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BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *BACILLUS PUMILUS* ISOLATED FROM COASTAL ENVIRONMENT IN COCHIN, INDIA

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ABSTRACT

Bacillus species constitute a diverse group of bacteria widely distributed in soil and the aquatic environment. In this study, *Bacillus* strains isolated from the coastal environment of Cochin, India were identified by detailed conventional biochemical methods, fatty acid methyl ester (FAME) analysis and partial 16S rDNA sequencing. Analysis of the data revealed that *Bacillus pumilus* was the most predominant species in the region under study followed by *B. cereus* and *B. sphaericus*. The *B. pumilus* isolates were further characterized by arbitrarily primed PCR (AP-PCR), antibiotic sensitivity profiling and PCR screening for known toxin genes associated with *Bacillus* spp. All *B. pumilus* isolates were biochemically identical, exhibited high protease and lipase activity and uniformly sensitive to antibiotics tested in this study. One strain of *B. pumilus* harboured cereulide synthetase gene *cesB* of *B. cereus* which was indistinguishable from rest of the isolates biochemically and by AP-PCR. This study reports, for the first time, the presence of the emetic toxin gene *cesB* in *B. pumilus*.

Key words: *Bacillus pumilus*; FAME; 16S rDNA; *cesB*; AP-PCR.

INTRODUCTION

The Gram-positive, aerobic, rod-shaped endospore-forming bacteria of the Genus *Bacillus* are the most widely represented organisms in the soil. Due to their ability to form spores and withstand a range of variable environmental conditions, *Bacillus* spp. adapt easily to diverse habitats (25). The diverse physiology of *Bacillus* spp. requires elaborate biochemical tests for their identification (30). Advances in chromatographic analysis of whole cell fatty acid methyl ester (FAME) profiles have made this technique sufficiently sensitive and reliable for grouping of *Bacillus* at species level (31). Further, nucleic acid based techniques such as 16S rDNA (3,34) and gyrase B (*gyrB*) sequence analysis have proved to be of immense value for phylogenetic analysis of bacteria (35). Based on the 16S rDNA sequence analysis, 5 groups have been identified within the genus *Bacillus*, of which the group 1 (*B. subitilis* group) comprises of *B. amyloliquefaciens*, *B. subtilis* and *B. pumilus* (3,12).

Several species of *Bacillus* inhabit coastal and marine environments, though it is hard to strictly classify them as indigenous to these habitats. Together with *B. cereus* and *B. subtilis*, *B. pumilus* is considered as a major component of marine bacterial communities (8,15,22,27). Recently, *B. pumilus* has also been reported to be the second most predominant *Bacillus* species in spacecrafts (17). This bacterium is highly resistant to extreme environmental conditions such as low or no nutrient availability, desiccation, irradiation, H_2O_2 and chemical disinfections (19). The ecological role of *B. pumilus* is emphasized by the fact that they do produce compounds antagonist to fungal and bacterial pathogens (4,6). Thus, *B. pumilus* is of considerable research interest to understand its physiological diversity, genetic relatedness with other *Bacillus* spp. and the possible presence of toxigenic factors. In the study reported here, we describe i) isolation and identification of *Bacillus* spp. from environmental samples by conventional methods, FAME and 16S rDNA sequencing and ii) further

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phenotypic and genetic characterization of *B. pumilus*, the predominant *Bacillus* group of bacteria in the coastal region under study.

MATERIALS AND METHODS

Isolation and biochemical characterization of *Bacillus* spp.

Sea water, sediment, fish and shellfish were collected off Cochin, West coast of India and processed for the isolation of *Bacillus* spp. Fish, shellfish or sediment samples were homogenized in phosphate buffered saline (PBS 0.05 M, pH 7.2), serially diluted in the same medium and spread plated on nutrient agar prepared in 50% seawater. One hundred micro liters of seawater samples were directly spread plated on the same medium and incubated at 30ºC for 24-48 h. The colonies that came up on agar plates were purified and stored at -80ºC in nutrient broth containing 30% glycerol. For taxonomic identification, the isolates were subjected to a series of biochemical tests (11), which included nitrate reduction, anaerobic growth, gas production from glucose, Voges-Proskauer (VP), growth at different NaCl concentrations, temperature and pH ranges, degradation of starch, casein, urea, tween 20, gelatin, chitin, acid production from arabinose, mannitol, xylose, glucose, lactose, citrate utilization and production of DNAse. The production of extracellular enzymes namely caseinase, chitinase, protease, alkaline phosphatase, gelatinase and lipase was studied following the protocol described by Smibert and Krieg (29)

Fatty acid methyl ester (FAME) analysis

Gas chromatographic analysis of whole cell fatty acid methyl ester (FAME) was performed for further identification and grouping of isolates. Fatty acid methyl ester extraction was performed using standard procedures (28). The fatty acid profiles generated were compared against an inbuilt Sherlock TSBA Library version 3.9 (MIDI Inc., DE, USA). A similarity index of >60% was used for clustering of isolates at species level.

Antimicrobial susceptibility assay

The inhibition of *B. pumilus* strains by various antibiotics was tested by standard disc diffusion technique (7). The cultures were grown in nutrient broth overnight and plated on Muller Hinton agar (Hi-Media, Mumbai). The following antibiotic discs with their concentrations indicated in parenthesis were used; amoxicillin (25 mcg), penicillin (10 mcg), ciprofloxacin (5 mcg), gentamycin (10 mcg), cotrimaxazole (25 mcg), chloramphenicol (30 mcg), bacitracin (8 mcg), tetracycline (30 mcg), kanamycin (30 mcg), erythromycin (15 mcg), vancomycin (30 mcg).

DNA isolation and purification

Pure genomic DNA was isolated following the method of Ausubel *et al*. (5). Briefly, the cultures were grown overnight in 3 ml nutrient broth with shaking at 30ºC. A 1.5 ml of the culture was centrifuged at 12 000 *g* for 10 min and the resultant pellet was resuspended in 567 µl 1× TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). Proteinase K and SDS were added to final concentrations of 100 µg/ml and 0.5% respectively, and incubated at 37ºC for 1 h. After incubation, NaCl (5 M) and CTAB/NaCl (10% w/v cetyl trimethyl ammonium bromide in 0.7 M NaCl) were added and incubated at 65ºC for 10 min. The mixture was extracted once each with an equal volume of chloroform-isoamyl alcohol (24:1) and phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated from the aqueous phase using 0.6 volumes of isopropanol and washed once with 70% ethanol. The DNA pellet obtained after final centrifugation was vacuum dried and dissolved in 50 µl 1× TE buffer. DNA quantification was done using a UV-1601 spectrophotometer (Shimadzu Corporation, Japan).

16S rDNA sequencing and AP-PCR

The 16S rDNA of 4 strains (NIOB 005, NIOB 133, NIOB 485 and NIOB 525) were PCR amplified using universal primers and PCR conditions described by Iwamoto *et al*. (16) (Table 1). The resultant 454 bp products were purified using a PCR purification kit (Qiagen, Germany) and sequenced. The sequences were subjected to homology search using BLAST programme (2) of the National Center for Biotechnology Information (NCBI).

AP-PCR was performed using primer CRA22 described by Neilan (18) (Table 1). All the reactions were carried out in 30 µl volumes consisting of a 10×buffer (100 mM Tris-HCl, 500 mM KCl and 20 mM $MgCl_2$), 200 µM concentrations of each of the four dNTPs, 30 picomoles of primer, 3 U of Taq polymerase (MBI Fermentas). All PCR amplifications were carried out in an eppendorf mastercycler (Eppendorf, Germany). In all the reactions, 300 ng of the pure genomic DNA was used. The amplification products were separated on a 2% agarose gel, stained with ethidium bromide and photographed. Amplification profiles obtained were analyzed and a dendrogram was generated using BioNumerics version 4.6 software (Applied Maths, Belgium)

PCR detection of cereulide synthetase genes

B. pumilus were screened for the presence of cereulide synthetase genes *cesA* and *cesB* using primers previously described (Table 1). The expected amplicons with *cesA* and *cesB* gene-specific primers were 188 bp and 635 bp respectively.

Nucleotide sequence accession numbers

The partial 16S rDNA and *cesB* sequences derived in this study have been deposited in GenBank under the accession numbers EU283326, EU283325, EU283323, EU283322, EU283320, EU283318, EU167933, EU283321, EU167932, EU167924, EU289221

Table 1. PCR primers used in this study.

Primer	Sequence (5'-3')	Target gene	Reference
EUB-F	gcacaagcggtggagcatgtgg	16S rDNA	16
EUB-R	gcccgggaacgtattcaccg		
CER1	atcataaaggtgcgaacaaga	Cereulide synthetase (*cesA*)	14
EMT1	aagatcaaccgaatgcaactg		
EM1-F	gacaagagaaatttctacgagcaagtacaat	Cereulide synthetase (*cesB*)	10
EM1-r	gcagccttccaattactccttctgccacagt		
CRA22	ccgcagccaa	Random primer	18

RESULTS

Taxonomic identification of *Bacillus* spp. isolated from coastal environment of Cochin

Eighty-two *Bacillus* spp. were isolated and identified by biochemical tests and fatty acid methyl ester analysis (FAME). These included *B. pumilus* (16), *B. cereus* (15), *B. sphaericus* (11), *B. subtilis* (10), *B. amyloliquefaciens* (8), *B. megaterium* (6), *B. lentimorbus* (5), *B. coagulans* (4), *B. licheniformis* (4), *B. circulans* (1), *B. flexus* (1) and *Bacillus* GC group 22 (1). The *Bacillus* GC group 22 corresponds to the gas chromatographic profile of a *Bacillus* species in the Sherlock TSBA Library version 3.9 (Microbial ID, MIDI Inc.), the 16S rDNA sequence of which does not match any known species of the genus *Bacillus*. In our study, *B. pumilus* was the most predominant species followed by *B. cereus* and *B. sphaericus*. Ivanova *et al*. (15) in their study found that *B. pumilus* and *B. subtilis* were the most abundant *Bacillus* spp. associated with marine sponges, ascidians, soft corals, and seawater.

Characterization of *B. pumilus*

Sixteen *B. pumilus* strains isolated from different sources (Table 2) exhibited uniform phenotypic properties (Table 3). Physiological tests revealed the production of detectable protease and lipase but not amylase, phosphatase, DNase, gelatinase and chitinase. The partial 16S rDNA sequences (≈ 500 bp) of *B. pumilus* determined in this study revealed 99-100% homology with *B. pumilus* 16S rDNA sequences in the GenBank. The antibiotic susceptibility profiles of *B. pumilus* were identical. All 16 isolates were uniformly inhibited by amoxicillin, ciprofloxacin, gentamycin, cotrimaxazole, chloramphenicol, bacitracin, tetracycline, kanamycin, erythromycin, vancomycin. All isolates exhibited resistance to penicillin.

AP-PCR typing of isolates

The random primer CRA 22, which consistently yielded 4-10 bands with *B. pumilus*, was chosen for typing of isolates by AP-PCR. The analysis of AP-PCR fingerprints revealed heterogeneity among *B. pumilus* isolates with 10 distinct patterns (Fig. 1). Despite this overall genetic diversity, near identical profiles were obtained between strains NIOB 485 and NIOB 525 (from crab), strains NIOB111 (fish) and NIOB 426 (from sediment), strains NIOB096 (from crab) and NIOB169 (from fish), strains NIOB163 (from fish) and NIOB431 (from starfish).

Table 2. Sources of *B. pumilus* strains isolated in this study.

Strain	Source
NIOB005	Sediment
NIOB014	Sediment
NIOB018	Sediment
NIOB026	Oyster
NIOB096	Crab
NIOB111	Fish
NIOB113	Fish
NIOB133	Fish
NIOB137	Fish
NIOB163	Fish
NIOB169	Fish
NIOB190	Fish
NIOB426	Sediment
NIOB431	Starfish
NIOB485	Crab
NIOB525	Crab

Detection of cereulide synthetase gene in *B. pumilus* by PCR

One isolate *B. pumilus* NIOB 133 isolated from an estuarine fish yielded 635 bp amplicon with *cesB*-targeted primers EM1-f and EM1-r (Fig. 2). The product of *cesB* PCR was sequenced and nucleotide sequence analysis of the PCR product revealed

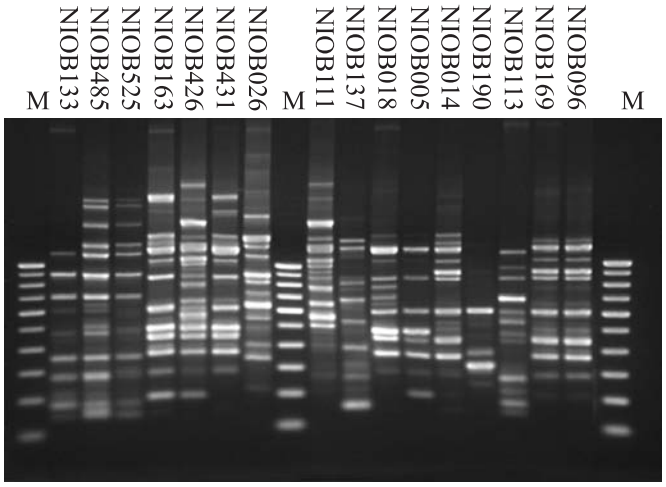

Figure 1. AP-PCR patterns of *B. pumilus* obtained with primer CRA22. M= 100 bp DNA ladder (GeneRuler, Fermentas).

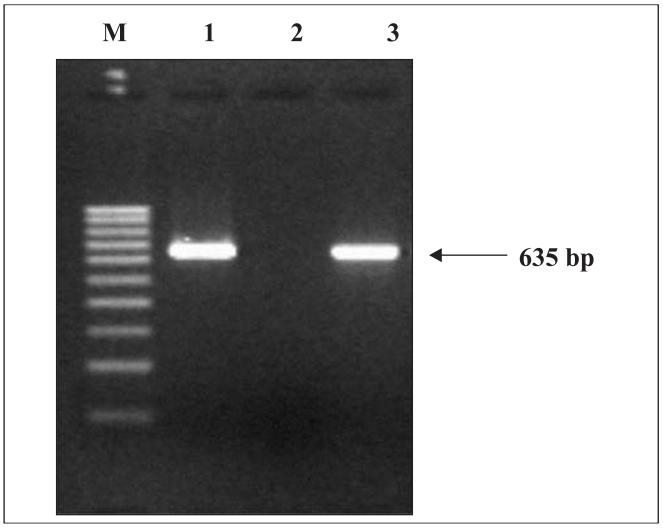

Figure 2. Detection of cereulide synthetase gene *cesB* in *B. pumilus* by PCR using primers EM1-F and EM1-r.
M= 100 bp DNA ladder (GeneRuler, Fermentas). 1: *B. pumilus* NIOB133 *cesB*$^+$; 2: *B. pumilus* NIOB 137 *cesB*$^-$; 3: *B. cereus* NIOB 020 (*cesB*$^+$ reference strain, environmental isolate).

Table 3. Growth and substrate utilization characteristics of *B. pumilus* observed in this study.

Characteristic	*B. pumilus* phenotype	Characte-ristic	*B. pumilus* phenotype
Amylase	+	Substrate utilization	
Protease	+	D-Glucose	+
Lipase	+	L-Arabinose	+
Phosphatase	-	D-Xylose	+
DNase	-	D-Mannitol	+
Gelatinase	-	Galactose	+
Chitinase	-	Fructose	+
Growth temperature	5-50ºC	Mannose	+
Growth ph	5-11	Nitrate	-
Nacl tolerance	10%	Adonitol	-
Oxidase	+	Dulcitol	-
Catalase	+	Sorbitol	-
Indole production	-	Inositol	-
Voges-Proskauer	+	Urea	-
Citrate utilization	-		

96% similarity with corresponding sequence of *cesB* of *B. cereus* (GenBank accession no. DQ889676) (26), while the deduced amino acid sequence showed 92% homology with a few amino acid mismatches (Fig. 3). This strain was negative by *cesA* PCR using primers CER1 and EMT1.

DISCUSSION

This study on the diversity of *Bacillus* spp. isolated from a coastal environment by biochemical assays, FAME analysis and 16S rDNA sequencing revealed that *B. pumilus* was the predominant species followed by *B. cereus*. *B. pumilus* belongs to *B. subtilis* group of aerobic spore-forming organisms, which has lately evoked considerable research interest due its involvement in cases of food-poisoning. Recently, a pumilacidin-producing *B. pumilus* has been implicated in a case of food poisoning (13). A study by Brophy and Knoop (9) reported experimental induction of enterocolitis in guinea pigs, while some compounds produced by *B. pumilus* were reportedly toxic to mice, eukaryotic cells and humans (20,21,24,32).

Sixteen environmental *B. pumilus* strains isolated from different sources (Table 2) exhibited uniform phenotypic properties (Table 3). The identity of these isolates was further confirmed by fatty acid methyl ester analysis with a similarity index of >60% (data not shown). The partial 16S rDNA sequences (500 bp) of these strains revealed 99-100% similarity with *B. pumilus* 16S rDNA sequences in the GenBank. Thus, a combination of conventional physiological tests and genetic analysis enabled unambiguous identification of *B. pumilus* from coastal environments of Cochin. None of the strains exhibited resistance to the antibiotics tested in this study except to penicillin. This observation is interesting, since penicillin

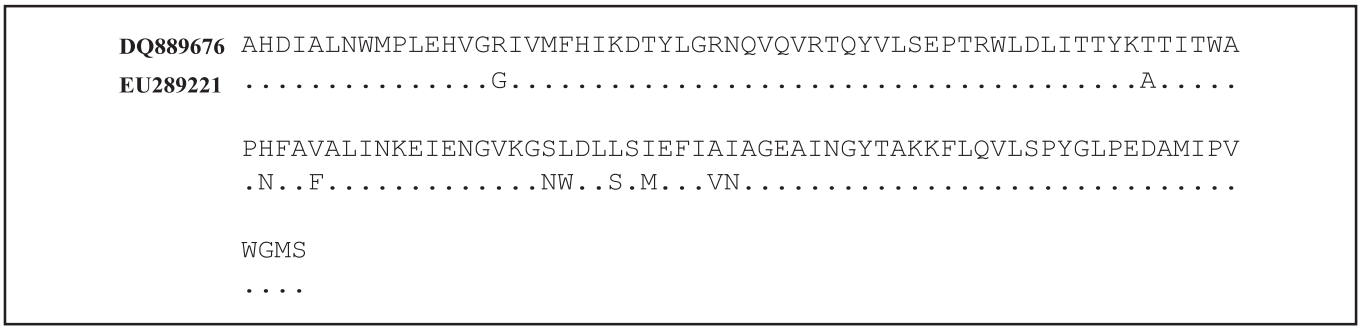

Figure 3. Alignment of deduced partial amino acid sequence of *cesB* derived in this study from *B. pumilus* strain NIOB 133 (EU289221) with the corresponding GenBank sequence of plasmid pCER270 (DQ889676). Dots indicate identical amino acids.

resistance has not been reported in *B. pumilus*. Studies on antibiotic resistance of *B. pumilus* are limited, since the organism is not considered infectious to humans and animals. However, some recent studies have revealed that several *Bacillus* species including *B. pumilus* can cause infections, ranging from skin infection to life threatening bacteremia in immunocompromised individuals (23,33). Thus, more studies need to be performed to understand the human health significance of *B. pumilus*, genetic basis of infections and resistance to antimicrobials.

The whole genome comparison of *B. pumilus* strains by AP-PCR demonstrated that *B. pumilus* were genetically diverse. Though the isolates shared several common amplification bands, overall heterogeneity among *B. pumilus* studied was apparent (Fig. 1). Despite this, some strains isolated from similar sources during different points time exhibited identical or near identical profiles. However, it was not possible to attribute isolates to a particular source solely based on the AP-PCR profiles, since some strains isolated from different sample types such as fish and sediment also exhibited identical AP-PCR profiles. The random primer CRA 22 used in this study has sufficient discriminating power and will be useful for studying genetic diversity among the *Bacillus* group of bacteria.

In order to understand the toxigenic potential of environmental strains of *B. pumilus*, the isolates obtained in this study were screened for the presence of cereulide synthetase genes *cesA* and *cesB* by PCR. Surprisingly, one strain (NIOB 133) was PCR positive for *cesB*-targeted gene (Fig. 2). This strain however, did not harbour *cesA* gene as revealed by *cesA*-specific PCR using primers CER1 and EMT1. Cereulide is a small heat stable cyclic dodecadepsipeptide produced by some strains of *B. cereus* which has high toxicity to humans (1,36). Cereulide is synthesized by a non-ribosomal peptide synthetase, encoded by the *ces* genes located on a 270-kb pXOI-like virulence plasmid named pCER270 (10,26), more often found associated with clinical isolates of *B. cereus*. A recent study has reported the presence of *cesA*, but not *cesB*, in *B. pumilus* and *B. licheniformis* isolated from bovine mastitis (20). However, the *cesB*-positive strain in our study lacked *cesA*. Therefore, it appears that cereulide-encoding genes may be distributed over a wide species range within the *Bacillus* group of bacteria in the environment. The *cesB*-positive *B. pumilus* in the present study was indistinguishable from the rest of the strains by biochemical assays or by its fatty acid profile. Further, the AP-PCR profile of this strain was similar to other non-toxigenic strains (Fig. 1). The production of toxin and the consequent ability of this strain to initiate emetic symptoms need to be established by suitable animal feeding experiments. Our study constitutes the first report on the presence of *cesB* in *B. pumilus*.

In conclusion, *Bacillus* spp. constitute key components of coastal-marine heterotrophic bacterial communities owing to their diverse and flexible physiological properties. Isolation and characterization of *Bacillus* spp. from these environments will help in identifying novel mechanisms of environmental survival, diverse metabolic activities, production of biotechnologically valuable compounds such as enzymes and antimicrobial substances and the presence of putative toxigenic factors. In our study, a combination of methods involving FAME, 16S rDNA sequencing and biochemical assays enabled complete identification of *B. pumilus*. Though, the biochemical and enzymatic properties of *B. pumilus* isolated in this study were uniform, intraspecific genetic diversity was evident from AP-PCR analysis. The detection of cereulide synthetase gene *cesB* in *B. pumilus* is significant, since *ces* genes were previously thought to be restricted to emetic strains of *B. cereus*. The organization of *ces* operon in *B. pumilus* is a subject for further study.

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RESUMO

Caracterização bioquímica e molecular de *Bacillus pumilus* isolado do ambiente costeiro de Cochin, Índia

As espécies de *Bacillus* constituem um grupo diversificado de bactérias amplamente distribuídas no solo e no ambiente aquático. Neste estudo, cepas de *Bacillus* isoladas do ambiente costeiro de Cochin, Índia, foram identificadas através de métodos bioquímicos convencionais, análise de ésteres metílicos de ácidos graxos (FAME) e sequenciamento de 16S rDNA. A análise dos dados revelou que *Bacillus pumilus* foi a espécie predominante na região estudada, seguido de *B. cereus* e *B. sphaericus*. Os isolados de *B. pumilus* foram caracterizados através da reação em cadeia da polimerase com primers arbitrários (AP-PCR), perfil de sensibilidade a antibióticos e triagem por PCR de genes de toxinas associadas com *Bacillus* spp. Todos os isolados de *B. pumilus* foram bioquimicamente idênticos, apresentaram elevada atividade de protease e lipase e foram uniformemente sensíveis aos antibióticos estudados. Um dos isolados de *B. pumilus* apresentou o gene *cesB* de *B. cereus*, que não foi não distinguível dos demais isolados por testes bioquímicos nem por AP-PCR. Este é o primeiro relato da presença do gene *cesB* da toxina emética em *B. pumilus.*

Palavras-chave: *Aspergillus flavus*, pimenta em pó, alterações nutricionais, aflatoxina.

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