Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading

Adhesion and cellsurface properties of wild species of spore formers against enteric pathogens

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ARTICLE INFO

Article history:
Received 24 August 2012
Received in revised form 31 October 2012
Accepted 5 December 2012
Available online 20 February 2013

Keywords:
Spore formers
Adhesion
Aggregation
Enteric pathogens

ABSTRACT

Objective: To investigate the adhesion potential and cell surface properties against enteric pathogens *Salmonella typhi*, *Salmonella para typhi* A and *Vibrio cholera*. **Methods:** Adhesion potentials of spore and vegetative phase were studied separately for the isolates. Hydrophobic nature was measured on the basis of affinity towards the xylene. Autoaggregation and coaggregation were studied on the basis of clumping of cells. *In vitro* adhesion studies were done on mucous which were prepared from infant child faeces. Biofilm production of superior adhesive isolate was confirmed by SEM analysis. **Results:** Spore and vegetative phases of isolates possessed a different rate of adhesion potentials on intestinal mucous, which indicated that cell surface properties were involved in adhesion process. Spores showed a higher hydrophobicity than their vegetative cells which remained less or non hydrophobic. Vegetative phases showed capabilities for autoaggregation and coaggregation. Spores were found to be more adhesive on intestinal mucous than vegetative phase. Among enteric pathogens *Vibrio cholera* registered higher adhesion potentials with supporting cell surface properties. Among the five sporeforming isolates, isolate BM-3 possess superior adhesion than enteric pathogens and also exhibited biofilm formation which enhances colonization potential. **Conclusions:** Spore and vegetative cell phases shows differences in adhesion potentials. Cell surface properties and adhesion studies reveals that isolate BM-3 can be selected as superior isolate which is capable for biofilm production. In short, isolate BM-3 possesses an enhanced adhesion potential than enteric pathogens towards intestinal mucous which is a desirable probiotic character.

1. Introduction

Probiotic bacteria have an important role in keeping the intestinal balance and protection against gastrointestinal pathogens. Probiotic application of spore-formers has not been established well in the world probiotic market although studies reveal its probiotic potential and efficacy[1]. Sporeformers can withstand harsh conditions in intestinal systems. The spore is quiescent cell form characterized by several protective layers surrounding the dehydrated cytoplasm that contains nucleoid[2]. Sporeformers with probiotics properties, can be used in the therapy of intestinal disorders of various origins. Criteria for selection of probiotics include lack of pathogenicity, tolerance to gastrointestinal conditions (acid and bile), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens. Microflora of the gastrointestinal tract plays a crucial role in the anatomical physiological and immunological development of the host[3]. Adherence of bacteria to intestinal epithelium is known to be a prerequisite for colonization and infection of the gastrointestinal tract by many pathogens[4]. Adhesion is a very important physical trait of probiotic bacteria which helps in the attachment and colonization in the host intestine. Probiotic bacteria can prevent the adhesion and invasion of gastrointestinal pathogens. Gastrointestinal pathogens have the ability to disturb intestinal microbial balance and are capable for subsequent pathogenesis. Enteric fever and cholera are the common health problem in India, accounting for more than 300 000 cases per year. So in the present study, adhesion and cell surface properties of five wild species of spore formers having basic

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probiotic properties[5] were compared with enteric pathogens *Salmonella typhi*, *Salmonella para typhi* A and *Vibrio cholera*. Best performing adhesive isolate was selected and further studied for its biofilm production capability.

2. Materials and methods

2.1.Microorganisms used in the study

Five spore formers isolated from different natural sources such as milk, soil, intestine of chicken, dry spices were used for this study. Our preliminary studies reveal that these five spore formers, possess basic probiotic characters such as acid, bile tolerance ,resistance in artificial gastric and intestinal fluid and antagonism to enteric pathogens such as *Salmonella typhi*, *Salmonella paratyphi* A and *Vibrio cholera*[5]. Organisms were designated as BM-3, CI-2, CD-4, S-7, and CD-1. Enteric pathogens *Salmonella typhi*, *Salmonella paratyphi* A and *Vibrio cholera* were obtained from MTCC Chandigarh.

2.2. Growth conditions

Vegetative cells of isolates were cultured in nutrient broth for 8 h while their spores were cultured in Difco sporulation medium for 48 h. Enteric pathogens, were separately cultured in nutrient broth for 6 h. All cultures were incubated at 37 ℃.

2.3. Adhesion properties

2.3.1. Hydrophobicity

Hydrophobicity tests of spore formers and enteric pathogens were performed according to Jayesh[6] with slight modifications. Isolates from respective culture medium were centrifuged at 3 000 *g* for 15 min at 4℃ and resuspended in the de ionized water. One mL of xylene was added to 3 mL of bacterial suspensions which was previously adjusted to an optical density of 0.5 at 610 nm. The suspensions were pre incubated for 15 min at room temperature and then thoroughly mixed in a vortex for 2 min. After the two layers had separated completely on standing for about 20 min at room temperature OD of lower aqueous phase were separately reanalyzed with a spectrophotometer at 610 nm and compared with initial OD at 610 nm. Percentage hydrophobicity was calculated as:

$$\frac{ODi-ODe}{ODi} \times 100$$

Where ODi is the initial bacterial suspension reading and ODe is the aqueous phase reading after microbial adhesion to xylene.

2.3.2. Auto aggregation

Autoaggregation assays were performed to spore formers and enteric pathogens according to Del Re[7]. Aggregation abilities of microorganisms were screened by visual observation.

2.3.3. Coaggregation

Coaggregation assays[7] were performed to sporeformers against enteric pathogens. The percentage of coaggregation was calculated using the equation of Handley[8].

2.3.4. Adhesion assay

Human intestinal mucous were obtained from faeces of healthy new-borns (15-36 months of age) according to the method of Khalil[9]. The crystal violet method[9] was used to determine adhesion ability. Adhesion assay were done both for enteric pathogens and sporeformers.

2.3.5.Selection of superior adhesive isolate and its confirmation for biofilm production

Most adhesive isolate was selected and biofilm production was confirmed by SEM studies as described by Lembke[10]. The biofilm of BM-3 on the glass pieces were fixed for 1 h in a solution containing 2.5% gluteraldehyde. The glass pieces were washed in 0.1 M sodium acetate buffer (pH 7.3). Samples were dehydrated through a graded series of ethanol, dried, coated with platinum and examined using JEOL 6390 (Japan).

3. Results

3.1. Hydrophobicity

Hydrophobic nature of spore formers and enteric pathogens were measured on the basis of its affinity towards the xylene. Spores of all species showed a higher hydrophobicity over their vegetative cells. Among the isolates, spores of BM-3 showed a higher hydrophobicity of 58.4%. Among enteric pathogens *Vibrio cholera* registered a high percentage of 52.2%. The hydrophobicity of spores formers and pathogenic strains to xylene were shown in Figure 1&2.

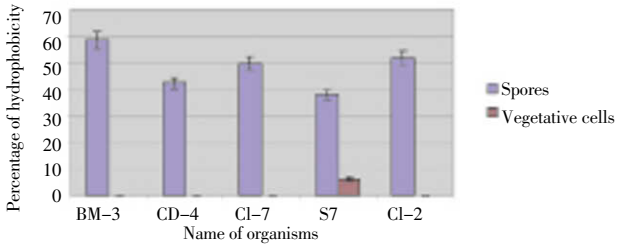

Figure 1. Hydrophobicity assay of spores and vegetative cells of sporeformers.

Table 1
Coggregation of vegetative cells.

Name of organisms	Percentage of coaggregation		
	Salmonella typhi	*Salmonella para typhi* A	*Vibrio cholera*
BM-3	6.40±0.15	3.80±0.92	4.70±0.38
CD-4	0	0	2.47±0.42
CI-7	0	0	3.53±0.31
S7	0	0	5.27±0.61
CI-2	2.67±0.64	1.27±0.35	1.60±0.20

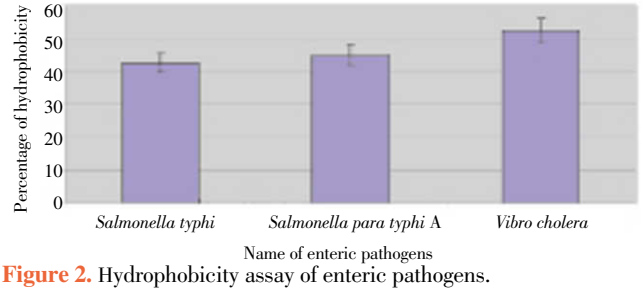

Figure 2. Hydrophobicity assay of enteric pathogens.

3.2. Auto aggregation

Autoaggregation nature of spore formers and enteric pathogens were measured on the basis of their sedimentation characteristics. Vegetative cells of isolates were found to be more autoaggregating than that of its spores. Spores of all isolates showed a moderate autoaggregation. Among the isolates vegetative cells of BM-3 showed highest autoaggregation of 38.4% whereas their spores shows an autoaggregation of 23.4%. Among enteric pathogens *Vibrio cholera* showed a higher autoaggregation of 26.0% (Figure 3-5).

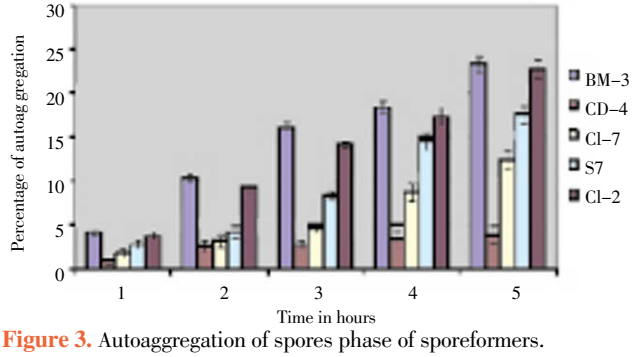

Figure 3. Autoaggregation of spores phase of sporeformers.

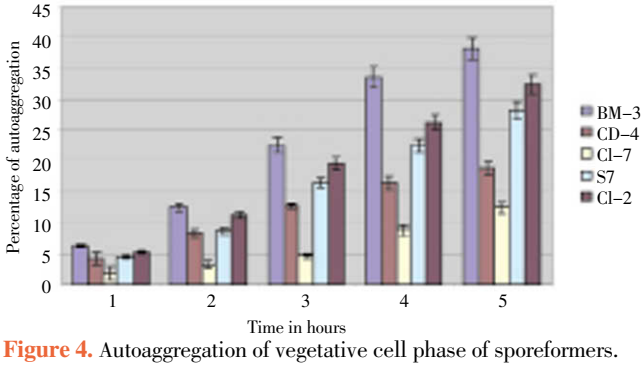

Figure 4. Autoaggregation of vegetative cell phase of sporeformers.

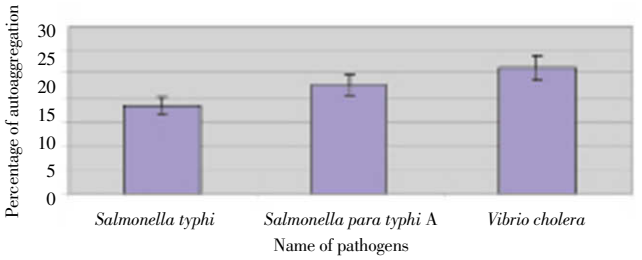

Figure 5. Autoaggregation of enteric pathogens.

3.3. Coaggregationz

Coaggregation was shown by the vegetative cells of isolates towards enteric pathogens. Spores of isolates were found to be non coaggregating with enteric pathogens. Results are expressed as the percentage reduction after 5 h in the absorbance of a mixed suspension compared with the individual suspension (Table 1).

3.4. Adhesion assay

Isolates and enteric pathogens were tested for their ability to adhere on intestinal mucous. Spores shown strong *in vitro* adhesion over its vegetative cells. Adhesion assay reveals that spore of BM-3 were more capable for adhesion in intestinal mucous than enteric pathogens (Figure 6, 7).

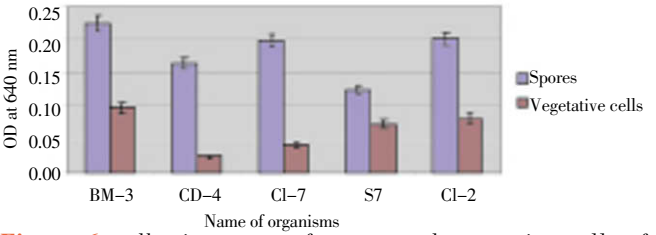

Figure 6. Adhesion assay of spores and vegetative cells of sporeformers.

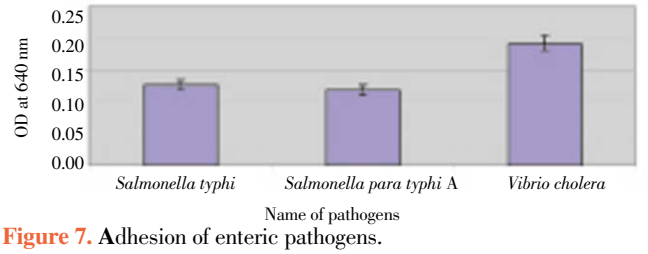

Figure 7. Adhesion of enteric pathogens.

3.5. Selection of superior adhesive isolate and its confirmation for biofilm production

Isolate BM-3 was selected as superior adhesive strain among the tested isolates BM-3 had an enhanced adhesion potential than enteric pathogens also. SEM studies confirmed the capability of BM-3 for its biofilm production. Scanning electron micrographs revealed biofilm formations on the surface of the glass pieces (Figure 8).

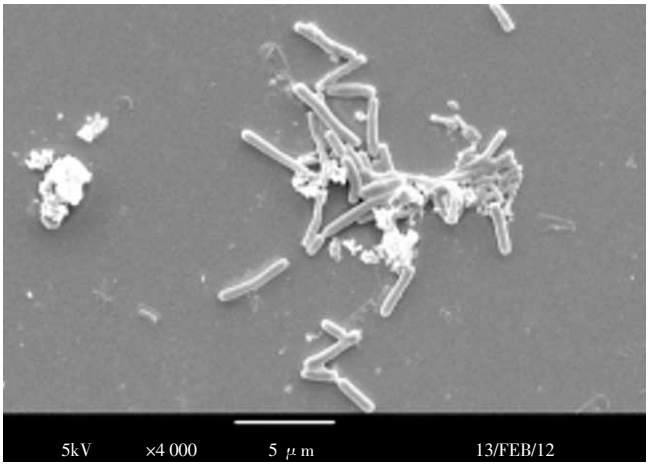

Figure 8. Scanning electron micrograph of biofilm produced by BM-3.

4. Discussion

Entericpathogens are of great importance because they cause infections both in man and animals. Use of spore formers as probiotics has not been established well because of their non-indigenous origin, and it is scarcely studied in the various vital aspects[11]. Adhesion and cell surface properties such as hydrophobicity, aggregation capabilities together with coaggregation properties with potential pathogens can be used for preliminary selection of probiotic bacteria. Hydrophobicity, autoaggregation and mucin adhesion are important attribute which help in the attachment of various substrata that explain the probiotic nature of the microorganism[1]. Five acid, bile tolerant strains possessed basic probiotic qualities[5] were selected and examined for its adhesion ability and aggregation properties against enteric pathogens. The bacterial adhesion to hydrocarbons has been extensively used for measuring cell surface hydrophobicity. Adherence of organisms to xylene, a non polar solvent, demonstrates hydrophobic nature of the isolates. Increased hydrophobic nature of spores over their vegetative cells may be due to presence of hydrophobic proteins present in spore coat. Doyle[12] found that agents which disrupt protein structure, modified the hydrophobicity of spores, suggesting that the spore coat also has hydrophobic sites. From this study, we can infer that spores of isolates had more hydrophobic sites than their vegetative cells. *Vibrio cholera* were found to be hydrophobic than enteric fever pathogens *Salmonella typhi*, *Salmonella para typhi* A. Spores of BM-3 showed increased hydrophobicity than enteric pathogens reveals the probiotic character of the strain to be used as probiotic. The concept of aggregation ability includes autoaggregation, characterized by clumping of cells of the same strain, and coaggregation, in which genetically distinct cells are involved. Many studies indicated that aggregation ability is related to cell adherence properties[4,13]. Present study does not support a positive correlation between aggregation properties and adhesion potentials. Cell surface properties such as hydrophobicity, aggregative properties changes according to its spore or vegetative phase. Autoaggregative pattern of isolates were found to be more in its vegetative cell phase rather than its spores. This may be due to the morphological peculiarities of vegetative cell wall. Coaggregation with gut pathogens may be useful for screening to identify potential probiotic strains. Coaggregation ability of probiotic strains helps in excluding the pathogens before to the proper adhesion to mucus of intestine. Among isolates vegetative phase of BM-3 possessed a better coaggregation with enteric pathogens *Salmonella typhi*, *Salmonella para typhi* A and *Vibrio cholera*. Non coaggregative pattern of spores of isolates clearly refers that cell wall structure, composition and physicochemical properties of bacteria play a key role in aggregation traits. Adherence of probiotic bacteria to intestinal mucosa is the first step in gut colonization[9] and therefore its an important criterion for in vitro probiotic selection.Through adhesion ability and colonization on tissues, probiotic microorganisms can prevent pathogen access by steric interactions or specific blockage on cell receptors[14]. A relationship between hydrophobicity and adhesion ability in Bifidobacterium has been reported[15]. Increased adhesive nature of BM-3 on intestinal mucosa may be due to the increased hydrophobic nature of its spores. Present study indicates a positive correlation between hydrophobicity and adhesion to intestinal mucous. Enteric pathogens had an inborn ability for proper adhesion in gastrointestinal tract and further pathogenesis. Cell surface properties and adhesion studies reveals that isolates BM-3 can be selected as superior adhesive strain against enteric pathogens.Bacterial biofilms are microbial depositions on surfaces in aqueous environments[16]. Biofilm production helps in making a dynamic equilibrium of the isolate in their niche. Biofilm production capability enhances adhesion and colonization trait of the isolate BM-3. Spore and vegetative phases of isolates possess a

different rate of adhesion potentials, which indicates that cell surface properties were involved in adhesion process. Present study reveals that isolate BM-3 possessed superior adhesion properties than enteric pathogens reveals its ability for gastrointestinal colonization. Isolate also shows coaggregation property towards enteric pathogens which helps in competitive exclusion of enteric pathogens. Isolate BM-3 possess superior adhesion and colonization potential which is a necessary probiotics character. So future in vivo studies are aimed at persistence of the strain in the gastrointestinal tract of Balb/c mice and nature of immunostimulation induced by these candidate bacteria.

Conflict of interest statement

We declare that we have no conflict of interest.

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