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Original Paper

Adherence of *Aeromonas veronii*, *Shigella flexneri* and *Escherichia fergusonii* with *Microcystis*-dominated bloom relates to their ability to utilize chitin

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Abstract

The study was undertaken to size-fractionate the bacterial population associated with *Microcystis* blooms of an eutrophic lake. Two bacterial cultures were isolated from the particulate-bound fraction, and biochemical tests, phylogenetic analysis and homology of 16S rRNA gene sequences revealed their high similarity with A*eromonas veronii* and *Shigella flexneri*. Another isolate with characteristics resembling *Escherichia fergusonii* was obtained in phytoplankton-free water samples. Since these three cultures were enriched on chitin-selective medium, their capability to utilize chitin as a sole C source was examined. The *in vivo* chitinase activity (as µg *N*-acetylglucosamine produced min^{-1} mL^{-1} culture) was 82.1, 28.5 and 18.5 for *A. veronii, S. flexneri* and *E. fergusonii*, respectively. The corresponding *N*-acetylglucosamine accumulation in medium was 421, 288 and 122 µg mL^{-1}. There was also a gradation in growth indices in the three bacteria, which corresponded to their chitin utilization ability. Notwithstanding differences in chitinase activity, the three strains utilized almost equally the exogenous *N*-acetylglucosamine. We propose that chitinase activity may have a role in affinity of the particular bacterial cell to phytoplankton extracellular polymeric substances and therefore, adherence ability.

Key words: *Aeromonas veronii,* chitin, cyanobacterial bloom, *Escherichia fergusonii*, extracellular polymeric substances, *N*-acetyl-glucosamine, *Shigella flexneri*, 16S rRNA sequence.
Abbreviations: CCA, colloidal chitin agar; CFU, colony forming unit; GlcNAc, *N*-acetyl-D-glucosamine; MCC, microbial culture collection; ONPG, ortho-nitrophenyl-β-galactoside.

Introduction

During the bloom event, complex bacteria-phytoplankton interactions change throughout the whole period of bloom formation, depending on the phytoplankton species, physiological status, environmental conditions and bloom stage (Zhou et al. 2018). Nonetheless, major lineages of Alphaproteobacteria, Gammaproteobacteria and Flavobacteria often dominate the phytoplankton assemblages (Buchan et al. 2014). In Scandinavian waters, cyanobacteria-associated bacterioplankton have been identified as belonging to the taxonomic divisions Bacteroidetes, Proteobacteria, Firmicutes, Planctomycetes, Verrucomicrobia, Acidobacteria, Chloroflexi and Thermomicrobia, suggesting extraordinary diversity of the heterotrophic population associated with phytoplankton (Eiler, Bertilsson 2004; Berg et al. 2009).

The bacterial association of *Microcystis*-dominant blooms has been extensively studied and some rather interesting differences in diversity indices between the particulate-associated and "free-living" bacterial communities were revealed (Jankowiak, Gobler 2020). Several studies have indicated that the *Microcystis*-adhered microbiome displays considerably less diversity of bacterial taxon than is found associated with free-living bacterioplankton. For example, association of members of rather limited taxonomic groups like Rhodobacterales, Sphingomonadales, Burkholderiales and Xanthomonadaceae was reported for *Microcystis* colonies (Shi et al. 2012). In contrast, much diverse taxa were prevalent in other habitats, and some of them could also be equally dominant in the cyanosphere. Furthermore, lack of Actinobacteria and dominance of Gammaproteobacteria are other characteristic features of cyanosphere communities (Shi et al. 2012; Parveen et al. 2013). More recently, it was reported that each *Microcystis* colony represent an oligotype comprised of same genotype of cells, which may differ from other colonies with respect to the 16S rRNA gene sequence (variable region; Smith et al. 2021). Each oligotype may attract oligotype-specific bacterial taxa, and thereby differ from other oligotypes. Thus, bacterial assembly is a dynamic feature related to the oligotypes of colonies that form the bloom.

The bacterial communities are attracted to phyto-plankton extracellular polymeric substances (EPS) that are

mainly comprised of amino sugars such as galactosamine and glucosamine, and the acidic sugars galacturonic acid, glucuronic acid and muramic acid (Durkin et al. 2009; Sperling et al. 2017; Mühlenbruch et al. 2018). Chitin and laminarin are the cell wall heteropolysaccharides found in mucilaginous cyanobacteria and entangled predatory zooplankton (Ahmed et al. 2007; Neogi et al. 2012), which are potent attractants for chitinase- and laminarinase-producing bacteria such as *Vibrio cholerae* (Anas et al. 2021). Among other bacterial genera, *Pannonibacter* and *Chryseobacterium* utilize various organic substrates in the cyanobacterial exudates, and consequently can establish strong association (Gao et al. 2020).

Earlier we showed firm attachment of *V. cholerae* with *Microcystis* dominant blooms, which during membrane filtration, co-sedimented with phytoplankton matter, while other bacteria separated as "free-living" forms in the filtrates (Chaturvedi et al. 2015). *V. cholerae* cells were also found to be embedded within extracellular matrix (ECM) (Islam et al. 2020). Since *V. cholerae* produces exo-chitinase (Anas et al. 2021), we believe that mobilization of chitin for nutrition can be a factor attributing to the strong adherence.

The aim of this investigation was to ascertain whether chitin utilization ability of bacterial taxa has any relationship with the adherence behaviour of the strains of such communities with *Microcystis* colonies.

Materials and methods

Chemicals

All chemicals used in this study were purchased from HiMedia (India) and Sigma-Aldrich (USA) and had analytical grade quality.

Survey region

A *Microcystis* spp. dominated permanent Lake, Gokalpur Tal, Jabalpur (23.0448°N, 79.8983°E) was selected for the collection of a cyanobacterial bloom. Water samples were collected at a depth of 0.3 m below the surface after skimming it across water surface using 70 µm plankton nets (courtesy Prof. T.R. Rao, Department of Zoology, University of Delhi, Delhi) and poured in 2 L wide-mouth sample bottles. About 200 to 500 mL water samples were allowed to stand for 6 h and then the buoyant colonies were collected using Pasteur pipettes, subsequently washed with distilled water and examined under a microscope to verify cyanobacterial dominance at 400 × magnification.

Size-fractionation of phytoplankton

In triplicates, about 100 mL of bloom samples from five phytoplankton-rich locations were filtered using polycarbonate membrane filters (5.0 µm pore size; 47 mm diameter; Axiva Sichem Biotech) under vacuum at ~380 mm Hg. The seston was twice washed with sterile distilled water. The filtrate that passed through the membrane and washings were pooled and filtered again under vacuum using a 47 mm diameter nitrocellulose membrane filter with 0.45 µm pore size; (Axiva Sichem Biotech). The membranes with recovered material were stored at −20 °C in Petri dishes.

Preparation of colloidal chitin

Colloidal chitin was prepared from commercial chitin (HiMedia) by the modified method of Jha and Modi (2017). Chitin powder (10 g) was suspended in 60 mL concentrated HCl and shaken on a rotary shaker for 60 to 120 min at 30 °C until chitin was completely homogenized. This suspension was then slowly mixed with 400 mL sterile double distilled water at 4 °C and then rapidly stirred to precipitate colloidal chitin, which was pelleted at 7000×g for 10 min at 4 °C. The pellet was washed several times with sterile distilled water, pH 3.0 to 3.5. The pH of colloidal chitin was neutralized with 1 M NaOH and re-precipitated by centrifugation as above. This preparation was washed with cold sterile water two to three times and stored airtight at 4 °C until further application.

Preliminary screening of chitin-utilizing bacteria

Seston collected after various filtration steps as above (n = 30) was the source to screen bacteria utilizing chitin as a sole carbon source, and for identification on selective media. The sediment trapped as seston (about 0.1 mL) was scraped into a fresh sterile Eppendorf tube. The volume was made up to 1 mL using autoclaved double distilled water. The seston suspension was diluted 1:100 with distilled water and was poured onto colloidal chitin agar (CCA) medium (Atlas, Parks 1997) consisting of the following (g L^{-1}): $(NH_4)_2SO_4$, 2.0; Na_2HPO_4, 1.1; KH_2PO_4, 0.7; $MgSO_4$ $7H_2O$, 0.2; $FeSO_4$, 0.001; $MnSO_4$, 0.001 and colloidal chitin 3.0; pH 7.0, adjusted with KOH. This was solidified using 1.5% bacteriological grade agar. After 48 to 72 h incubation at 37 °C, the plates were observed for appearance of clear zones around growing colonies. The well-defined colonies were hand-picked using sterile toothpicks and inoculated by slightly piercing onto second CCA plates with squares marked at the bottom, referred to as master plates. Accordingly, for 15 water samples (five locations in triplicates) and two membrane filters, growing colonies from 30 master plates were re-inoculated to identical CCA medium at the same positions, and were referred to as replica plates.

Then, the colonies were transferred from 30 replica plates to 30 Petri dishes with each of three selective media: (a) thiosulfate-citrate-bile salts sucrose (TCBS) agar, (b) bismuth sulphite (BS) agar and (c) eosin-methylene blue (EMB) agar. The colony characteristics were recorded and the cultures were subjected to biochemical tests. Frequency of total number of colonies appearing in selective media with typical colony characteristics (see below) per 100 colonies picked as inoculum from respective replica

CCA plates was recorded. For colony enumeration, a colony counter was used. The colonies from the source replica plates were identified at a preliminary level using characteristics of cultures and using standard biochemical tests. The following colony characteristics were recorded: (a) on TCBS – yellow mucoid shiny and pointed colonies, 2 to 3 mm diameter and tiny green rounded colonies, 2 to 4 mm; (b) on EMB – greenish, metallic sheen in reflected light, bluish black centre in transmitted light, translucent, amber colonies, mucoid grey-brown centre in transmitted light, bright pink colonies in mucoid texture, and purple colonies with pinkish edges; and (c) on BS – brown, grey or black colonies, 2 to 3 mm diameter after 24 h, usually without or somewhere with metallic sheen.

Biochemical tests

The shortlisted chitin selected bacterial isolates from CCA plates were subjected to biochemical tests according to the Bergey's Manual of Systematic Bacteriology and manufacturer's instructions: IMViC, Triple Sugar Iron test (TSI), urease, catalase, ortho-nitrophenyl-β-galactoside (ONPG), L-lysine, L-arginine, and H_2S production test (Holt et al. 1994; Aneja 2014).

Isolation of chitin-utilizing bacteria

The colonies with a certain identifiable taxon of bacteria were marked on the replica CCA plates and transferred by streaking in duplicate to next batch of CCA plates. The growing colonies were subsequently transferred as above and incubated for at least seven generations on CCA plates. The colonies appearing after the seventh generation of growth on CCA plates were again examined for identifiable characteristics on selective media as above. Based on the colony appearance and biochemical tests, some presumptions were made on the identity of the chitin-utilizing strains.

Identification of isolates using 16S rRNA gene sequence analysis

Some representative chitin-utilizing bacterial isolates were subjected to partial 16S rRNA genome sequencing at the facility of the National Centre for Microbial Resource, Pune (India). Isolates were designated as GKP-E1, GKP-S2 and GKP-VC2. Genomic DNA was isolated by the standard phenol/chloroform extraction method (Sambrook et al.1989). PCR amplification of the 16S rRNA gene was carried out using universal primers 16F27 (5'-CCA GAG TTT GAT CCT GGC TCA G-3') and 16R1492 (5'-TAC GGC TAC CTT GTT ACG ACT T-3'). The amplified 16S rRNA gene PCR product was purified by polyethylene glycol-NaCl precipitation and directly sequenced on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. Essentially, sequencing was carried out from both ends using additional internal primers, such that each position was read at least twice. Assembly was carried out using the Lasergene package followed by identification using the EzBioCloud database according to Yoon et al. (2017). The 16S rRNA sequences were blasted against available sequences in the GenBank database.

Phylogenetic analysis

A phylogenetic (neighbour-joining) trees of the strains were generated by the software package MEGA version 5 to determine similarity of the sequences of the sstrains used in this study to cluster of their closely related species retrieved from GenBank. Analysis of 16S rRNA gene phylogeny was performed using published sequences from the Eztaxon database (Kim et al. 2012). Further, the topology of the trees was checked by neighbour-joining, maximum likelihood, and maximum parsimony methods.

After verifying the identity of the selected chitin-utilizing bacteria, they were deposited in NCCS, Pune (India) and the repository's accession numbers obtained.

Preparation of bacterial samples for analysing chitin utilization and growth parameters

The colonies were transferred to 10 mL chitin-containing broth (Atlas, Parks 1997) and allowed to grow for 48 h at 37 °C on an orbital rotary shaker (Remi Instruments, India) maintained at 120 rpm. After growth was attained as visible turbidity, culture aliquots were diluted by 10^{-2} to 10^{-4} and spread on CCA plates to count colony forming units (CFU). Protein concentration in the bacterial cells in chitin broth was determined according to Lowry et al. (1951). The cells were retrieved at different time intervals (0, 6, 12, 24, 48 and 72 h) and used for protein determination and for CFU analysis as above. Bovine serum albumin was used as a standard protein.

Thereafter, the cultures were centrifuged at 10 000 × g for 15 min at 4 °C and the cell-free supernatant was used for determining *N*-acetyl-D-glucosamine (GlcNAc) concentration, as also for chitinase assay.

Determination of in vivo *chitinase activity*

The method of Imoto and Yagishita (1971) was used to estimate chitinase activity. In this assay, 0.5 mL culture filtrate was added to 1 mL of substrate solution, which contained 0.5% suspension of the colloidal chitin in 0.1 M acetate buffer, pH 4.5. After proper mixing, the above reaction mixture was kept at 37 °C for 30 min. The reaction mixture was centrifuged at 10 000 × *g* for 10 min. The resulting supernatant (about 1.5 mL) was transferred to a fresh tube, and to this 2 mL of Schale's reagent was added. The mixture was heated in a boiling water bath for 15 min. After cooling, the absorbance of the mixture was recorded at 420 nm against water using a Beckman DU spectrophotometer. One unit of chitinase activity is defined as the amount of enzyme that produced 1 μmol GlcNAc min^{-1} with Schale's method, using a calibration curve for GlcNAc (Horn, Eijsink 2004).

Determination of GlcNAc content

To determine the total GlcNAc content in cell-free supernatant, 670 µL Schale's reagent was added to 500 µL culture supernatant. The mixture was incubated in a boiling water bath for 15 min, and quickly chilled on an ice bath. Absorbance was recorded at 420 nm against water as blank and the amount of GlcNAc was determined from a standard calibration curve (see above) (Schales, Schales 1945).

Determination of GlcNAc utilization

The chitin-utilizing pure cultures of bacteria were acclimatized to grow with GlcNAc as a sole carbon source as follows. The colonies were transferred to M9 medium containing 0.125% GlcNAc (Ghosh et al. 2011; Wang et al. 2018) with the following composition: 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 19 mM NH_4Cl, 9 mM NaCl, 2 mM $MgSO_4$ $7H_2O$, 0.1 mM $CaCl_2$, 0.03 mM thiamine-HCl and GlcNAc 0.125% (w/v); pH 7.0. The bacteria were allowed to grow for 48 h at 37 °C and the content of GlcNAc in the spent medium was measured at 420 nm at 0, 6, 12, 24 and 48 h using Schale's method as above.

Statistical analyses

One way analysis of variance (ANOVA) test followed by Tukey's post hoc treatment (Spence et al. 1974) was used to test for significant differences in frequencies of different groups of bacteria separated by the two membrane filters. Here, the frequency refers the number of colonies of each individual identifiable bacterial taxon on selective medium per one hundred colonies that were selected as inoculum in the replica CCA plates.

For comparison of physiological attributes between the three isolates, ANOVA was applied to test for significant differences between pairs of treatments, $p < 0.05$ was regarded as statistically significant. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, USA).

Results

It was hypothesized that superior chitin utilizing bacteria display better attachment to the *Microcystis*-dominant phytoplankton material. For this purpose, phytoplankton-adhered and "free" bacteria were segregated by a "size-fractionation" technique using two different pore-size membrane filters. Upon further chitin-selection and probing of the isolated colonies using selective media (TCBS, EMB and BS-agar), a clear-cut demarcation of the bacterial taxa between the two seston material was noticed.

In the 5.0 µm cut-off membrane there was a dominance of presumed *Vibrio cholerae* / *Aeromonas* / *Citrobacter* that was able to grow on TCBS with overlapping colony characteristics – yellow mucoid shiny and pointed colonies, 2 – 3 mm diameter. As shown in Table 1, the average frequency of the presumed *V. cholerae* / *Aeromonas* spp. against total bacterial colonies on CCA plates was 39.5 ± 9.5% of 5.0 µm membrane fractions, and 5.8% ± 2.8 on the 0.45 µm membrane ($F = 46.7$; $p = 0.0001$). Therefore, the presumed *V. cholerae* / *Aeromonas* spp. was a dominant cyanobacterial assemblage in all sets from five sampling locations and was significantly more abundant in the 5.0 µm seston as compared to the 0.45 µm fractions, according to the Tukey's post hoc test ($p = 0.001$).

Randomly, TCBS grown colonies showed negative arginine dehydrolase, positive indole, positive lysine decarboxylase and negative urease tests. These attributes initially suggested that they were *V. cholerae*. However, variable citrate utilization and positive VP, and catalase tests hinted that non-*Vibrio* bacteria were prevalent in 5.0 µm seston. The positive lysine decarboxylase test ruled out possibility of *Cirobacter*. Positive catalase pointed out towards the presence of suspected *Aeromonas* (Table 2).

In a completely opposite trend, the average frequencies of *Escherichia* spp. / some unknown bacteria, demonstrating growth on EMB plates with typical green-metallic sheen around the colonies, were very low in the 5.0 µm membrane

Table 1. Comparative account of the frequencies (number of colonies on enrichment medium against CCA medium) of bacteria from *Microcystis*-dominant bloom samples filtered by 5.0 µm and 0.45 µm membrane filters. Values are the mean ± SD of three replicates. Values in the same column with different letters are significantly different from each other ($p < 0.05$, Tukey's post-hoc test)

Sampling location	Pore size of membrane filter	Frequency of presumptive *V. cholerae* / *Aeromonas* sp. against CCA plates	Frequency of presumptive *Shigella* sp. / *Salmonella* sp. against CCA plates	Frequency of presumptive *E. coli* / *E. fergusonii* against CCA plates
S1	5.0 µm	56.4 ± 13.8 a	20.0 ± 2.7 a	0.5 ± 0.7 a
S2		37.9 ± 8.6 a	32.9 ± 2.4 a	0.3 ± 0.5 a
S3		41.9 ± 13.9 a	24.6 ± 12.0 a	0.9 ± 1.4 a
S4		29.7 ± 1.9 a	24.3 ± 10.9 a	2.2 ± 8.8 a
S5		31.8 ± 10.6 a	20.1 ± 3.5 a	7.3 ± 5.2 a
S1	0.45 µm	4.2 ± 1.3 b	38.4 ± 13.7 a	16.3 ± 1.5 b
S2		9.1 ± 4.3 b	35.8 ± 15.0 a	22.8 ± 5.4 b
S3		1.5 ± 2.2 b	31.1 ± 12.7 a	18.9 ± 1.5 b
S4		5.7 ± 4.2 b	25.3 ± 9.2 a	27.9 ± 5.0 b
S5		8.6 ± 6.3 b	28.9 ± 16.7 a	22.8 ± 6.1 b

Table 2. Cultural and biochemical characteristics of representative colonies from population of presumptive *V. cholerae* / *Aeromonas* spp. from CCA to TCBS plates, presumptive *E. coli* / *E. fergusonii* from CCA to EMB plates and presumptive *Shigella* spp. / *Salmonella* spp. from CCA to BSA agar plates. +, indicates that 80% or more of strains were positive; –, indicates that 20% or less of strains were negative; v, indicates that between 21 and 79% of strains were positive; A/A, yellow (acid) slant, yellow (acid) butt; K/A, red (neutral or alkaline) slant, yellow (acid) butt

Test parameter	Presumptive *E. coli* / *E. fergusonii*	Presumptive *Shigella* spp. / *Salmonella* spp.	Presumptive *V. cholerae* / *Aeromonas* spp.
Colony characteristics			
Growth on media	EMB	BSA	TCBS
Colony characteristics	Colonies 2 – 3 mm in diameter grow with a metallic sheen	Colonies 2 – 3 mm in diameter after 24 h	Yellow colour, pointed colonies 2 – 3 mm in diameter
Colour of colony	Greenish metallic sheen in reflected light, blue centre in transmitted light	Black brown colonies sometimes grey usually without metallic sheen	Yellow colour mucoid, shiny colony
Partial confirmatory biochemical test			
Arginine dehydrolase test	v	–	–
Indole	+	v	+
Methyl red	+	+	–
Voges-Proskauer	–	–	+
Citrate	–	–	v
H_2S	–	–	–
Catalase	–	+	+
ONPG	+	–	+
Urease	–	–	–
Lysine	+	–	+
Triple sugar iron agar test	A/A	K/A	K/A

filter seston (f = 2.3 ± 2.6%). It was dominant (f = 21.7 ± 3.9%) in precipitates of 0.45 µm membrane filters (F = 68.4; $p < 0.0001$). The p-value corresponding to the F-statistic of one-way ANOVA was lower than 0.05, indicating significant differences between the two fractions (Table 1).

The IMViC test (positive indole and methyl-red tests while negative VP and citrate utilization tests) of EMB colonies indicated the presence of *Escherichia* and ruled out other genera like *Enterobacter*. Additional biochemical tests, such as positive ONPG, negative urease and positive lactose fermentation in MacConkey agar medium further confirmed *Escherichia* being the likely genus (Table 2).

The frequency of presumed *Shigella* spp. / *Salmonella* spp. / unknown bacteria that can grow on BS-agar with black-brown centred mucoid colonies was f = 24.4 ± 4.7% for samples from 5.0 µm membrane filter seston, and 31.9 ± 4.7% in the 0.45 µm samples. There was no significant difference (F = 5.13; p = 0.0533) in the CFU counts of presumed *Shigella* spp. / *Salmonella* spp., verified by the Tukey's test ($p > 0.05$), between the two membrane filters (Table 1).

The result of IMViC, urease and ONPG tests of BS-agar colonies pointed towards suspected *Salmonella*, but the TSI test was variable, with poor to negative H_2S production and the lysine decarboxylase test was negative, suggesting *Shigella* (Table 2).

The characteristics shown above were not sufficient to ascertain unambiguously the generic or species level identity of the screened colonies. Therefore, seven colonies from each of the membranes were sub-cultured on CCA medium for seven generations. During this period, only three cultures maintained good growth and exhibited cultural characteristics and biochemical attributes of the source colonies of initial replica CCA plates. These were designated as: (a) GKP-VC-2, originally characterized as *V. cholerae* / *Aeromonas* spp., (b) GKP-S2, *Shigella* spp. / *Salmonella* spp., both from 5.0 µm membrane filters, and (c) GKP-E1, *Escherichia* spp. collected from 0.45 µm membrane filters. These isolates were deposited with the National Centre for Microbial Resource, Pune, India, with accession numbers: MCC4600, MCC4603 and MCC4602, respectively.

After unambiguously proving the axenicity of the strains, their taxonomic position was determined by 16S rRNA gene sequence comparison. The EzBioCloud database consists of a hierarchical taxonomic system containing phyla, classes, orders, families, genera, species and subspecies. This classification was based primarily on the maximum likelihood phylogeny for partial 16S rRNA gene sequence data, where a 97% similarity cut-off was used for the recognition of phylotypes. The strain GKP-VC-2 (MCC4600), GKP-S2 (MCC4603) and GKP-E1

(MCC4602) showed 97 to 100% sequence similarity with strains belonging to the genera *Aeromonas, Shigella* and *Escherichia* after phylogenetic assessment. Taxa without their type or representative 16S rRNA gene sequences were not included in the database.

Among the isolates, strain GKP-VC-2 (MCC4600) placed perfectly and strongly in the *Aeromonas* cluster (Fig. 1). To strengthen the finding, other very closely related genera were included in the analysis, but as is evident, the strain clearly fell within the *Aeromonas* cluster. To further cross-check this finding, the topology of the tree was checked by neighbour-joining, maximum likelihood, and maximum parsimony methods and in all the methods, the strain again lied firmly inside the *Aeromonas* cluster. This serves as strong phylogenetic proof that the strain was indeed a member of the genus *Aeromonas*.

There were however some ambiguities in the cases of strains GKP-S2 (MCC4603) and GKP-E1 (MCC4602). The 16S rRNA gene tree that included the sequence of GKP-S2 clustered in a small sub-cluster of *Shigella flexneri* ATCC 29903 and *Shigella sonnei* CECT 4887 (Fig. 2). Other taxa in the cluster belonged to the genera *Shigella* and *Escherichia*. This confusing clustering was most probably a result of limited resolution that the 16S rRNA gene provides in the case of very closely related bacterial taxa. This also explains the need for physiological and biochemical assays in order to ascertain the identity of our strains/sequences using multiple methodologies.

Using the 16S rRNA gene, the strain GKP-E1 clustered in an intermixed cluster of both *Shigella* and *E. fergusonii* (Fig. 3). This complicated clustering was a result of the low divergence of the 16S rRNA gene along with possible homoplasy. This explains the reason why 16S rRNA gene phylogenetic inferences must be assessed with complementary physiological and biochemical data for establishing the identity of any particular bacterial isolate.

Chitinase activity of pure cultures of chitin-utilizing bacteria *A. veronii, S. flexneri* and *E. fergusonii* was carried out after brief acclimatization to chitin-containing mineral medium. Prior to assays, the reaction was optimized and it was found that maximum chitin hydrolysis was observed with 0.5% (w/v) colloidal chitin, pH 7.0 and a growth period of 48 h at 37 °C under constant stirring. The highest chitinolytic activity, 82.1 nmol GlcNAc min^{-1}

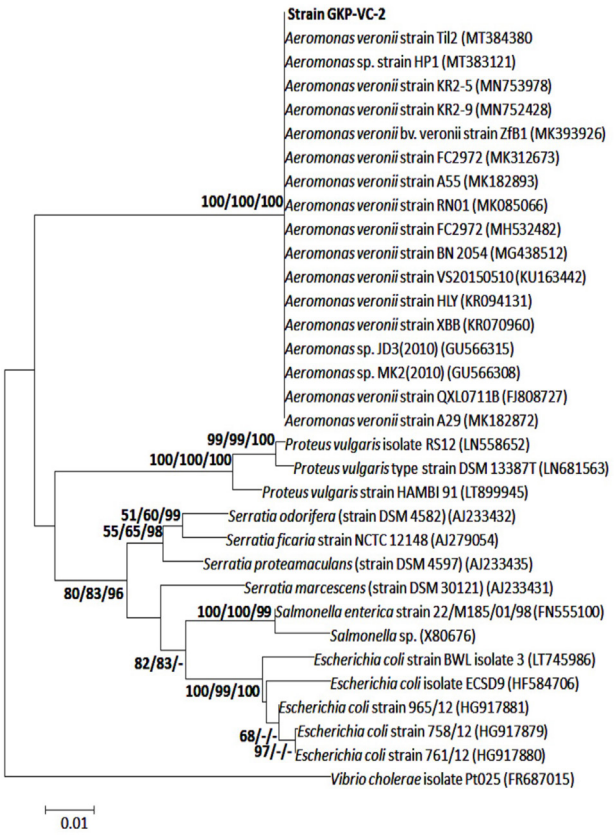

Fig. 1. Phylogenetic tree based on the 16S rRNA gene of strain GKP-VC-2 along with all the closely related taxa, with the bootstrap values representing neighbour-joining, maximum likelihood, and maximum parsimony methods, respectively. Bar, 0.01 changes per nucleotide position. No bootstrap values < 50 are shown.

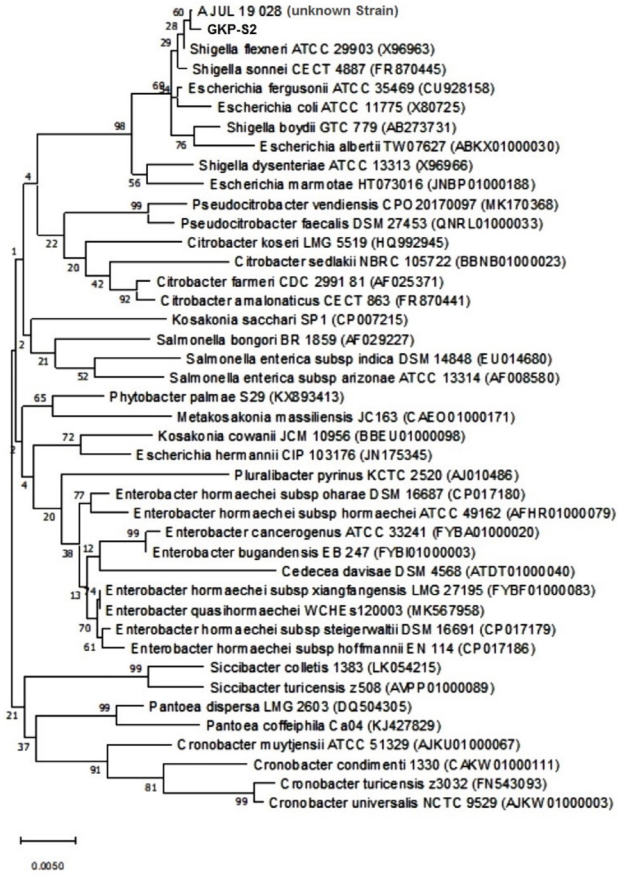

Fig. 2. Phylogenetic tree based on the 16S rRNA gene of strain GKP-S2 along with all the closely related taxa, with the bootstrap values representing neighbour-joining, maximum likelihood, and maximum parsimony methods, respectively. Bar, 0.001 changes per nucleotide position. No bootstrap values < 50 are shown.

mL^{-1}, was observed in *A. veronii* cultures and the activity was sustained up to 48 h in chitin broth. Further, *S. flexneri* exhibited an activity of 46.16 nmol GlcNAc min^{-1} mL^{-1} lasting for 48 h, and *E. fergusonii* displayed the least activity – 28.49 nmol GlcNAc min^{-1} mL^{-1}, which lasted up to 24 h (Fig. 4A). Thereafter, the enzyme activity gradually decreased in subsequent growth periods, and here too, *E. fergusonii* had the lowest activity – 18.55 nmol GlcNAc min^{-1} mL^{-1} after 72 h. In pair-wise comparison of the three strains, the highest mean values of chitinase activity at corresponding time points was significantly different (Tukey's post hoc pair-wise comparison $p < 0.05$; ANOVA $F = 5.89$, $p = 0.0267$).

After measuring the chitinase activity, the concentration of the chitin degradation product, GlcNAc, was determined during the incubation period as specified. As shown in Fig. 4B, *A. veronii* showed maximum GlcNAc concentration at 48 h (421.22 µg mL^{-1}). In the case of *S. flexneri* the concentration was 287.78 µg mL^{-1}, while *E. fergusonii* had the lowest concentration (121.57 µg mL^{-1}) at 24 h. The mean values of the highest GlcNAc concentration in growth medium significantly differed between the strains ($F = 5.99$ and $p = 0.0256$).

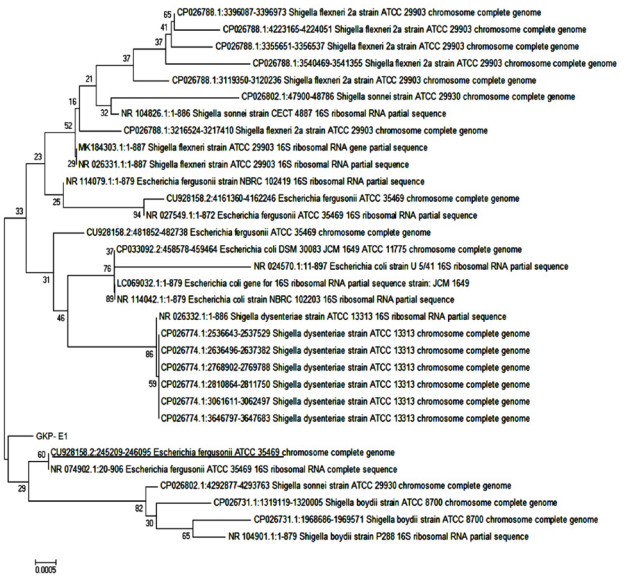

Fig. 3. Phylogenetic tree based on the 16S rRNA gene of strain GKP-E1 along with all the closely related taxa, with the bootstrap values representing neighbour-joining, maximum likelihood, and maximum parsimony methods, respectively. Bar, 0.0005 changes per nucleotide position. No bootstrap values < 50 are shown.

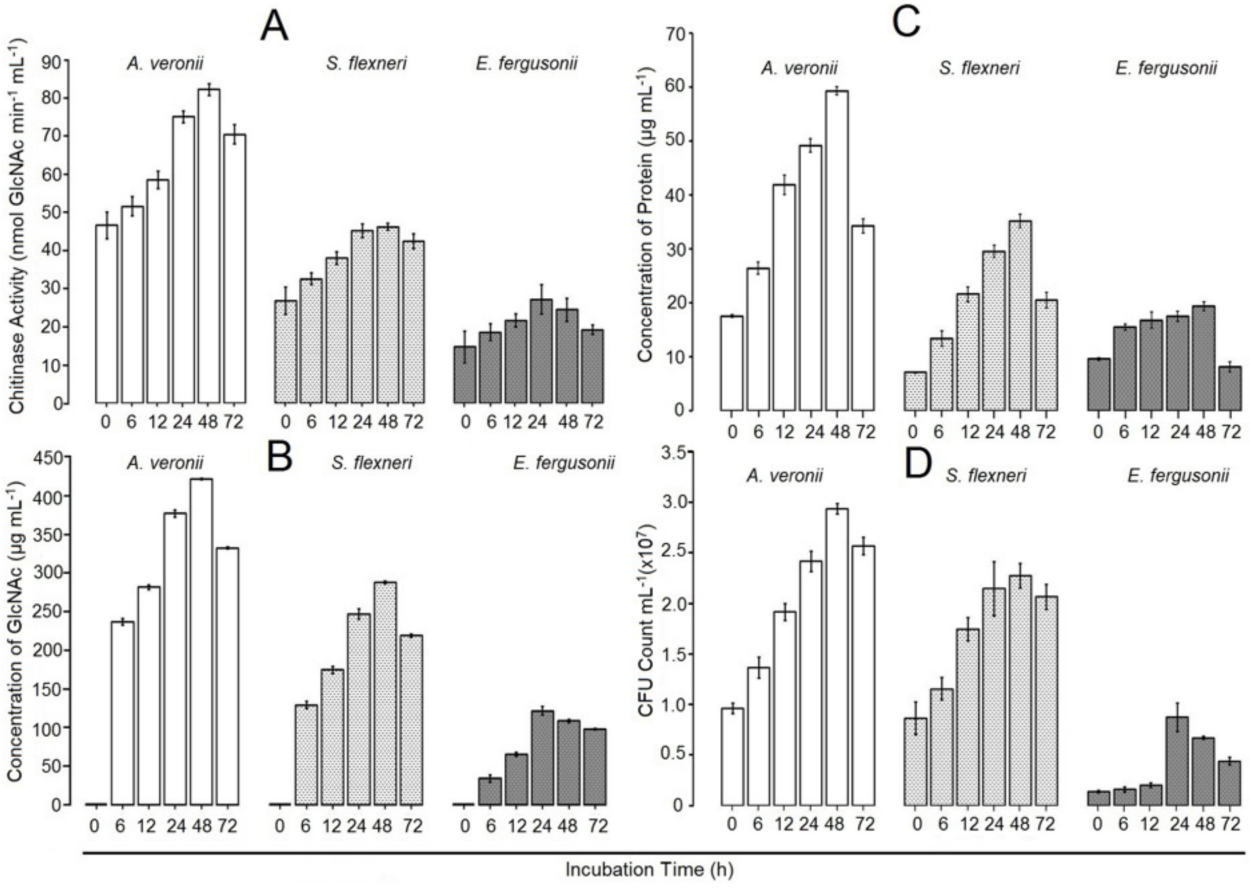

Fig. 4. Time kinetic changes in chitinase activity (A), N-acetylglucosamine content in culture filtrate (B) cellular protein content (C) and CFU per mL (D) of bacterial isolates *A. veronii* CECT 4257, *S. flexneri* ATCC 29903 and *E. fergusonii* ATCC 35469 using colloidal chitin as a substrate at different time intervals. Bar represents mean ± SD of three independent experiments in triplicate analysis ($n = 9$).

Growth of the bacteria in terms of cellular protein concentration was determined in the time points shown in Fig. 4C. Between zero time and 48 h, *A. veronii, S. flexneri* and *E. fergusonii* cells grew with an increase in protein biomass by 41.8, 28.11 and 9.8 μg mL^{-1}, respectively. These mean protein values were found to significantly differ between the three strains (F = 5.94; p = 0.0261). Growth was also recorded as net CFU counts during the time course of incubation. As shown in Fig. 4D, *A. veronii* (2.93×10^7 CFU mL^{-1}) had the best growth, followed by *S. flexneri* (2.27×10^7 CFU mL^{-1}), both peaking at 48 h, and then by *E. fergusonii* (0.87×10^7 CFU mL^{-1}) with maximum growth at 24 h. The numbers of culturable bacteria determined by CFU counts were significantly different among the strains (F = 5.91, p = 0.0264).

After ascertaining positive chitinase activity, GlcNAc accumulation in the medium and concomitant growth of the isolated bacteria, we tested for the ability to assimilate the chitin degradation product, GlcNAc. GlcNAc was supplemented as a sole C source in the M9 mineral medium. Within 72 h of incubation, at conditions otherwise standardized for chitinase production, about 1084.9 μg mL^{-1} GlcNAc was utilized by *A. veronii* within 24 h. *Shigella flexneri* utilized 1042.13 μg mL^{-1} within 48 h, while *E. fergusonii* consumed 1031.63 μg mL^{-1} within 48 h (Fig. 5A). Hence, unlike chitin, the net GlcNAc utilization was almost similar among the three strains, though *A. veronii* had the lowest assimilation. The net amount of GlcNAc utilized by each strain did not significantly differ at P > 0.05 (F= 3.34; p = 0.088).

The absolute growth of the three bacteria while consuming GlcNAc as the sole C source was also monitored under the above conditions. As can be seen (Fig. 5B), the net CFU per ml count towards the end of incubation (24 h) was the highest (3×10^7) in the case of *A. veronii*. For *S. flexneri* the count was 2.97×10^7 and for *E. fergusonii* – 2.08×10^7. The mean absolute growth as bacterial cell number during GlcNAc utilization also did not significantly differ between the strains (F = 4.44 and p = 0.0503). Hence, there was not much difference in net growth using GlcNAc in the medium. The corresponding gain in cellular protein content of the cultures towards the end of the growth period also showed a somewhat similar trend with *A. veronii* and *S. flexneri* accumulating similar protein content, whereas *E. fergusonii* accumulated a slight lower amount (no significant difference).

Discussion

In this communication, "size fractionation" of firmly bound vis-à-vis loosely associated bacterial communities sampled from Microcystis blooms displayed differential abilities to utilize chitin. The bacteria that utilized chitin at high rates preferred to colonize within the mucilaginous milieu of planktonic assemblages, and co-filtered in the phytoplankton rich fraction. These populations preferentially grew on TCBS agar with biochemical characteristics typical of Aeromonads (Abbott et al. 2003; Park et al. 2003; Beaz-Hidalgo et al. 2015; Dong et al. 2017). Moreover, upon repeated chitin-subculture, an ability to utilize chitin was short-selected, which gave us isolated chitinophilic bacterium showing phylogenetic placement of *A. veronii* as plausible species.

Interestingly, there was no lineage of any other *Aeromonas* species as described by Beaz-Hidalgo et al. (2015), making it very clear that the given strain is *A. veronii*. It is noteworthy that *A. veronii* contain several genes that potentially encode for chitinases. The substrate specificity of *A. veronii* B565 was determined by Zhang et al. (2014) against various substrates, such as β-1,3-1,4-glucan, colloidal chitin and shrimp-shell. A putative chitinase gene, *ChiCD3*, was identified in *A. veronii* CD3 (Liu et al. 2011). This gene was extensively distributed among the genome-sequence of *A. veronii* CYJ202, *Aeromonas salmonicida* subsp. *salmonicida* A449 and *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966. Another chitinolytic enzyme was

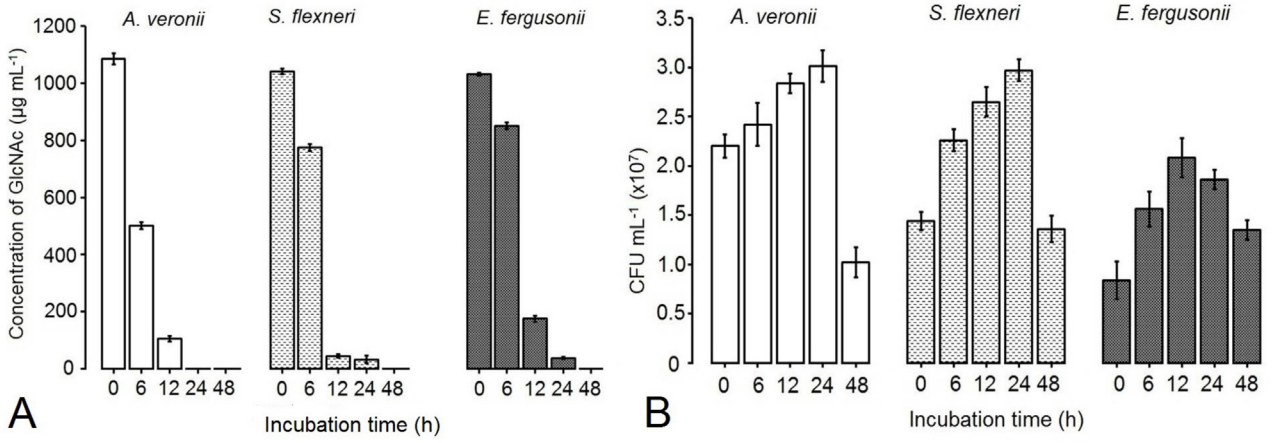

Fig. 5. *N*-acetylglucosamine utilization (A) and CFU per mL (B) of the bacterial isolates *A. veronii* CECT 4257, *S. flexneri* ATCC 29903 and *E. fergusonii* ATCC 35469 in M9-GlcNAc (0.125%) supplemented broth up to indicated incubation period.

characterized in the planktonic bacteria *Aeromonas* sp., *Aeromonas hydrophila* and *Aeromonas salmonicida* with capability to hydrolyse biopolymer (GlcNAc)2 and colloidal chitin (Brzezinska, Donderski 2001).

It was demonstrated that a population of bacteria showing characteristics of *Escherichia* spp. (Farmer et al. 1985; Maheux et al. 2014; Adesina et al. 2019) separated from phytoplankton material in "free-state". It should be noted that *Escherichia* species and *Shigella* species are very similar such that conventional 16S rRNA gene-based phylogenetic analysis cannot distinguish them (Fukushima et al. 2002), and thus in this study, identity of *E. fergusonii* could be confused with that of *Shigella boydii* or other *Shigella* species making an intermixing cluster. Therefore, we also relied on biochemical traits that might distinguish isolated *Escherichia* from the *Shigella* strain (Fig. 2, 3). Among others, one striking difference could be observed for growth on EMB plates. While *E. fergusonii* clearly demonstrated lactose fermentation and thus a green metallic sheen appeared, *S. flexneri* was lactose-non-fermenting or slow fermenting, not expected to produce the sheen, and indeed this was the observation. Perhaps more biochemical and serological data are needed, because most of the modern molecular tools are still not appropriate to differentiate them.

The strain characterized as *E. fergusonii* utilized chitin at rates significantly lower than that of *A. veronii*. Apparently, the nutritional dependence of *Escherichia* on the phytoplankton community is not strong. Chitinase genes and growth on chitin C has rarely been reported in any *Escherichia* spp. It was also reported that *A. veronii* could utilize *Microcyctis aeruginosa* derived EPS and did adhere to the ECM, but *E. coli* failed to establish any such relationship (Weiss et al. 2019).

In the study, *Shigella* was rated as an intermediate class of chitin-utilizing bacterioplankton that fractionated between "particulate- and free-states" (Table 1), indicating that such populations display both firm- and loose adherence. Interestingly, for the present *Shigella* isolated from the particulate fraction of the blooms, chitinase activity was moderate, yet sufficiently high to establish bondage with *Microcystis* colonies. We are yet to examine the chitinase connection in the loosely bound *Shigella* strains.

To conclude, the bacterial population belonging to the "classical" Vibrionaceae family, notably *Aeromonas-Vibrio* due to high chitinophilic activity, demonstrated strong adherence with *Microcystis* blooms, whereas *Escherichia* spp. (Enterobacteriaceae) with subdued enzymes easily released out of the cyanosphere. Strong association of potentially pathogenic bacteria like *Aeromonas, Shigella, Vibrio, Sphingomonas, Pseudomonas* and *Microcystis* spp. (Dziallas, Grossart 2011; Chaturvedi et al. 2015; this study) may have health implications because some of these pathogens may use plankton as a reservoir for propagation in the endemic state (Islam et al. 2020).

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