pathogenic *Aeromonas hydrophila* are major challenges in the highly lucrative aquaculture of *Cyprinus carpio*.

A novel *Bacillus cereus* (NRRL 100132) strain was previously isolated as a biological agent for *C. carpio* and its outstanding capability in enhancing water quality and reducing *Aer. hydrophila* growth was demonstrated in both *in-vitro* and *in-vivo* studies (Lalloo *et al.* 2007). This *B. cereus* is a water additive and was shown to be safe for use. The functionality of the micro-organism was also demonstrated across a range of physiological conditions,

prevalent in aquaculture (Lalloo *et al.* 2008). Spore-forming *Bacillus* spp. are attractive as biological control agents as they possess antagonistic effects on pathogens, can improve water quality and are ubiquitous in natural environments (Wolken *et al.* 2003; Hong *et al.* 2005). Spores are physiologically robust and can be formulated into stable commercial products which are tolerant to the environmental conditions required in their application (Gross *et al.* 2003; Lalloo *et al.* 2009).

The success of strategies using biological agents and adoption of this technology by the aquaculture industry depends on an understanding of the beneficial characteristics and mechanism of action (Verschuere *et al.* 2000; Vine *et al.* 2006). However, studies showing the mode of action for antagonism of *Aer. hydrophila* by *Bacillus* spp. are limited, while no studies on the mode of action of *B. cereus* as a biological agent against this pathogen have been reported (Kumar *et al.* 2006; Newaj-Fyzul *et al.* 2007). Potential mechanisms of biological agents against pathogens include competition for adhesion sites, production of enzymes, immune stimulation, synthesis of antimicrobials, competitive exclusion and bioremediation (Verschuere *et al.* 2000; Sanders 2003; Hong *et al.* 2005). The basis of competitive exclusion is through competition for chemicals or for available energy or by intrinsic growth rate advantage (Verschuere *et al.* 2000; Holzapfel *et al.* 2001; Irianto and Austin 2002; Hong *et al.* 2005). Many of these mechanisms only apply to probiotics added to feed, but the latter three are relevant to waterborne additives such as *B. cereus*.

The bioremediation capability for ammonium, nitrite, nitrate and phosphate waste removal by *B. cereus* NRRL 100132 was well elucidated previously (Lalloo *et al.* 2007). Likely modes of action by our *B. cereus* isolate in antagonism of *Aer. hydrophila* are the production of inhibitory compounds and competitive exclusion. Fastidious heterotrophs such as *Bacillus* spp. often demonstrate a high utilization of organic carbon (Verschuere *et al.* 2000). Some are also capable of synthesizing low molecular weight chelating compounds called siderophores which facilitate competitive uptake of iron for growth (Verschuere *et al.* 2000; Winkelmann 2002). As both carbon and iron are essential requirements for growth by most organisms, limitations can result in growth attenuation (Braun and Killmann 1999). In this study, we investigated the contribution of direct inhibition by production of extracellular inhibitory compounds and competitive exclusion through growth rate advantage, competition for key nutrients such as organic carbon and iron as potential modes of action involved in the inhibition of the important fish pathogen, *Aer. hydrophila* by our novel *B. cereus* aquaculture biological agent.

Materials and methods

Micro-organisms used in the study

Our novel isolate *B. cereus* (NRRL 100132) and a test pathogen Aer. *hydrophila* (ATCC 7966) was cultured and stored as described previously (Lalloo *et al.* 2007).

Detection of antimicrobial activity of *Bacillus cereus* NRRL 100132

The production of antimicrobial compounds by *B. cereus* NRRL 100132 was assessed by culturing the strain in 2 l Braun Biostat B fermenters (Sartorius BBI Systems, Melsungen, Germany) as previously described (Lalloo *et al.* 2009). Airflow was maintained at 1 v v^{-1} m^{-1}, and agitation speed was ramped from 500 rev min^{-1} to a maximum of 1000 rev min^{-1} to maintain oxygen saturation above 30%. All materials used in this study were obtained from Merck (Darmstadt, Germany) unless otherwise stated.

Fermenters were sampled during early exponential, mid exponential and the sporulation phase. The growing culture (fermentation broth sample), intracellular cell fraction and extracellular supernatant were evaluated for the presence of inhibitory compounds. The extracellular fraction was the resultant supernatant after centrifugation of the whole broth at 13 000 ***g***. The resultant cell pellet was washed, re-suspended in saline (0·9% m v^{-1} NaCl) and ultra-sonicated at a frequency of 20 kHz s^{-1} at 192 watts on ice for 12 min (12×48 s cycles of sonication with a 12-s pause between cycles) and then re-centrifuged. The supernatant of this cell preparation was used as the intracellular fraction. Cell preparations (100 μl) of growing culture, intracellular fraction or extracellular supernatants were loaded into wells (10 mm) on nutrient agar plates prespread with *Aer. hydrophila* (ATCC 7966) culture. Plates were incubated (12 h, 32°C) and visualized for zones of inhibition.

Co-culture of *Bacillus cereus* and *Aeromonas hydrophila* in shake flasks

Stored cryo-cultures (2 ml) of *Aer. hydrophila* and *B. cereus*, prepared according to Meza *et al.* (2004), were used to inoculate triplicate 1-l Erlenmeyer flasks, containing synthetic pond water (SPW) growth medium and the culture flasks incubated (Lalloo *et al.* 2007). Similarly, a negative control co-culture study devoid of iron was conducted. Samples were taken two hourly, and cell counts were performed using a Thoma® bacterial counting chamber (Hawksley & Sons, London, UK) for both organisms.

Comparison of growth rate between *Bacillus cereus* and *Aeromonas hydrophila* under nutrient limitation

The impact of nutrient limitation on growth of *B. cereus* or *Aer. hydrophila* was assessed by lowering the concentration of one media component (glucose, nitrite, nitrate, ammonia, iron or phosphate) in SPW to 10% of base case. De-ionized water was the negative control and SPW was the positive control.

Media were prepared by combining amino acid, vitamin, trace element, nutrient and ion solutions. Each media formulation contained 20 μl of an amino acid solution (45 mg l^{-1} each of the following: alanine, arginine, aspartic acid, glutamic acid, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine and valine), 20 μl of a vitamin solution (Lalloo *et al.* 2009) and 20 μl of a trace element solution ($CaCl_2$ 3·4 mg l^{-1}, $MgCl_2{\cdot}4H_2O$ 2·6 mg l^{-1}, H_3BO_3 5·0 mg l^{-1}, Na2$MoO_4{\cdot}2H_2O$ 0·3 mg l^{-1}, $CoCl_2{\cdot}6H_2O$ 0·4 mg l^{-1}). The nutrient solution (glucose 10·0 g l^{-1}) and ion solution [$NaNO_2$ 0·6 g l^{-1}, KNO_3 0·85 g l^{-1}, $FeC_6H_6O_7$ 0·16 g l^{-1}, $(NH_4)_2SO_4$ 0·93 g l^{-1} and H_3PO_4 3·8 g l^{-1}] were added as 20 μl aliquots to the media. Once all media components were added, the volume of each well was made up to 200 μl with de-ionized water. All solutions were sterilized by filtration through 0·22-μm filters.

Cultures of *B. cereus* NRRL 100132 or *Aer. hydrophila* (ATCC 7966) were grown (Lalloo *et al.* 2007) to 1×10^5 cells per ml, and an inoculum volume of 10 μl was used to inoculate the respective micro-titre wells (six wells per organism per test). Plates were incubated at 32°C for 24 h on a microtitre plate shaker set at 100 rev min^{-1}, and absorbance was measured and recorded every hour at 660 nm (Abs_{660}) using a BioTek Power wave^{HT} microtitre plate reader (BioTek Instruments Inc., Vermont, USA). Growth rates were determined from plots of the natural logarithm of Abs_{660} over time, conforming to linearity ($r^2 > 0{\cdot}9$). The growth rates obtained for both *B. cereus* and *Aer. hydrophila* were compared (ANOVA) to assess the impact of the individual component limitations on the growth of the two organisms (Table 1).

Table 1 Assessment of different cell preparations for the growth attenuation of *Aeromonas hydrophila* by *Bacillus cereus*

	Growing culture	Intracellular fraction	Extracellular supernatant
Mid exponential phase	+	–	–
Early stationary phase	+	–	–
Sporulation phase	+	–	–

–, No inhibition observed; +, presence of inhibition.

Measurement of glucose and iron uptake rates

Cryopreserved cultures of *B. cereus* or *Aer. hydrophila* were used to inoculate 1-l Erlenmeyer flasks containing 100 ml of sterile SPW in triplicate and incubated as previously described. Samples were taken on an hourly basis and analysed for iron and glucose concentrations. Iron concentrations were determined using a Spectroquant® kit 1.14549.0001 (Merck). Glucose concentrations were determined using an HPIC (CarboPac™, PA1 column; Dionex, Sunnyvale, MA, USA). Uptake rates were calculated from plots of concentration of iron or glucose against time for each micro-organism.

Measurement of siderophore production

Bacillus cereus (NRRL 100132) was used to inoculate 100 ml of sterile SPW in 1-l Erlenmeyer flasks and incubated as previously described. Flasks were sampled two hourly, and the cell and spore concentrations were determined, from which the sporulation ratio was calculated (Monteiro *et al.* 2005). Qualitative siderophore production using a modified chrome azurol S (CAS) assay (Milagres *et al.* 1999) and semi-quantitative siderophore production using the CAS universal siderophore assay (Schwyn and Neilands 1987) were assessed. The qualitative assessment of siderophore production in the culture medium was visualized by a colour change from blue to orange on modified CAS agar plates. For the semi-quantitative assay, the amount of siderophore present in the test sample was reported as a percentage relative to a control sample of which the siderophore concentration was known.

Results

Inhibition of growth by production of an antibacterial compound

Zones of inhibition of *Aer. hydrophila* growth were observed during the exponential, early stationary and sporulation phases when viable cells were tested in plate well assays. However, plate well assays testing intracellular extracts or extracellular supernatants did not show any antagonism of *Aer. hydrophila* by *B. cereus* during the entire growth cycle (Table 1).

Investigation into competitive exclusion in co-culture studies

Co-culture experiments were conducted by cultivating *B. cereus* and *Aer. hydrophila* together in shake flasks. *Bacillus cereus* displayed a typical growth profile

($\mu = 0{\cdot}96$), but there was a drastic decrease in the cell density of the pathogenic *Aer. hydrophila* population. When *B. cereus* cell concentration peaked, the pathogen had decreased by more than 70% of the starting concentration (Fig. 1a). In co-culture studies devoid of iron (Fig. 1b), a drastic decrease in *Aer. hydrophila* cell numbers was observed. *Bacillus cereus* cell number also decreased, although the initial death rate was more gradual than that of *Aeromonas hydrophila*.

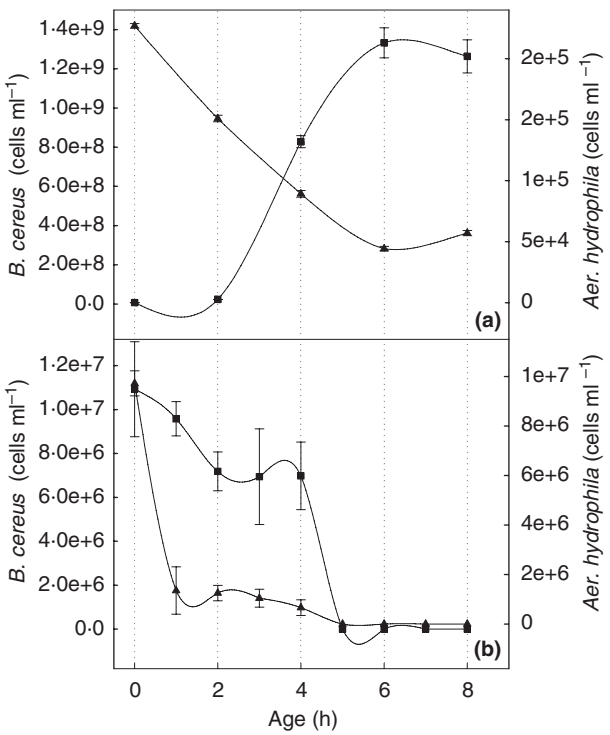

Figure 1 Cell concentration during co-cultivation of *Bacillus cereus* (■) with *Aeromonas hydrophila* (▲) in SPW (a) and SPW devoid of iron (b). SPW, synthetic pond water.

Effect of individual nutrient components on antagonism against the pathogen measured by differential growth rates

Bacillus cereus had a significantly higher growth rate in comparison with *Aer. hydrophila* when cultivated in SPW as a positive control ($P = 0{\cdot}003$), SPW with low iron concentration ($P < 0{\cdot}001$) and SPW with low glucose concentration ($P < 0{\cdot}05$) (Table 2). When media contained reduced concentrations of ammonia, nitrite or nitrate, there was no significant difference in growth between the two organisms ($P > 0{\cdot}05$) (Table 2). Neither of the micro-organisms grew in treatments where phosphate was limited (Table 2).

Evaluation of iron and glucose uptake rates by *Bacillus cereus* and *Aeromonas hydrophila*

During separate batch cultivations under identical conditions, *B. cereus* and *Aer. hydrophila* demonstrated classical exponential growth curves. Trends for glucose uptake from the growth media were linear ($r^2 > 0{\cdot}9$), but not for iron uptake by either of the micro-organisms (Fig. 2). *Bacillus cereus* had an overall iron uptake rate of 0·60 mg l^{-1} h^{-1} and a glucose uptake rate of 0·30 g l^{-1} h^{-1}. These uptake rates were significantly higher ($P < 0{\cdot}01$) than that of *Aer. hydrophila* for iron (0·43 mg l^{-1} h^{-1}) and glucose (0·14 g l^{-1} h^{-1}), respectively.

Evaluation of the production of siderophores

In the qualitative siderophore plate assay, *B. cereus* colony-forming units with orange halos were observed during the exponential growth and sporulation phases (data not shown). This observation was confirmed in the *B. cereus* culture study, where siderophore production was assessed. A maximum growth rate of 0·7 h^{-1} and cell concentration of *c.* $7{\cdot}00 \times 10^7$ cells per ml was achieved (Fig. 3a). The culture reached a high sporulation ratio at *c.* 12 h of growth (Fig. 3b). There was a gradual increase

Table 2 Growth rate assessment of *Bacillus cereus* and *Aeromonas hydrophila* cultivated under nutrient limitation

Treatment	*B. cereus* μ_{max}	Std. dev	*Aer. hydrophila* μ_{max}	Std. dev	Difference in growth rate	*P*-value
Synthetic pond water	0·041	0·001	0·032	0·000	0·009	0·003
De-ionized water	0·000	0·000	0·000	0·002	0·000	0·374
Low glucose	0·033	0·001	0·031	0·001	0·002	0·045
Low nitrite	0·035	0·004	0·031	0·006	0·004	0·473
Low nitrate	0·032	0·001	0·036	0·004	−0·003	0·210
Low ammonia	0·033	0·002	0·036	0·002	−0·003	0·266
Low iron	0·044	0·001	0·033	0·002	0·011	0·001
Low phosphate	0·000	0·000	0·000	0·000	0·000	$^{n}/_{a}$

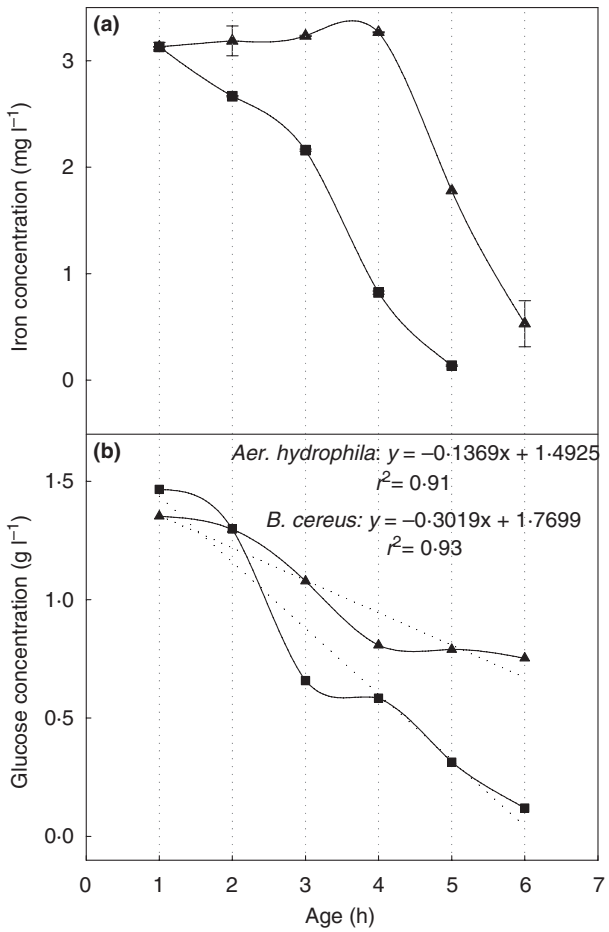

Figure 2 Iron (a) and glucose (b) uptake rates by *Bacillus cereus* (■) and *Aeromonas hydrophila* (▲).

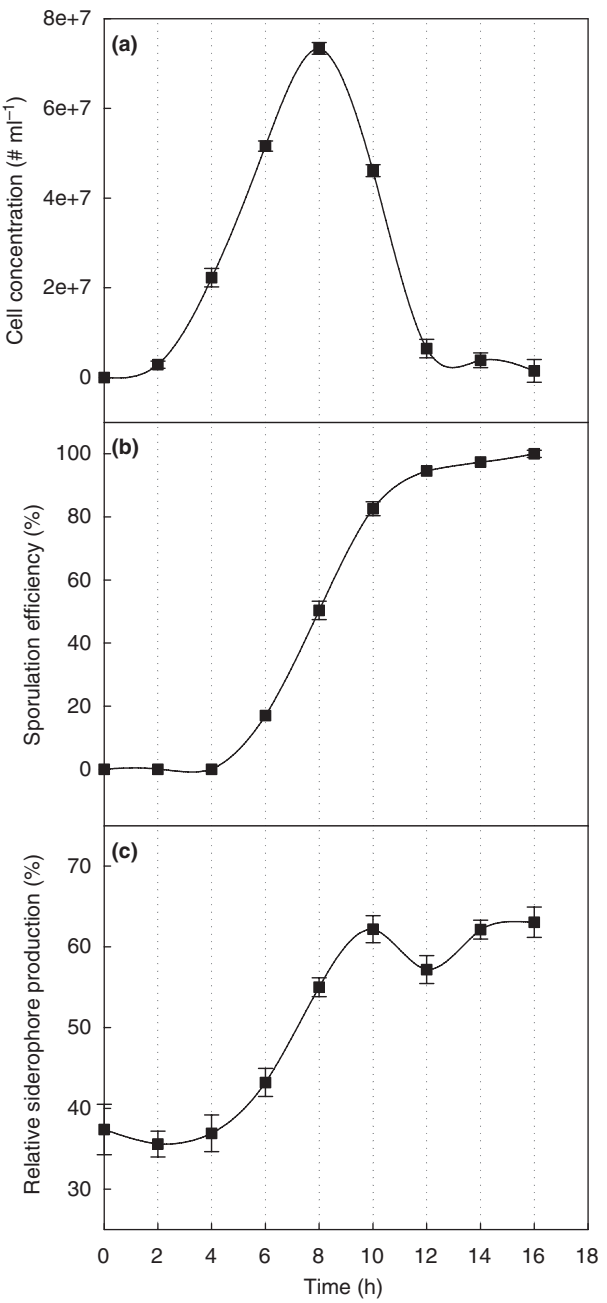

Figure 3 Growth data based on cell concentration (a), sporulation ratio (b) and relative siderophore production (c) by *Bacillus cereus* cultivated in synthetic pond water.

in the production of siderophores during the course of the cultivation (Fig. 3c), reaching a maximum relative sideropohore production of 65% as the culture entered the stationary phase. After completion of sporulation, the siderophore concentration remained at a constant high level.

Discussion

The mode of action of a novel *B. cereus* isolate as a biological agent in aquaculture for the inhibition of pathogenic *Aer. hydrophila* was investigated. The production of antimicrobial compounds by *B. cereus* was excluded as a mode of action based on the absence of growth inhibition of pathogenic *Aer. hydrophila* by intracellular or extracellular fractions of *B. cereus* (Table 1). In contrast, actively growing *B. cereus* cells caused growth inhibition of *Aer. hydrophila.* Although production of antimicrobial compounds is a common mode of action exploited for attenuation of a selected target pathogen in an environment (Fredrickson and Stephanopoulos 1981; Hong *et al.* 2005), this mechanism did not apply to *B. cereus* NRRL 100132. Similar to our findings, Brunt and Austin (2005) showed that their *Bacillus* obtained from the digestive tract of carp also inhibited the growth of pathogenic *Lactococcus garvieae* and *Streptococcus iniae* without showing any signs of antibiosis, thus indicating an alternate

mode of action other than production of antimicrobial compounds.

Competitive exclusion through an intrinsically higher growth rate and competitive uptake of essential nutrients was identified as a mode of action involved in the antagonism of *Aer. hydrophila* by *B. cereus*, based on co-culture data (Fig. 1). Co-cultivation of *B. cereus* together with *Aer. hydrophila* in SPW resulted in a decline of more than 70% in the cell density of the pathogenic organisms in a remarkably short time period (Fig. 1a).

Competitive exclusion was partly attributed to a substantially higher growth rate of *B. cereus* ($0{\cdot}96\ h^{-1}$) in comparison with *Aer. hydrophila,* where cell death was observed. These findings further confirmed our previous work where pathogen decline was proven in *in vitro* and *in vivo* studies, when *B. cereus* was administered as a biological agent (Lalloo *et al.* 2007). Several previous studies have reported higher growth rate as a likely mechanism of biological agents in the inhibition of other micro-organisms (Moriarty 1998; Pinchuk *et al.* 2001; Patterson and Burkholder 2003).

In addition to the intrinsically higher growth rate, competition for the essential nutrients, glucose and iron, contributed to the mechanism of competitive exclusion of *Aer. hydrophila* by *B. cereus*. Competitive exclusion by an intrinsically higher growth rate is often linked to competitive uptake of essential nutrients such as iron and glucose (Rico-Mora *et al.* 1998; Verschuere *et al.* 2000). As *B. cereus* and *Aer. hydrophila* are both heterotrophic, competition for organic substrates as both carbon and energy sources could be expected, although this mode of action for the inhibition of *Aer. hydrophila* by *B. cereus* has not been demonstrated previously (Verschuere *et al.* 2000). In nutrient limitation studies, *B. cereus* had a significantly higher growth rate than *Aer. hydrophila* in both SPW and SPW with limited glucose or iron (Table 2). We further confirmed these observations in glucose and iron uptake studies (Fig. 2), which indicated a significantly higher uptake ($P < 0{\cdot}001$) of glucose ($0{\cdot}30\ g\ l^{-1}\ h^{-1}$) and iron ($0{\cdot}60\ mg\ l^{-1}\ h^{-1}$) by *B. cereus* in comparison with *Aer. hydrophila* for glucose ($0{\cdot}14\ g\ l^{-1}\ h^{-1}$) and iron ($0{\cdot}43\ mg\ l^{-1}\ h^{-1}$), respectively. When *Aer. hydrophila* iron uptake rates were evaluated, a 4-h lag was observed, whereas *B. cereus* uptake was immediate (Fig. 2). Furthermore, when grown in co-culture media devoid of iron (Fig. 1b), death of the biological agent and the pathogen confirmed the essential requirement for iron by both micro-organisms. Results also indicated that *B. cereus* had a slower death rate and was thus more resilient to iron deficiency than *Aer. hydrophila*. These results indicated that competition through higher growth coupled with the competitive uptake of glucose and iron were key modes of action for antagonism by *B. cereus* (Verschuere *et al.* 2000; Patel *et al.* 2009).

The mechanism of competitive exclusion by competition for iron uptake was facilitated by siderophore production by the *B. cereus* isolate. The strain of *B. cereus* exhibited a growth-associated increase in siderophore concentration during the exponential phase of growth (Fig. 3c). Most importantly, the siderophores remained in the medium during and postsporulation. These results correlated with the work conducted by Patel *et al.* (2009), where siderophore production increased during the exponential phase of growth and remained stable during the sporulation phase, with a similar level of siderophore production to the present *B. cereus* isolate. A qualitative assay revealed a large number of colony-forming units with orange halos (data not shown), confirming the presence of siderophores (Milagres *et al.* 1999). Prior research conducted by Park *et al.* (2005) and Wilson *et al.* (2006) also specifically demonstrated the ability of *B. cereus* to produce siderophores. Studies carried out by Smith and Davey (1993) and Gram *et al.* (1999) demonstrated a positive correlation between the production of siderophores and a decrease in pathogen prevalence. Although *Aer. hydrophila* is itself capable of synthesizing low molecular weight siderophores, termed 'amonabactins', production of the siderophore is thought to be inducible and regulated by extracellular iron concentration (Chart and Trust 1983). *Bacillus cereus* was able to produce siderophores immediately at the start of batch culture (Fig. 3), thereby decreasing the iron concentration to very low levels within the first 5 h (Fig. 2) thus starving *Aer. hydrophila* of iron.

The modes of action for attenuation of growth of pathogenic *Aer. hydrophila* by the *B. cereus* isolate, in particular competitive exclusion by growth rate, competition for essential nutrients such as glucose and iron, and siderophore production, increase its attractiveness as a probiotic and biological agent for aquaculture. The siderophore-producing capability of the *B. cereus* isolate addresses the severe shortage of probiotics able to facilitate competitive exclusion based on iron competition (Patel *et al.* 2009). The absence of antimicrobial activity is beneficial for application of the *B. cereus* isolate as a biological agent, as the presence of antimicrobial substances in aquaculture systems is undesirable because of increased virulence in disease-causing pathogens, negative acceptance by consumers and carryover to the environment (Barker 2000; Jana and Jana 2003). Lack of information on modes of action of biological agents limits the adoption of biological solutions to address the challenges of aquaculture, ultimately perpetuating the use of chemotherapeutic agents (Moriarty 1997; Moriarty 1998; Balcázar *et al.* 2006). The modes of action described here,

combined with previously demonstrated *in vitro* and *in vivo* functionality, the ability to reduce the concentration of waste ions in reticulated aquaculture, physiological tolerance to environmental conditions and bio-safety (Lalloo *et al.* 2007, 2008) renders the *B. cereus* isolate NRRL 100132 as an ideal biological agent to address the many challenges facing modern day intensive aquaculture.

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