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Characterization of quorum sensing system in *Clostridium chauvoei*

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ABSTRACT

Clostridium chauvoei causes fatal black quarter infection in cattle and buffaloes. The quorum sensing (QS) system, a bacterial cell to cell communication process, of the pathogen was characterized in the current study. The results indicated that *C. chauvoei* lacked *luxS* (autoinducer-2) based quorum sensing as detected by the sensor strain *Vibrio harveyi* BB170. This was supported by absence of *luxS* gene in *C. chauvoei* genome. However, the genomic analysis indicated the presence of *agrBD* system in all three genomes of *C. chauvoei* available at the NCBI database. The AgrD, which synthesizes QS messenger auto-inducing peptide, was a 44 amino acid protein which shared 59% identity and 75% similarity with AgrD of *C. perfringens* strain 13 and 56% identity (20% coverage) with *Staphylococcus aureus* N315. The functional cysteine amino acid was conserved in all the strains. The genomic organisation further suggests the presence of diguanylate cyclase, a gene responsible for synthesis of secondary messenger cyclic di-GMP, at 3' immediate downstream of *agrD* gene. The real time expression analysis for *agrD* gene indicated that expression was better at 37 °C (1.9–3.7 fold increase) compared to a higher temperature of 40 °C. However, stable expression was observed at different growth stages (log and early stationary phase) with 0.8–1.4 fold changes in expression pattern. The results indicate the presence of a constitutively expressed *agrBD* quorum sensing system in *C. chauvoei*.

1. Introduction

Clostridium chauvoei, a Gram-positive, spore forming, anaerobic bacterium, is the causative agent of fatal blackleg disease. The disease is characterized by sudden death, inflammation of skeletal and cardiac muscles, toxemia and high mortality amongst cattle, sheep and other ruminants. In humans, *C. chauvoei* has been reported to be involved in fulminant gas gangrene [1] and neutropenic enterocolitis [2]. *C. chauvoei* secretes many exotoxins, including α toxin (oxygen stable haemolysin), β toxin (DNase), γ toxin (hyaluronidase), δ toxin (oxygen labile haemolysin), neuraminidase (sialidase) and *C. chauvoei* toxin A (CctA) [3].

Quorum sensing (QS) is a bacterial cell to cell communication process. It involves secretion of extracellular signaling molecules called autoinducers (AIs). Common classes of QS molecules include N-acyl homoserine lactone or AI-1 in Gram-negative bacteria, accessory gene regulator (*agr*) mediated oligopeptide in Gram-positive bacteria, and autoinducer-2 (AI-2) in both Gram-negative and Gram-positive bacteria [4,5]. Bacteria use quorum sensing for biofilm formation, secretion of virulence factors, antibiotic production, bioluminescence, sporulation and transfer of genetic material [5,6].

The *luxS* gene synthesizes AI-2 signal in a wide range of Gram-negative and Gram-positive bacteria [4,5]. Among clostridial pathogens, the AI-2 signal has been detected in *C. perfringens* [7], *C. botulinum* [8,9] and *C. difficile* [10,11]. The *luxS* mutant in *C. perfringens* showed a reduced production of alpha (phospholipase and sphingomyelinase activity), kappa (collagenase) and theta (oxygen labile hemolysin) toxins [7].

Extensive study on the peptide-based signaling system, called

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agr, has been done in *Staphylococcus aureus* [12]. A homolog of *S. aureus agrBD* has been characterized in many *Clostridium* species, such as *C. perfringens* [13–17] and *C. difficile* [18]. In *S. aureus*, *agrD* gene encodes precursor oligopeptide AgrD, which is processed to a final autoinducing peptide (AIP) by an AgrB-dependent mechanism [12]. The AIP bind and activate AgrC, which in turn activates AgrA by phosphorylation [5]. The activated AgrA activates the expression of regulatory RNAIII resulting in toxins secretion [5] and expression of *agrBDCA* operon, which switch the entire population into high density quorum sensing mode [19]. In clostridial species, *agr* system regulates sporulation [8,15,20], and production of several toxins, such as neurotoxin in *C. botulinum* [8], alpha, kappa and theta toxins in *C. perfringens* type A [13], epsilon toxin in *C. perfringens* type D [14]and toxin production and colonization in *C. difficile* [18]. However, no information is available regarding the quorum sensing system in *C. chauvoei*. In the present study, hence, we characterized the quorum sensing system in *C. chauvoei* using sensor strain *Vibrio harveyi* BB170 and genomics approach.

2. Materials and methods

2.1. Strains, media and culture conditions

C. chauvoei strain ATCC 10092 was used for growth kinetics and expression analysis of quorum sensing gene *agrD*. The primers used in the present study are presented in Table 1. The strain ATCC 10092 was cultured in ATCC® Medium 2107 (modified reinforced clostridial agar/broth medium pre-reduced, from HiMedia, India) and incubated at 37 °C under anaerobic conditions. The bacterium was identified by 16–23s rRNA spacer PCR [21]. *Vibrio harveyi* BB170 was used as the sensor strain for detecting autoinducer-2 and *V. harveyi* BB120 was used as a positive control. These bacteria were routinely grown on Zobell marine agar (HiMedia, India) at room temperature.

2.2. Growth kinetics study

A 10 ml aliquot of ATCC medium 2107 in serum vial was inoculated with 0.5 ml of a 12 h broth culture of *C. chauvoei* and incubated under anaerobic condition at 37 °C and 40 °C. The samples were collected in duplicates at 2 h intervals during 2–16 h (log to early stationary phase) and at 8 h intervals during 16–48 h (late stationary phase). The absorbance at 600 nm was recorded using a spectrophotometer (Lab India, UV3000). The generation time was calculated as per Powell [22].

2.3. Quantification of autoinducer 2 (AI-2)

Clostridium chauvoei culture was collected at 8 h, 12 h and 24 h, and centrifuged at 6000 × g for 10 min at 4 °C. The supernatant was filtered (0.45 μm filter) and preserved at −60 °C till further use. The AI-2 was quantified using the sensor strain *V. harveyi* BB170 by the method of Bassler and coworkers [23,24]. Briefly, a single colony of the sensor strain *V. harveyi* BB170 was transferred to 10 ml autoinducer bioassay medium [25]. The culture was incubated overnight at 28 °C, 100 rpm till absorbance at 600 nm reached 1.0. A 10 μl sensor strain culture was transferred to 50 mL fresh autoinducer bioassay medium resulted in 1: 5000 dilution. A 180 μl of diluted sensor strain was incubated with 20 μl cell-free culture supernatant of *C. chauvoei*. *V. harveyi* BB120 cell-free culture supernatant was used as a positive control. Background luminescence (negative control) was measured by replacing culture supernatant with 20 μl sterile ATCC 2107 medium. Plate was covered with aluminium foil and incubated at 37 °C for 3 h at constant shaking of 100 rpm. The luminescence was measured using spectra Max M5 plate reader (Molecular Devices, USA). All the samples were run in triplicate. The autoinducer was quantified as fold change in luminescence over negative control using the formula:

$$\text{Autoinducer signal} = \frac{\text{Luminescence produced by sensor strain in presence of cell free culture supernatant}}{\text{Luminescence produced by sensor strain with sterile medium}}$$

2.4. Detection of agrBDCA system and luxS in C. chauvoei

Genome sequences of *C. chauvoei* strains ATCC 10092, JF4335 and 12S0467 were downloaded from NCBI database and analyzed for quorum sensing genes *luxS* and *agrBDCA* system. The *agrB*, *agrD* and *luxS* genes of *C. perfringens* strain 13 and *agrBDCA* operon of *S. aureus* N315 were also downloaded from NCBI database. *C. chauvoei* genomes were re-annotated using Prokka [26] and RAST [27] programs. These annotations were manually curated to find various components of *agr* systems. The putative genes were further searched using HMMER [28], Pfam [29] and NCBI BLASTP programs [30]. The percentage similarity and identity were calculated against the genes of *C. perfringens* strain 13 and *S. aureus* N315. These proteins were further characterized for presence of signal peptide, transmembrane helix and cellular localisation by SignalP [31], TMHMM [32] and PSORTb [33]. Multiple sequence alignments of Agr proteins were carried out using T-Coffee [34] and conserved

Table 1
Primers used for characterisation and expression analysis.

Target gene	Primer sequence	Amplicon size (bp)	References
Characterisation of *C. chauvoei*			
16-23S rRNA	IGCSC (F): GAAATTGCACATGAATTAAA 23UPCH (R): GGATCAGAACTCTAACCTTTCT	522	[21]
cctA	F: CGCGAACAGATTGGAGGTATAAAAAGAATATTAATGCTT R: GTGGCGGCCGCTCTATTAATCATTAAAACGATTATATTC	983	Unpublished lab data
agrD	14F: AGACATTTAGGTTCTTCTGGTTG 116R: AGGTATTAATGGCTGTAGCTG	103	This study
recA	391F: GCAGAGGCTTTAGTACGTTCAG 512R: CTTGCTTGAAGACCTACGTGAG	122	This study

substitution was generated using Boxshade server of Swiss Institute of Bioinformatics (https://embnet.vital-it.ch/software/BOX_form.html). The neighbor-joining phylogenetic tree was constructed at 1000 bootstrap iterations using MEGA6 [35].

Based on the *in silico* results, primers were designed for *agrD* gene at Primer3Plus server [36]. The presence of *agrD* gene in *C. chauvoei* ATCC 10092 was verified by PCR. The 25 μl reaction mixture consisted of 2.5 μl 10X buffer, 1 μl each of forward and reverse primers, 0.5 μl dNTPs, 0.5 μl $MgCl_2$, 2.5 U Taq DNA polymerase, 18 μl nuclease free water and 1 μl of 10 ng DNA template. The PCR assay conditions were initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension of 72 °C for 30 s. Final extension was carried out at 72 °C for 10 min. The amplified product was visualized and photographed using gel documentation system (Bio-Rad, USA).

2.5. RNA extraction from C. chauvoei and cDNA synthesis

The samples were collected at 8 h (log phase) and 12 h (early stationary phase) at two different temperatures (37 and 40 °C) in ATCC medium 2107. The RNA was extracted from duplicate samples as per Bakker and coworkers [37] with minor modifications. Briefly, 4.5 ml of *C. chauvoei* culture was pelleted, washed with PBS and the pellet was mixed with 200 μl of freshly prepared lysis solution (200 mM tris-HCl pH 7.0, EDTA 100 mM, lysozyme 10 mg/ml). After 10 min incubation at 37 °C, added 1 mL Aquazol, mixed vigorously and incubated for 5 min at room temperature. The phase was separated by adding 200 μL ice cold chloroform. After 5 min, the suspension was centrifuged at 12,000 rpm at 4 °C for 10 min. A 500 μL upper aqueous phase was transferred to a new Eppendorf tube. The RNA was precipitated using 2.5 vol ice cold ethanol and the pellet was washed twice with 70% ethanol. After a brief air drying, the pellet was dissolved in nuclease free water. The RNA concentration was measured in a Nanophotometer (Eppendorf, Germany). The quality of RNA was checked by the method of Aranda et al. [38] using bleach gel technique. Briefly, the bleach solution having 4% sodium hypochlorite (HiMedia, India) was used for treating agarose solution and gel casting apparatus. A 1.2% agarose (w/v) gel was prepared in 1 × TAE buffer (tris acetate 40 mM, EDTA 1 mM, pH 8.2) with 1% bleach (1 ml bleach solution in 99 ml TAE buffer). To allow the complete degradation of RNase, the solution was mixed intermittently for 6 min, before boiling. The gel was casted in tray pre-treated with 1% bleach for 1 h. After running the gel at 100 V/cm for 45 min, the gel was visualized on Bio-Rad Gel documentation system.

A quantified fraction of extracted RNA (10 μg) was subjected to in-solution DNase treatment using DNase I (Machery Nagel, Germany) as per manufacturer's protocol. The efficacy of DNase treatment was checked using 16S rDNA PCR with genomic DNA as positive control and nuclease free water, in place of DNA or RNA, as negative control. The RNA concentration was measured in a Nanophotometer (Eppendorf, Germany). The cDNA was synthesized using 1 μg of DNase I treated RNA by Revert Aid first strand synthesis kit (Thermo Scientific) using manufacturer's protocol.

2.6. Real time expression analysis

Primers used for expression analysis of quorum sensing gene *agrD* and reference gene *recA* were designed at Primer3Plus server [36]. The primers characteristics were further assessed by oligoanalyzer [39]. The real time qPCR expression analysis was carried out in duplicates using Maxima SYBR Green/ROX qPCR Master Mix (2 ×) (Thermo scientific) in Step-one Real-time PCR system (Applied BioSystems) using manufacturer's protocol with slight modifications. Briefly, the 10 μl reaction mixture consisted of 5.0 μl master mix, 0.4 μl each of forward and reverse primers, 2.0 μl cDNA (20 ng) and 2.2 μl nuclease free water. The real-time PCR cycle parameters were as follows: initial denaturation for 30 s at 95 °C, followed by 40 cycles of denaturation for 10 s at 95 °C, and annealing and extension for 30 s at 60 °C. Fluorescence measurement was taken after each annealing step. A melting curve analysis was performed from 60 to 95 °C to detect potential nonspecific products. The assay conditions were as follows: initial denaturation for 15 s at 95 °C, followed by 40 cycles of annealing for 1 min at respective annealing temp, and denaturation for 15 s at 95 °C. The PCR efficiency was calculated by running the real time PCR reaction at three DNA template concentration viz., 1 ng, 10 ng and 100 ng. The PCR reaction efficiency was calculated using formula $10^{-1/\text{slope}} - 1$ [40]. The relative quantification was carried out using *recA* as reference gene. The data on expression was analyzed at 40 °C with respect to lower temperature 37 °C and in early stationary phase with respect to late log phase using $2^{-\Delta\Delta CT}$ method [41].

2.7. Statistical analysis

Growth kinetics of *C. chauvoei* and expression analysis of *agrD* gene were carried out in duplicates at 37 °C and 40 °C. The growth at different temperatures was analyzed at each sampling time by paired T-test. The expression analyses of *agrD* gene and AI-2 production were carried out using two-tailed Student's T-test and analysis of variance (ANOVA), respectively. Level of significance was declared at $p < 0.05$) and $p < 0.01$). Before analysis, data were checked for normality by probability plots and homogeneity of variances by Levene's test. All analyses were performed using statistical software package SAS v.9.3 program (SAS Institute, Cary, NC, USA).

3. Results

3.1. Growth kinetics study of C. chauvoei

Growth kinetics study indicated that *C. chauvoei* remained under log phase from 4 to 12 h and early stationary phase started by 12 h (Fig. 1). By late log to early stationary phase, *C. chauvoei* growth was higher by 0.3 (absorbance at 600 nm) at 37 °C compared to 40 °C with significantly higher growth ($p < 0.05$) observed at all the sampling times. The generation time of the bacterium was 91.5 min and 90.8 min at 37 °C and 40 °C, respectively.

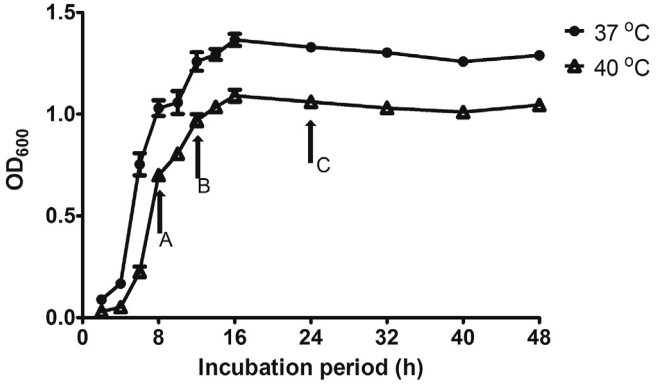

Fig. 1. Growth kinetics of *C. chauvoei*. A. Late log phase; B. Early stationary phase; C. Mid to end stationary phase. A significant difference ($p < 0.05$) in growth was observed at each time point between 37 °C and 40 °C.

3.2. Quantification of autoinducer 2

We observed that *C. chauvoei* did not produce AI-2 signal molecule as no significant difference in AI-2 level was observed between *C. chauvoei* and negative control (data not shown). Moreover, BLASTP analysis did not find LuxS protein homolog in three isolates of *C. chauvoei* (ATCC 10092, JF4335 and 12S0467).

3.3. Confirmation of agr-based quorum sensing in C. chauvoei

The PCR amplification with primers specifically designed for *agrD* gene produced a 103 bp targeted PCR product in *C. chauvoei* ATCC 10092 (data not shown). The *agr* system was genetically characterized against known *agr* system of *C. perfringens* and *S. aureus* (Table 2). The *in silico* analysis of *C. chauvoei* strains ATCC 10092, JF4335 and 12S0467 revealed the presence of *agrBD* locus. The autoinducing pro-peptide of 44 amino acids was similar to *C. perfringens* strain 13 (44 amino acids) and *S. aureus* N315 (47 amino acids). Overall, the AgrD protein shared 100% coverage, 59% identity and 75% similarity with AgrD of *C. perfringens* and 20% coverage, 56% identity with *S. aureus* (Table 2). Interestingly, the functional cysteine amino acid and neighbouring alanine amino acids in mature autoinducing peptide were conserved in all the three species (Fig. 2A). A high level of divergence between AgrD of *Clostridium* species and *S. aureus* was reflected by phylogenetic analysis (Fig. 3A).

The analysis of sequenced genomes of *C. chauvoei* indicated that it encodes 212 amino acid AgrB protein compared to 214 and 187 amino acid proteins in *C. perfringens* and *S. aureus*, respectively. The AgrB of *C. chauvoei* showed 43% identity and 66% similarity with *C. perfringens* and only 31% identity (20% coverage) with *S. aureus* (Table 2). Further, the functional amino acid required for endopeptidase activity of AgrB was conserved at 77 (histidine) and 84 (cysteine) positions in all the three species (Fig. 2B). The prediction by Hidden Markov model suggests the presence of 4–5 transmembrane helix with its localisation in cytoplasmic membrane in *Clostridium* and *Staphylococcus aureus* (Table 3).

The genomic organisation of *agr* operon in *C. chauvoei* and *C. perfringens* indicated that they have identical organisation with two putative genes located 5' upstream of *agrB* and diguanylate cyclase at downstream of *agrD* (Fig. 4). Though, these two hypothetical proteins have not yet been experimentally characterized, the BLASTP analysis suggests their similarity to AgrC and AgrA proteins of *S. aureus* (Table 2). This is supported from multiple sequence alignments (Supplementary Fig 1). The putative AgrA protein in *C. chauvoei* had DUF 5317 domain with four transmembrane helix regions and made up of 181 amino acids in comparison to 238 amino acids in *S. aureus*. On the contrary, the putative protein showing similarity to AgrC is highly truncated with similarity restricted to N-terminal (Supplementary Fig 2).

3.4. Expression analysis of quorum sensing genes

The bleached agarose gel electrophoresis after DNase treatment reflects the presence of three discrete bands in all the RNA samples (Fig. 5A). The reference gene *recA* had stable expression across temperature and culture duration (Ct, 19.9 to 21.7). The PCR efficiency of *agrD* gene was 103%. The expression analysis of *agrD* indicates that its expression is significantly higher at 37 °C ($p < 0.05$) compared to higher temperature 40 °C with 2.1-and 3.7-fold higher expression at log ($p = 0.08$) and early stationary phases ($p = 0.06$), respectively (Fig. 5B). However, no significant difference ($p > 0.05$) in expression of *agrD* was observed between log to early stationary phase.

4. Discussion

Autoinducer 2 is considered as universal quorum sensing signal in bacteria [42]. It plays a significant role in inter-species and intergeneric communications and has been reported in several Gram-negative and Gram-positive bacteria [43]. The *luxS* synthase synthesizes quorum sensing signaling molecule AI-2 [42]. Several clostridia, such as *C. perfringens* [7], *C. botulinum* [9] and *C. difficile* [10] have the *luxS* signaling system. In the present study, the analysis indicated that *C. chauvoei* did not produce AI-2 signal as the level of AI-2 in *C. chauvoei* supernatant did not differ significantly with negative control. Further, the genome search indicated the absence of *luxS* gene in *C. chauvoei*. The genome search in many other pathogenic clostridia, such as *C. septicum, C. tetani, C. novyi* and *C. haemolyticum,* showed absence of *luxS* gene (data not shown). The reason behind absence of *luxS* gene-based quorum sensing in several clostridial species is not yet clear.

Autoinducing peptide (AIP) serves as a cell signaling molecule in Gram-positive bacteria [13,14,44]. The *agrBDCA* locus has been

Table 2
BLASTP analysis of Agr proteins in *Clostridium chauvoei*.

C. chauvoei strain	Strand	Accession number	Gene location	*Number of amino acid	*C. perfringens* strain 13				*Staphylococcus aureus* N315			
					Coverage	Identity (%)	Similarity (%)	E value	Coverage	Identity	Similarity	E value
AgrB												
ATCC10092	+	WP_079481528.1	BTM21_RS02620	212	98	43	66	3e-46	20	31	52	5e-04
JF4335	–	SLK20774.1	CCH01_19680	212	98	43	66	3e-46	20	31	52	5e-04
12S0467	–	ATD55624.1	BTM20_10405	212	98	43	66	3e-46	20	31	52	5e-04
AgrD												
ATCC10092	+	WP_021876277.1	BTM21_RS02625	44	100	59	75	8e-12	20	56	66	0.036
JF4335	–	SLK20773.1	CCH01_19670	44	100	59	75	8e-12	20	56	66	0.036
12S0467	–	ATD55623.1	BTM20_10400	44	100	59	75	8e-12	20	56	66	0.036
**Putative AgrC												
ATCC10092	+	WP_021876279.1	BTM21_RS02615	155	98	42	67	3e-44	53	27	55	0.35
JF4335	–	SLK20776.1	CCH01_19690	155	98	42	67	3e-44	53	27	55	0.35
12S0467	–	ATD55625.1	BTM20_10410	155	98	42	67	3e-44	53	27	55	0.35
**Putative AgrA												
ATCC10092	+	WP_079481529.1	BTM21_RS02610	181	97	53	74	4e-68	40	28	55	0.20
JF4335	–	SLK20778.1	CCH01_19700	181	97	53	74	4e-68	40	28	55	0.20
12S0467	–	ATD55626.1	BTM20_10415	181	97	53	74	4e-68	40	28	55	0.20

*The no. of amino acids for different agr proteins in *C. perfringens* strain 13 and *S. aureus* N315 were 214 and 187 (agrB), 44 and 47 (agrD), 153 and 427 (agrC), and 188 and 238 (agrA) respectively.
**Putative AgrC and putative AgrA have not been experimentally characterized in *C. perfringens*.

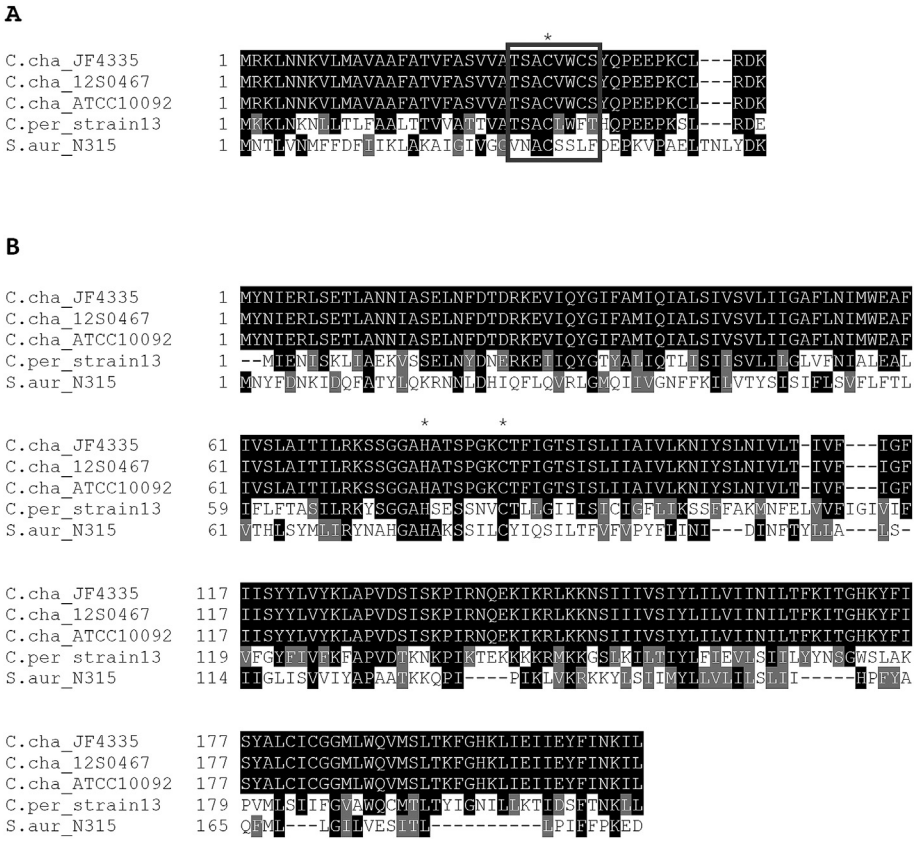

Fig. 2. Multiple sequence alignment of AgrD protein (A) and AgrB protein (B) of *C. chauvoei* with *C. perfringens* and *S. aureus*. The box in agrD is showing thiolactone ring with functional conserved cysteine amino acid. The conserved histidine and cysteine amino acids are active sites in agrB protein.

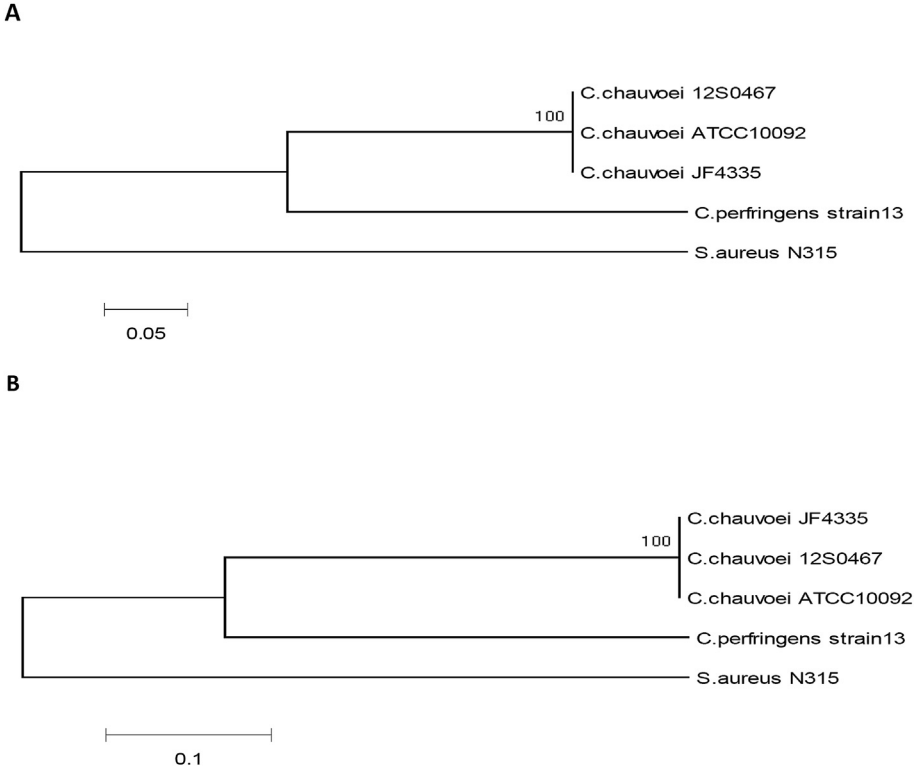

Fig. 3. Phylogenetic analysis of AgrD protein (A) and AgrB protein (B) of *C. chauvoei* strains with *C. perfringens* and *S. aurues*. Both agrD and agrB proteins were 100% identical in the three isolates of *C. chauvoei*.

Table 3
Characterisation of agr proteins.

Bacterial Strains*	Protein ID	Locus tag	No. of amino acid	Transmembrane helix (TMHMM)	Localisation (pSORTB)	Signal peptide (SignalP)
AgrD pro-peptide						
C. chauvoei JF4335	SLK20773.1	CCH01_19670	44	1	CM	Yes (between 29 and 30 AA)
C. perfringens strain 13	WP_003449588.1	CPE_RS08070	44	1	Unknown	Yes (between 29 and 30 AA)
S. aureus N315	WP_001094921.1	SA_RS10575	47	0	CM	No
AgrB						
C. chauvoei JF4335	SLK20774.1	CCH01_19680	212	5	CM	No
C. perfringens strain 13	WP_003455887.1	CPE_RS08075	214	5	CM	No
S. aureus N315	WP_001105696.1	SA_RS10570	187	4	CM	No
Putative AgrC						
C. chauvoei JF4335	SLK20776.1	CCH01_19690	155	4	CM	No
C. perfringens strain 13	WP_003449794.1	CPE_RS08080	153	4	CM	No
S. aureus N315	WP_001800932.1	SA_RS10580	427	6 (N-terminal)	CM	No
Putative AgrA						
C. chauvoei JF4335	SLK20778.1	CCH01_19700	181	4	Unknown	No
C. perfringens strain 13	WP_003449620.1	CPE_RS08085	188	4	CM	No
S. aureus N315	WP_000688492.1	SA_RS10585	238	0	Cytoplasmic	No

CM, Cytoplasmic membrane.
All the four agr proteins (agrB, agrD, putative agrC and putative agrA) are 100% conserved among *C. chauvoei* isolates. Hence, *C. chauvoei* JF4335 are representative.

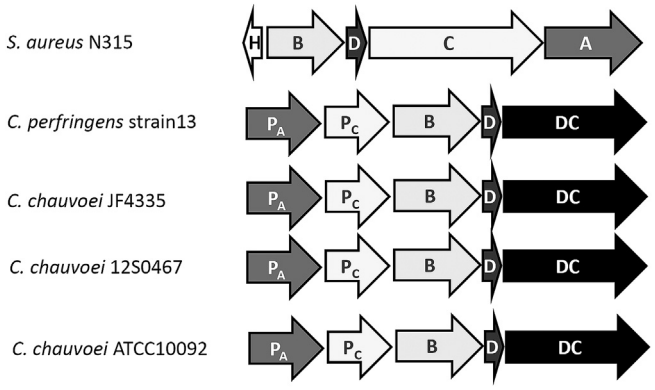

Fig. 4. Agr gene organisation in *C. chauvoei* with respect to *S. aureus* and *C. perfringens*. A:*agrA*; B:*agrB*; C:*agrC*; D:*agrD*; H: Delta haemolysin; DC: Diguanylate cyclase; P_A: Putative *agrA*; P_C: Putative *agrC*. *agrA* and *agrC* in *C. chauvoei* and *C. perfringens* have not yet been characterized experimentally. Diguanylate cyclase is responsible for synthesizing secondary messenger cyclic di-GMP.

well-characterized in *S. aureus* [19]. The *agrD* is the structural gene, which synthesizes AIP. The latter is secreted as pro-peptide and modified by AgrB to be secreted as mature AIP of eight amino acids [12,19]. In variance with *agrBDCA* operon of *S. aureus*, *agrBD* locus has been reported in *C. perfringens* strain 13 [13]. In the present study, the genomic analysis of *C. chauvoei* strains ATCC 10092, JF4335 and 12S0467 revealed the presence of *agrBD* locus. The *in silico* analysis further indicated the presence of autoinducing pro-peptide of 44 amino acids. It slightly differed from autoinducing pro-peptide of *S. aureus*, which has 47 to 56 amino acids [19,44]. It has been earlier reported that cysteine amino acid at 5th residues forms thiolactone bond, which is crucial for autoinducer function [44,45]. Therefore, conserved cysteine residue and presence of high identity with *C. perfringens* AIP signal indicates the presence of AIP signal in *C. chauvoei*.

The production of AIP is dependent on catalytic activity of AgrB protein. The catalytic active site on AgrB is located at 77th (histidine) and 84th (cysteine) residues with complete abolition of activity by a substitution of either amino acid [46]. In the present study, these two amino acids were conserved in all the three species. Further, the protein was more conserved at N-terminal compared to C-terminal, the later often provide group specificity [5]. Contrary to *agrBDCA* operon of *S. aureus*, *agrBD* operon has been experimentally characterized in *C. perfringens*. The genomic organisation of *C. chauvoei* and *C. perfringens* suggests the presence of two hypothetical genes at 5' upstream of *agrBD* operon (Fig. 4) with high level of identity specially noticed in AgrA protein (Table 2, supplementary Fig 1). In *agr* based quorum sensing system, the role of AgrA protein is crucial as it activates the expression of regulatory RNAIII in *S. aureus* resulting in toxins secretion [5]. However, the role of these putative proteins needs to be validated.

Many bacteria use intracellular cyclic di-GMP as a signaling molecule, which is synthesized by two molecules of GTP by diguanylate cyclase [47]. It has been reported that quorum sensing system in *V. cholerae* activates diguanylate cyclase and promotes biofilm formation [48]. The genomic analysis of *C. chauvoei* as well

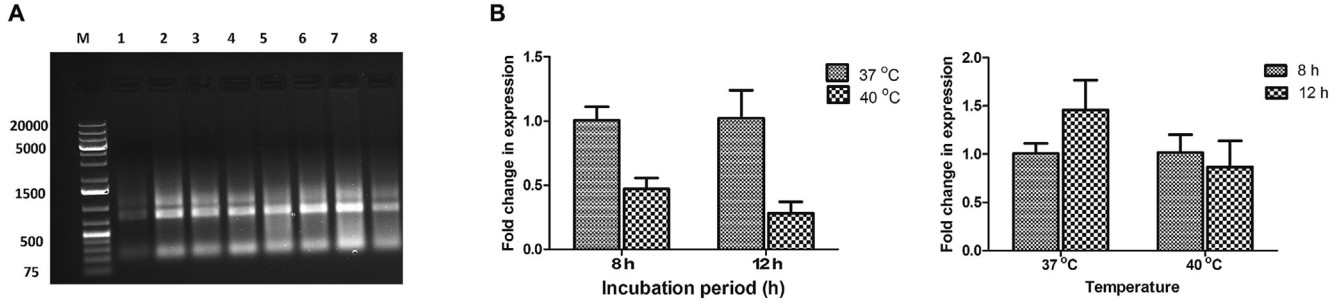

Fig. 5. Expression analysis of quorum sensing genes. A. Agarose gel electrophoresis of RNA extracted from *C. chauvoei* grown at different growth conditions. Lane M: 1 Kb DNA marker; Lane 1 & 2: 8 h (37 °C); Lane 3 & 4: 12 h (37 °C); Lane 5 & 6: 8 h (40 °C); Lane 7 & 8: 12 h (40 °C). B. Expression analysis of quorum sensing gene (*agrD*) in *C. chauvoei* grown at different growth conditions.

as *C. perfringens* suggests the presence of diguanylate cyclase in the immediate vicinity of *agr* operon. Though, it is purely speculative, we suspect that these two messenger systems, *viz., agr* and cyclic di-GMP are interacting and playing role in virulence of *C. chauvoei*.

C. chauvoei is an obligate anaerobic pathogen. Genomic analysis indicates that the bacteria lack ability to synthesize some of the vital amino acids such as arginine, histidine, leucine, glutamate and aspartate [3,49]. As the target hosts of *C. chauvoei*, such as cattle and buffaloes, have body temperature higher than 37 °C, a marginally higher temperature (40 °C) was evaluated for growth kinetics and *agrD* expression. The results indicated that growth as well as expression of quorum sensing autoinducer signal *agrD* had marked reduction at higher temperature 40 °C.The work on *C. difficile* has revealed that their toxins A and B were best expressed at 37 °C and were reduced at 42 °C [50]. Similarly, Mattar et al. [51] reported that 37 °C was the ideal temperature for DNase production in *C. septicum*. Overall, our findings indicate the presence of *agrBD* quorum sensing system in *C. chauvoei*. However, its role in pathogenicity needs to be investigated.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.anaerobe.2018.06.006.

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