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Adhesion Capacity of *Bacillus thuringiensis* Spores and its Relation with Biofilm Formation

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Abstract

Spores of *Bacillus thuringiensis* (Bt), used as bioinsecticide, represent a potential contamination risk of the equipment used for food processing and could also induce potential gastrointestinal symptoms in special cases of susceptible individuals. The objective of this work was to explore the *in vitro* adhesion capacity of Bt spores and its relation to the ability to form biofilms. The adhering capacity of spores of 65 Bt strains was determined in stainless steel tubes, as well as their ability to form biofilms on polystyrene microplates. We analyzed the relation of these two features. All strains showed a variable adhering capacity to stainless steel, ranging from 0.04% to 1.21% of the initial spore load of ~1.7×10^7CFU/mL. The, 92.3% of the strains were able to form biofilms at 96 h. Was observed that 23 strains showed high capacity, 19 moderate, and 18 low to form biofilms. Twenty two of the 65 strains showed the lowest levels for both parameters (r = 0.46). Our data suggest that the adhesion capacity does not necessarily predict either the capacity to form biofilms or their magnitude.

Keywords: *Bacillus thuringiensis*; Adhesion; Biofilms

Introduction

Among sporulated Gram-positive bacteria, several members of the *Bacillus* genus have been isolated in diverse environments of the production and processing industry of food for human consumption [1]. Of the *cereus* group species, *B. anthracis* and *B. cereus* are known pathogens in humans, whereas the entomopathogenic species *B. thuringiensis* (Bt) that is used in the production of different bioinsecticides is considered safe for humans and mammals in general [2]. However, a potentially pathogenic effect for mammals is still possible [3,4,5]. For instance, the persistence of spores in some products can, in principle, induce contamination of the equipment used in their processing, as has been suggested for *B. cereus* [6]. Consequently, this situation can lead to gastrointestinal symptoms in individual having an attenuated immuneresponse [7].

It is known that Bt spores and those of other *Bacillus* species, presenting exosporium, have a higher hydrophobicity, conferring them a higher adhesive potential to diverse materials [8,9], such as those used in industrial food processing [1,10-13]. To this respect, there are several reports on the *in vitro* capacity of these bacteria to adhere to stainless steel laminae and/or synthetic polymers [14-18].

Bacterial adhesion to a surface is a two-phases process: in the first phase, planktonic bacteria are moved to a surface by the effect of physical forces such as Brownian motion, van der Waals attraction forces, gravitational forces, the effect of surface electrostatic charge and hydrophobic interactions; in the second phase of adhesion, molecular irreversible reactions between bacterial surface structures and substratum surfaces become predominant [19,20]. Adhesion is an essential stage for the formation and subsequent growth of biofilms [19,21,22].

Biofilms are defined as microbial communities embedded in a polymer layer or matrix adhered to an inert surface or substrate, or to living cells or tissues [23,24]. Several members of the *Bacillus* genus are capable of forming biofilms *in vitro* at variable magnitudes dependent on the incubation time [6,25]. According to Auger et al. [25], less than 50% of the *B. cereus* and Bt strains showed capacity to form biofilm in their assays. Formation of biofilms has been related also to the resistance of these species to diverse antibiotics and disinfectants [26,27] found that the latent spores of *B. cereus* were more efficient in starting and forming biofilms as compared to bacterial cells in vegetative or sessile state. These same authors evidenced that the production of biofilm in cultures with spores depends on the growth curves, which does not occur in cultures in suspension, and could be related to the resistance of the biofilm to adverse factors.

Despite the existence of important collections of Bt isolated from different regions of Mexico, few studies have focused on the possible associations and distribution of features that could contribute to the analysis and a better understanding of the relations at the level of species and subspecies of the very

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variable lineages of the *Bacillus* genus. Considering that diversity (or clonality) evidence can be obtained through several types of markers and that the correlation among results from different methodologies allows resolving doubts and clarify scenarios, we used both biofilm formation and adherence assessments in our study.

Based on the close genetic relation of Bt with the other members of the *cereus* group and, hence, their shared phenotypical characteristics [28], the present study was performed to explore the adhesion capacity of Bt spores to stainless steel tubes and its possible relation with the capacity to form biofilms on polystyrene plates.

Methods

Bacteria and cultures

We studied a group of 63 strains of *B. thuringiensis* of environmental origin isolated from diverse sources (Table 1).

Table 1: Characteristics of Bacillus thuringiensis strains analyzed in this study.

Strain[a]	Source[b]	Location[c]	Date[d]	Strain[a]	Source[b]	Location[c]	Date[d]
BT8	CL	MX	Apr-89	IB72	CO	OAX	Aug-91
BT10	CL	GTO	Jun-89	IB74	AS	OAX	Aug-91
BT11	AS	GTO	Jun-89	IB76	CO	OAX	Aug-91
BT14	CL	GTO	Jun-89	IB80	GR	MOR	Jun-91
BT20	AS	GTO	Jun-89	IB82	CO	ZAC	Jun-91
BT21	AS	GTO	Jun-89	IB85	AS	OAX	Aug-91
BT22	AS	GTO	Jun-89	IB86	SC	OAX	Aug-91
BT23	CL	GTO	Jun-89	IB88	AS	GTO	Jun-91/
BT25	CL	GTO	Jun-89	IB90	SC	GTO	Jun-91
BT26	CO	GTO	Jun-89	IB91	GR	GTO	Jun-91
BT28	CO	GTO	Jun-89	IB94	GR	GTO	Jun-91
BT34	CO	GTO	Jun-89	IB97	GR	GTO	Aug-91
BT35	CO	GTO	Jun-89	IB100	GR	GTO	Jun-91
BT36	CO	QRO	Jun-89	IB115	CO	TAB	Aug-91
BT38	CO	GTO	Jun-89	IB120	CO	TAB	Aug-91
IB4	CO	MOR	Jun-91	IB126	CO	OAX	Aug-91
IB8	GR	MOR	Jun-91	IB130	GR	TAB	Aug-91
IB9	CO	MOR	Jun-91	IB135	GR	TAB	Aug-91
IB10	AS	MOR	Jun-91	IB136	CO	TAB	Aug-91
IB13	CO	PUE	Jun-91	IB152	AS	GTO	Jun-91
IB18	AS	MOR	Jun-91	IB155	CO	OAX	Aug-91
IB19	CO	OAX	Aug-91	IB157	AS	TAB	Aug-91
IB23	CO	PUE	Jun-91	IB162	CO	TAB	Aug-91
IB28	CO	MOR	Jun-91	IB163	CO	OAX	Aug-91
IB29	SC	GTO	Jun-91	IB164	AS	CHI	Aug-91
IB31	GR	MOR	Jun-91	IB177	CO	TAB	Aug-91
IB33	CO	MOR	Jun-91	IB182	CO	TAB	Aug-91
IB35	GR	MOR	Jun-91	IB189	CO	CHI	Aug-91
IB42	AS	MOR	Jun-91	IB195	AS	TAB	Aug-91
IB43	SC	MOR	Jun-91	IB213	CO	PUE	Jun-91
IB52	SC	MOR	Aug-91	IB214	AS	TAB	Aug-91
IB57	SC	MOR	Jun-91				

[a]BT strains from CINVESTAV Irapuato, and IB strains from Institute of Biotechnology, UNAM
[b]Isolated from: CL, crops leftover; AS, agricultural soil; CO, corn; Gr, grass; SC, sugar cane
[c]Location (Mexican States): MX, State of Mexico; GTO, Guanajuato; QRO, Queretaro; MOR, Morelos;PUE, Puebla; OAX, Oaxaca; ZAC, Zacatecas; TAB, Tabasco; CHI, Chiapas.
[d]Collecting date

We included the reference strains 10792 and 33679 (H3: 3a, 3b) of the ATCC (American Type Culture Collection).Fifteen of the studied strains were kindly donated by the Bioinsecticides Laboratory of the Department of Biotechnology and Biochemistry, from CINVESTAV-Irapuato, in Guanajuato, Mexico, and 48 were provided by the Department of Molecular Microbiology, from the Institute of Biotechnology-UNAM Cuernavaca, in Morelos, Mexico.

Strains were received desiccated in sterile filter paper; they were rehydrated in Soy Trypticase Broth (STB, Bioxon) and incubated at 30°C for 7 days. Strains were individually harvested in sterile cryovials containing STB supplemented with 2% fat-free milk (Difco) plus 15% glycerol (JT Baker), and stored at -70°C.

To prepare the working solutions, loops of each frozen stock were re-sown directly on 1.5% Trypticase Soy Agar (TSA) plates, streaked, and incubated at 30°C for 7 days. They were immediately harvested individually in polystyrene tubes containing 3 ml of sterile water with 0.1% Tween-80, and kept refrigerated until used.

Spores adhesion

The method described by Wijman et al. [6] with some modifications was used. Briefly, fresh spore suspensions were prepared in 10 mM potassium phosphate buffer (pH 7.4) with 0.1% Tween 80, adjusted to an OD_{545} of 0.3, and immediately 2 ml aliquots of each standardized suspension were placed in sensitized stainless steel tubes with ultra pasteurized fat-free milk (4 replicates). Then, 20 µL aliquots were taken from each tube to make serial decimal dilutions with STB on microplates, and plated on TSA with 2% agar (2% TSA). Tubes were closed with rubber sterile stoppers and incubated at 30°C for 4h under agitation at 60 rpm. Supernatants were discarded; tubes were rinsed three times under aseptic conditions with sterile deionized water. Immediately, 2 mL of 10 mM sodium phosphate buffer (pH 7.4) with 0.5% Tween 80 were added to the tubes, and placed in an ultrasonic bath (Branson 2800) at 40 kHz for 10 min at room temperature; the tubes were placed into an ice bath. The resulting spore suspensions were serially diluted in STB and plated in 2% TSA. Both the microplate and the 2% TSA preparations were incubated at 30°C during the night. Finally, strain colonies were counted per plate/dilution. These experiments were done in duplicate.

Biofilms formation

We followed the methodology proposed by Wijman, et al. [6] and Stepanovic, et al. [24] with minor modifications García, et al. [29]. Spore suspensions were standardized at an OD_{545} of 0.6 with STB and immediately 200 µL aliquots of each strain suspension were placed in series of 12 adjacent wells in 96-well microtiter polystyrene plates, and incubated at 30°C during 96 h. A series of wells with STB was included as control in each plate.

After incubation, supernatants were removed by mild aspiration with a vacuum pump; microplates were washed with 220 µL/well of 1X phosphate buffered saline (PBS), fixed with 220 µL/well of 100% methanol during 5 min at room temperature (RT); stained with 220 µL of 1% crystal violet per well for 15 min at RT, rinsed twice with 220 µL/well of sterile distilled water and air dried at RT. Finally, the crystal violet retained in the biofilms was solubilized with 220 µL/ well of 33% glacial acetic acid and OD_{620} readings were made in an automated microplate reader (Multiskan Ex, Thermo Electron).

Data analysis

Counts of colonies from the adhesion assay were used to calculate the average of colony forming units per milliliter (CFU/mL) at the start and end of the assay.

Mean OD_{620} values from replicates were calculated for all strains and controls at each time of incubation. The mean OD_{620} values were considered acceptable if their corresponding coefficients of variation (CVs) were less than 20%. The mean OD_{620} value of controls was used to set three cut-off values according to Stepanovic, et al. [24], and subsequently to categorize the bacteria, as Null-, Weak-, Moderate-, or Strong-Biofilm forming strains.

Statistical analyses of data were performed by means of parametric and/or non-parametric tests, as required, with the Software SPSS statistics v. 20 (IBM Corp.)

Pearson product-moment correlation coefficient was calculated.

Results

Adhesion capacity

Of all the strains, 26.2% (17/65) reached adhesion values below 0.2%; 33.8% (22/65) showed adhesion values ≥ 0.2 and < 0.5%; whereas 32.3% (21/65) showed values ≥ 0.5 and <1.0%. Only five strains (7.7%) showed adhesion values < 0.3%. All strains analyzed revealed adhering capacity to stainless steel in the presence of fat-free milk, but at a variable magnitude. In average (1.15-2.11 CFU), the initial load of spores in the tubes was of 1.68×10^7 CFU/mL, whereas the average (3.55-5.83 CFU) of adhered spores was 4.7×10^4 CFU/mL, representing a difference of approximately 2.5 $\log_{10}$ units. These data indicated that the percentages of adhering spores were below 1.5% of the initial loads and showed a large variability, ranging from 0.04% to 1.21%. The total distribution of the adhesion percentages is shown in Figure 1.

No significant difference was observed in the average values of adhesion percentage between the CINVESTAV-Irapuato strains (strains BT) and those of the Institute of Biotechnology-UNAM (strains IB). The strains of each institution showed adhesion percentage values that were heterogeneously distributed in the range of values.

Biofilms

Under the used experimental conditions, biofilms were formed as rings of adherent material in the air-liquid interface of the wells of the microplate, with variable thickness and density. Sixty out of the 65 strains (92.3%) showed variable capacity to form biofilms at 96 h of incubation with an OD_{620} range of 0.27 to 3.59 (Figure 2).

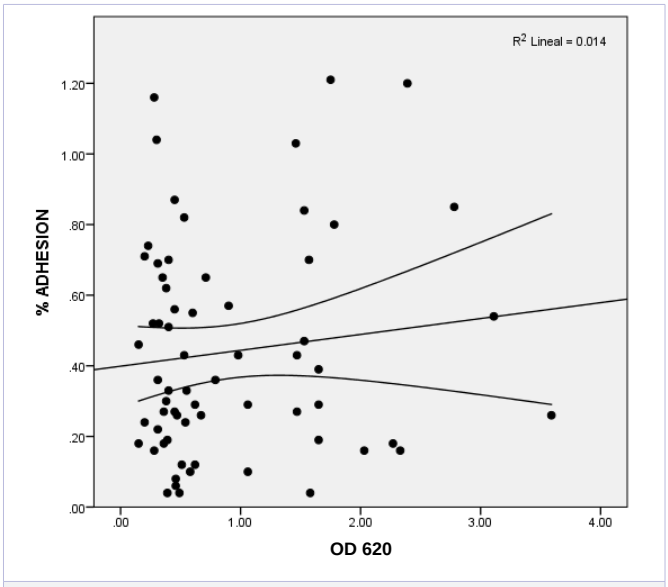

Figure 1: Distribution of the adhesion percentages with respect to the OD620 according to the capacity to form biofilms of the 65 Bt strains. The insert shows the pattern of aggregated distribution of the subpopulation that presented low values for both parameters.

According to the defined categories of biofilms formation, 35.4%, 29.2%, 27.7%, and 7.7% of the strains revealed high, moderate, low, and null capacity, respectively. The distribution of the capacity to form biofilms between the BT and IB strains was similar ($P < 0.05$).

The two reference strains included in the study showed dissimilar capacities to form biofilms: strain ATCC 10792 was highly producing (OD_{620} = 3.59) and strain ATCC 33679 was a moderate producer (OD_{620} = 0.51).

Regarding the possible correlation between adherence and the capacity to form biofilms, the Person product-moment correlation coefficient for the whole data set of 63 strains was r = 0.12 (negligible relationship). However, the subset data of strains showing an aggregated distribution pattern (Figure 1) for the lower levels of both adherence and capacity of forming biofilms showed a moderate correlation value of r = 0.46.

Discussion

There are several reports on the capacity of diverse members of the *Bacillus* genus, particularly *B. cereus* and *B. subtilis,* to form biofilms in *in vitro* models [6,27]. Bacterial adhesion to a surface is an essential step for the formation of biofilms [19].

Although the information on the role of biofilms formation by the entomopathogenic Bt species in nature is scarce, it could be considered to be a potential resistance and persistence mechanism in susceptible insects [25].

The widespread use of spores with their toxic insecticide proteins (Cry or Cyt) as bioinsecticides to control relevant insect pests for agricultural crops [30] also implies a potential contamination source in the industrial processing and packaging lines of some agricultural products [11,31].

In this study, we determined the adherent capacity of *B. thuringiensis* spores to stainless steel tubes, as an indicator of the Bt spores potential to persist adhered in industrial environments involved in the processing of vegetal origin food.

Our results indicate that Bt spores adhere to stainless steel surfaces at magnitudes that could be considered low with respect to the initial load in the order of 2 to 3 logarithms. The modified method used herein was useful to distinguish the adhering capacities of the studied Bt populations. These results agree with

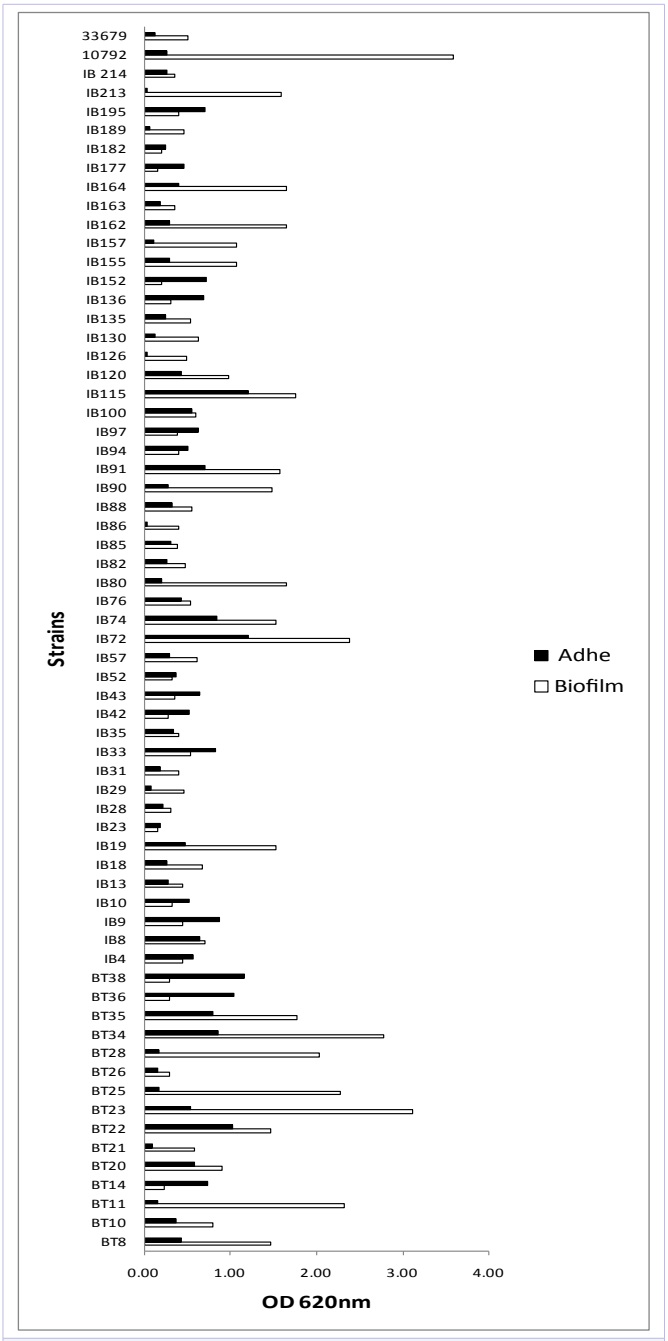

Figure 2: Distribution of the capacities to form biofilms at 96 h and adherence at 4 h by the Bt strains.

those of [6] for *B. cereus* regarding its adherence capacity.

In the adhesion model used in this study, it is assumed that only the reversible adhesion of Bt spores was fostered, since an ionic regulator without carbon sources was used and the contact time was relatively short. We selected to use the Bt spores suspension to perform both the adhesion and the biofilms formation assays in this work based on the findings [27], as they observed a greater efficiency in adhesion and biofilms formation of *B. cereus* spores, in contrast to planktonic and/or sessile cells. Besides, they also observed that the sporulated phase of growth presents a more prolonged lag phase in the growth curve in biofilms than in suspension, which could have implications in the resistance of biofilms to stressing factors.

The capacity of the Bt strains to form biofilms in this study was heterogeneous, their categorizing practically showed similar proportions of high, moderate, and low producing strains. Such a distribution had already been observed in a smaller group of strains [29]. Other studies with different *Bacillus* species have also shown variable capacities to form biofilms *in vitro* [6,25,32]. It is important to point out that more than 90% of the studied Bt strains were able to form biofilms. This could represent a contamination risk in the processing plants of agricultural products exposed to Bt-based bioinsecticides regardless of the innocuity of the bacterium.

The whole group of strains studied here showed full diversity richness. The subgroup from the CINVESTAV (BT) collection revealed greater diversity than that from the IBT-UNAM collection. This could be related to the fact that the CINVESTAV strains were isolated from neighboring localities; thus, it is more likely that genetic transfer and recombination among strains could have occurred [28].

Under our experimental conditions, incubation for 96 h at 30°C was optimal for the formation of biofilms by most Bt strains, coinciding with the late stationary growth phase. These data agree with the more efficient biofilms formation by *B. cereus* strains during nutrients depletion, generally in the transition from the exponential phase to the early stationary phase [33-35].

Our results suggest that other events different from those analyzed herein, such as genotypical and phenotypical switching, could be playing a significant role in the formation and differentiation of the biofilm.

Results obtained by Verplaetse, et al. [36], agree with our in terms of a high level of heterogeneity. These authors suggest that, in biofilm, the activity of three regulators is concomitant and happens in different subpopulations at the same time.

B. thuringiensis is ubicuitous in the soil even in extreme latitudes; however, there is no explanation for this. Very few investigations of microbial ecology have been carried out related to soil survival. To achieve this, we would have to widen the knowledge of our Bt subset obtained from the soil, by identifying the genes required by Bt, such as the trasporter BMB171-CO350 [37].

The signal(s) that trigger the change from planktonic growth to biofilms are still unknown; but, there is a consensus that formation of biofilms is fostered in conditions of environmental stress and nutrients deficiency [27,36,38].

On the other side, the initial adhesion of spores and subsequent formation of biofilms correlate with the production of high percentages of spores during the biofilm formation cycle [27]. The aforementioned can impact negatively the food processing industry, given the constant release of spores from the biofilm and their dissemination to form new biofilms.

Furthermore, Thomas et al. [39] established that the microorganisms isolated from any niche, such as medical, environmental, aquatic, or industrial, can exert different adhesion mechanisms, not only due to the variations in substrate, nutrients, ionic strengths, pH values, and temperature, but also because the phenotype and genotype (expression of structural components and adhesion proteins to surfaces) of the bacteria have undergone different adaptation processes along time through selective pressures.

In conclusion, we suggest taking into account the adhesion capacities and biofilms production, as reported here, for the routine phenotypical characterization of Bt populations. A moderate correlation between the capacity for biofilm formation and adherence was observed in the subset with the lowest values for both parameters. More studies should be done correlating these parameters among populations of strains of environmental origin and strains associated to contamination of the equipment used in the processing of some food products.

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