

RESEARCH

Open Access



An RpoN-dependent PEP-CTERM gene is involved in floc formation of an *Aquincola tertiaricarbonis* strain

Ming Xia^{1,2}, Dianzhen Yu^{3,4}, Han Chen^{3,5}, Jingcheng Dai⁵, Na Gao⁶, Shuyang Li⁵, Xuezhi Bi⁷ and Dongru Qiu^{5*}

Abstract

Background The floc is a characteristic of microbial aggregate growth, displaying cloudy suspensions in water. Floc formation has been demonstrated in a series of bacteria and the floc-forming bacteria play a crucial role in activated sludge (AS) process widely used for municipal sewage and industrial wastewater treatment over a century. It has been demonstrated that some exopolysaccharide biosynthesis genes and the sigma factor (*sigma54* or *rpoN*) were required for floc forming in some bacteria. However, the mechanism underlying the floc formation stills need to be elucidated.

Results In this study, we demonstrate that a TPR (Tetratricopeptide repeats) protein-encoding gene *prsT* is required for floc formation of *Aquincola tertiaricarbonis* RN12 and an upstream PEP-CTERM gene (designated *pepA*), regulated by RpoN1, is involved in its floc formation but not swarming motility and biofilm formation. Overexpression of *PepA* could rescue the floc-forming phenotype of the *rpoN1* mutant by decreasing the released soluble exopolysaccharides and increasing the bound polymers.

Conclusion Our results indicate that the wide-spread PEP-CTERM proteins play an important role in the self-flocculation of bacterial cells and may be a component of extracellular polymeric substances required for floc-formation.

Keywords Bacterial floc formation, PEP-CTERM proteins, Flocculation, RpoN sigma factor, *Aquincola tertiaricarbonis*

Background

It is long known that the activated sludge bacteria including *Zoogloea*, *Thauera* and *Pseudoduganella* species are capable of forming flocs in which bacterial cells are self-flocculated by an extracellular matrix composed of secreted extracellular polymeric substances (EPS) including exopolysaccharides and other biopolymers [1, 2]. Another floc-forming bacterium, *Aquincola tertiaricarbonis* RN12, was isolated from a Chinese water supply well in which nuisance brown precipitates frequently occurred and cause public concerns among the local inhabitants [3]. Certain *Aquincola* strains are capable of degrading gasoline-related contaminants [4–7]. In addition, *Aquincola* is one of the predominant proteobacteria in a membrane bioreactor (MBR) treating antibiotics-containing wastewater [8]. Since the RN12 strain could readily form flocculent precipitates under laboratory

*Correspondence:

Dongru Qiu
qiu@ihb.ac.cn

¹ School of Life Sciences, Hubei Engineering Research Center for Protection and Utilization of Special Biological Resources in the Hanjiang River Basin, Jiangnan University, Wuhan 430056, China

² Hubei Key Laboratory of Environmental and Health Effects of Persistent Toxic Substances, Jiangnan University, Wuhan 430056, China

³ University of Chinese Academy of Sciences, Beijing 100049, China

⁴ Institute for Nutritional Sciences, SIBS, Chinese Academy of Sciences, Shanghai 200031, China

⁵ Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, Hubei Province, China

⁶ Fisheries Research Institute, Anhui Academy of Agricultural Sciences, Hefei 230031, China

⁷ Bioprocessing Technology Institute, Agency for Science, Technology and Research, Singapore 138668, Singapore



© The Author(s) 2022. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

cultivation conditions and could be manipulated genetically, we have used this strain as a model to study the mechanisms underlying the formation of brown precipitates found in a water supply well.

It is believed that bacterial flocs may confer bacterial resistance to the predation of protozoa and other invertebrates [9] and may also be related to other important traits such as denitrification and nitrogen fixation. We have conducted genome sequence and annotation, as well as molecular genetics analysis on the floc-formation of *A. tertiaricarbonis* RN12 strain [3]. A large gene cluster for EPS biosynthesis and a gene encoding the alternative sigma factor RpoN1, one of the four paralogues, are required for the floc-formation of *A. tertiaricarbonis* RN12 strain [3]. The bacterial cells of the isolated mutants are planktonic rather than flocculated. Interestingly, the biosynthesis of exopolysaccharides remained in the rpoN1-disrupted mutant, but most of the EPS was released and dissolved in the culture broth rather than bound to the bulk of cells [3]. Consistently, the transcription of exopolysaccharide biosynthesis genes seems not to be regulated by RpoN1 [3]. These results indicate that RpoN1 may regulate the expression of certain gene(s) involved in the self-flocculation of bacterial cells but not in the biosynthesis and secretion of EPS required for floc-formation.

By using phylogenetic profiling methods, a family of special proteins, termed PEP-CTERM domain containing proteins were found and described as a series of proteins that have a short C-terminal homology domain, including a conserved Pro-Glu-Pro (PEP) motif, and a predicted N-terminal signal peptide for secretion and sorting [10]. All the bacteria encoding these putative PEP-CTERM proteins belong to the gram-negative bacteria with an outer membrane and encoding a gene cluster for production of exopolysaccharide. However, the possible relationships between these proteins and EPS remained largely unknown [10]. Interestingly, a TPR protein-encoding gene (*prst*) is frequently present around the PEP-CTERM domain protein gene, whose functions remained to be further studied [10].

In the present study, we have isolated and analyzed more transposon mutants defective in floc formation. It has been shown that a PEP-CTERM gene is regulated by the global transcription regulator RpoN1 and could mediate the floc formation. These results provide insights into the regulation of floc formation and the underlying mechanisms in *Aquicola*, *Zoogloea* and other floc-forming bacteria.

Table 1 General characteristics of the *Aquicola tertiaricarbonis* RN12 strain genome

	chromosome 1	chromosome 2	plasmid A	plasmid B
Genome size (bp)	3,831,859	5,141,032	129,637	32,782
GC content (%)	69.34	69.52	65.83	59.62
Number of rRNAs	6	3	0	0
Number of tRNAs	40	11	0	0
Number of protein coding genes	3541	2808	127	30

Results and discussion

Complete genome of *Aquicola tertiaricarbonis* RN12 strain

Both Illumina Hiseq and Pacific Biosciences platforms were used to complete the whole genome sequencing of *Aquicola tertiaricarbonis* RN12 strain. Four double stranded circular DNAs, including two chromosomes, termed chromosome 1 and chromosome 2, and two plasmids, the plasmid A (pA) and the plasmid B (pB), were identified (Table 1 and Fig. 1). The size of chromosome 1 was 3,831,859 base pairs (bp) and the G + C content was 69.34%, while the number of rRNAs operons, tRNAs and protein-coding genes were 6, 40, and 3541, respectively. On the other hand, the size of chromosome 2 was 5,141,032 bp, and the G + C content was 69.52%, while the number of rRNAs operons, tRNAs and protein-coding genes were 3, 11, and 2808, respectively. The plasmid A was 129,637 bp in size with the G + C content of 65.83%, while the plasmid B was 32,782 bp in size with the G + C content of 59.62%. The whole genome of this bacterium was 7,110,107 bp in size. Interestingly, the bacteriochlorophyll biosynthesis, light-harvesting proteins and the photosynthetic reaction center related genes were encoded on the chromosome 2, suggesting that the *A. tertiaricarbonis* RN12 strain might have originally been of the photosynthetic ability by itself, which was lost during bacterial evolution and adaptation to specific habitats as consequence of spontaneous mutations and/or transposon/insertion sequence insertional inactivation of certain photosynthesis related genes. Either chlorophyll synthesis or photosynthesis was not observed under light cultivation. It may be that the aerobic anoxygenic photosynthesis (AAP) was not occurred under the energy excess conditions [11]. Similarly, a large exopolysaccharide biosynthesis and secretion gene cluster, similar to that of another floc-forming strain *Zoogloea resiniphila*

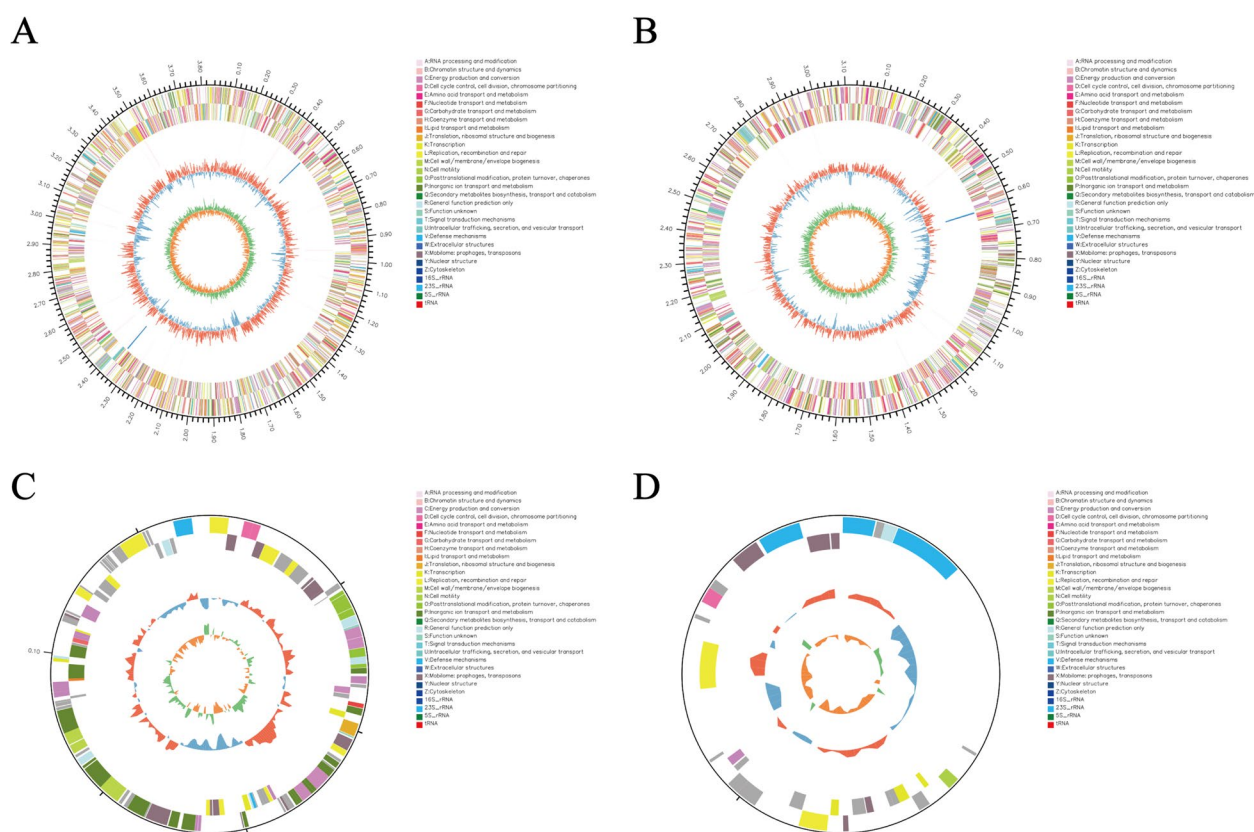


Fig. 1 Circular genome maps of the chromosome 1 (A), the chromosome 2 (B), the plasmid A (C), and the plasmid B (D) in *Aquincola tertiarycarbonis* RN12 strain, from outside to inside indicate the following: Circle 1: indicator of genome size; Circle 2 and 3: coding sequences in plus and minus strand, different colors represent the functional classification of COG with different CDS; Circle 4: the distribution of rRNA and tRNA; Circle 5: G + C content (the outer circle shown in red is greater than the average content, and the inner circle shown in green is less than the average content); Circle 6: GC skew (green represents the GC skew > 0, orange represents the GC skew < 0)

MMB [2], had been found in the genome of *A. tertiarycarbonis* RN12.

Identification of the genes required for floc formation

Large scale transposon mutagenesis was conducted on the wild type *A. tertiarycarbonis* RN12 strain by using the *mariner* transposon delivery vector pminiHimar RB1 as previously described [2, 12, 13]. A series of floc-formation-deficient mutants, whose liquid cultures were turbid and planktonic rather than flocculated as observed in the cultures of the wild type RN12 strain, had been isolated and the transposon insertion site was mapped in most of the floc-deficient mutants. The homogenous turbid cell cultures composed of planktonic single cells were visualized by light microscope. In addition to the previously described exopolysaccharide biosynthesis gene cluster [3], two other loci had been identified to be required for floc formation in our study. An experimentally uncharacterized gene encodes a PEP-CTERM sorting domain protein (TIGR02595, designated *pepA* hereafter) and was

located upstream of the mapped *prst* gene (Figs. 2 and 3A), which encodes a putative PEP-CTERM system TPR-repeat lipoprotein (TIGR02917).

Another mapped locus encodes a glycosyltransferase gene designated *epsB2* (TIGR03013), which is a PEP-CTERM system associated sugar transferase and may be a cytoplasmic membrane bound undecaprenyl-phosphate galactose phosphotransferase (Supplemental Fig. S1), and a Fis-type two-component system termed PrsK (TIGR02916, putative PEP-CTERM regulatory system histidine kinase) and PrsR (TIGR02915, PEP-CTERM-box response regulator transcription factor) (Fig. 3B). The *EpsB2* protein is a homolog of the *EpsB* glycosyltransferase found in *Methylobacillus* sp. strain 12S, which is also associated with a PEP-CTERM system, but of a distinct type [10, 14]. Interestingly, these proteins are part of the computationally predicted PEP-CTERM/exosortase system, analogous to the LPXTG/sortase system common in Gram-positive bacteria [10]. The transposon insertion had been mapped to the *epsB2* gene in multiple floc-deficient mutants (Supplemental Table. S2),

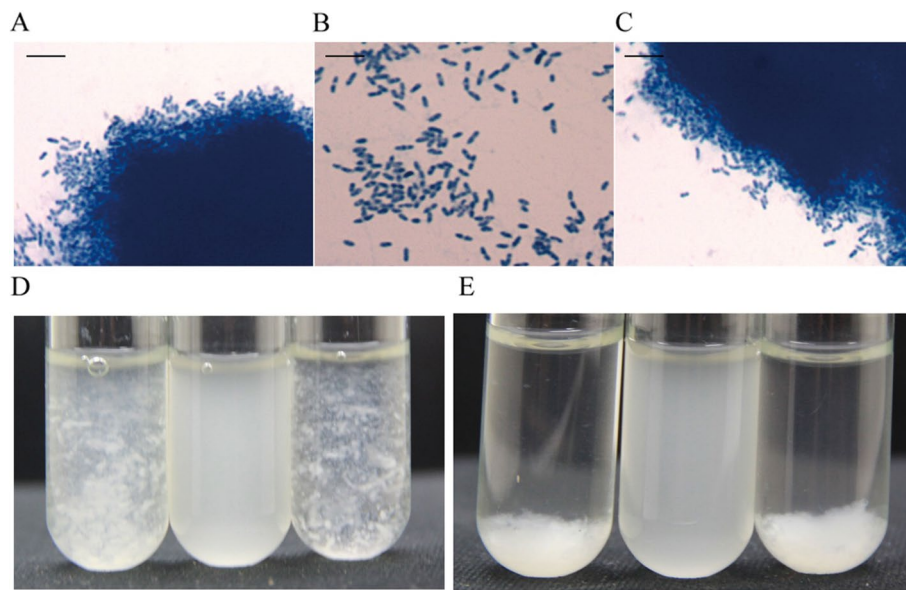


Fig. 2 Genetic complementation analyses of the *prsT* disruptant RN12M51 of *A. tertiarycarbonis*. **A** Wild type RN12 strain with pBBR1MCS-5 empty vector visualized by light microscope after staining with methylene blue. **B** Floc-formation deficient mutant RN12M51 with pBBR1MCS-5. **C** Floc-forming RN12M51 complemented by pBBR1MCS-5-*prsT* construct. **D** Agitated bacterial cultures from panels A, B and C, from left to right. **E** Settled bacterial cultures from panels A, B and C, from left to right. **A** to **C** were visualized by light microscope after staining with methylene blue ($\times 1000$ magnification); Bars: 10 μ m

suggesting its essential role in exopolysaccharide biosynthesis and floc formation. Its downstream gene is *prsK*, encoding sensory transduction histidine kinases, whose gene disrupted mutant is RN12M47 (Fig. 3B). The cellular role of the *prsT* and *epsB2* genes was further confirmed by the genetic complementation analyses in which the plasmid borne *prsT* (Fig. 2) or *epsB2* (Supplemental Fig. S2) gene could restore the floc-forming phenotype to the specific mutant. Previously we also identified a large gene cluster required for the floc formation of RN12 and the activated sludge bacterium *Zoogloea resiniphila* MMB, respectively [2, 3]. However, the plasmid borne *prsK* or *prsK-prsR* cassette failed to recover the floc-forming phenotype to the RN12M47 mutant due to an unknown reason (Supplemental Fig. S3). The predicted orthologues for the genes of these two gene clusters identified in the *A. tertiarycarbonis* RN12 strain are also present in the closely related proteobacterial genomes of *Rubrivivax gelatinosus* IL144 and *Leptothrix cholodnii* SP-6 (Supplemental Table S3).

Transcription of *pepA* was dependent on RpoN1 (sigma⁵⁴)

An obvious intergenic region (nearly 85 nt) could be found upstream of the open reading frame (ORF) of *pepA*, indicating that there might be complex gene transcriptional regulatory motifs. There is also a putative “stem-and-loop” transcriptional terminator between the ORF of

pepA and the downstream *prsT* gene (Fig. 3A). The transcriptional start site of *pepA* was determined by a primer extension analysis, demonstrating that the conserved $-24/-12$ motifs GG and GC were present upstream of its ORF (Fig. 4D). Furthermore, the transcription of *pepA* was compared in the wild type RN12 strain (carrying pBBR1MCS-2 empty vector), the *rpoN1* mutant RN12T4 (carrying pBBR1MCS-2) and the complementation strain carrying the pBBR1MCS-2-*rpoN1* construct by using real-time PCR (Fig. 4C) and RT-PCR (Figs. 4A and B). It was clearly demonstrated that high levels of transcription of *pepA* were dependent on the presence of a wild type *rpoN1* gene, which was either present on the chromosome of the wild type strain or encoded in the plasmid in the RN12T4 mutant ($p < 0.001$). On the other hand, the transcription of downstream *prsT* gene was lower and seemed to be not affected by the disruption of *rpoN1* ($p < 0.001$) (Supplemental Fig. S4). These results are consistent with previous bioinformatics prediction that certain PEP-CTERM genes are dependent on RpoN sigma factor and probably the accessory PrsK-PrsR two-component system as well [10]. A previously predicted enhancer sequence could also be found upstream of this preliminarily mapped RpoN-recognized promoter. More experiments are needed to clarify the regulatory mechanism underlying the expression of PEP-CTERM genes.

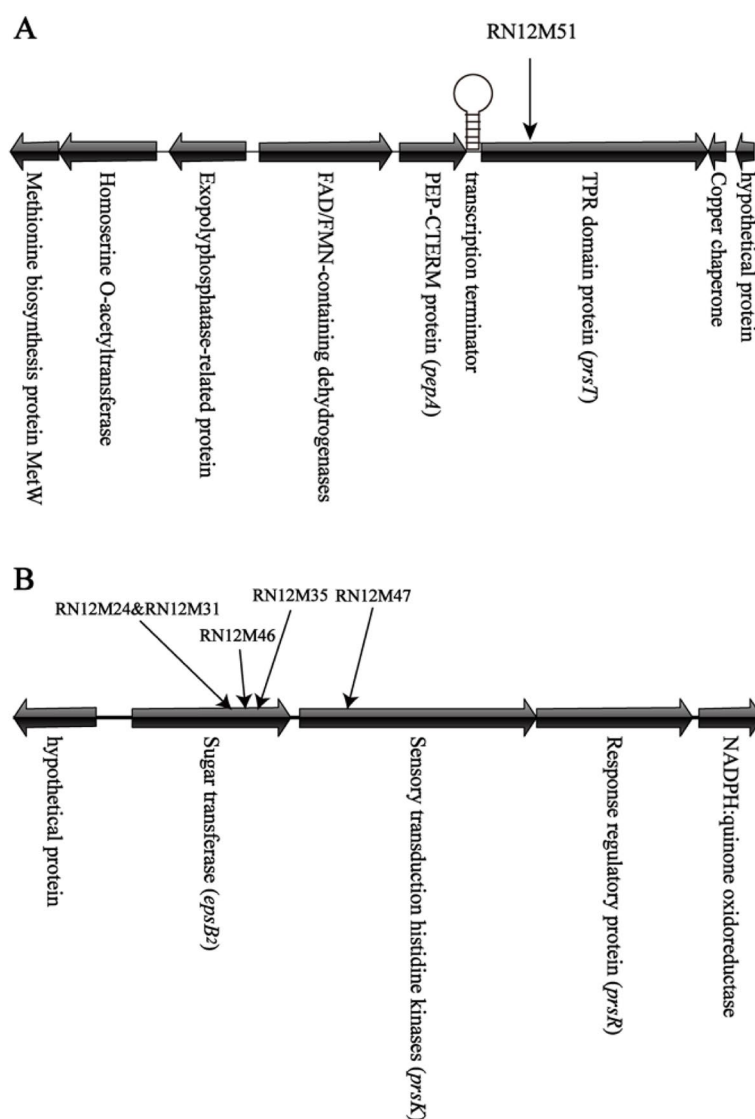


Fig. 3 The two gene clusters required for floc formation of *Aquincola tertiarycarbonis* RN12 strain. **A** Organization of the chromosomal region adjacent to the TPR lipoprotein encoding *prsT* gene which was disrupted by transposon insertion (black arrow) mapped in the floc-deficient transposon mutant M51. **B** Organization of the chromosomal region adjacent to the sugar transferase encoding *epsB2* gene and a sensory transduction histidine kinase gene *prsK* which was disrupted by insertion (black arrows) of a *mariner* transposon mapped in the transposon mutants deficient in bacterial floc formation

Overexpression of *pepA* rescued floc formation by decreasing soluble exopolysaccharides in the *rpoN1*-disrupted mutant

We wondered if overexpression of the PEP-CTERM genes such as *pepA* could rescue the floc-forming phenotype of the *rpoN1*-disrupted mutant RN12T4 (Fig. 5). The *PepA* PEP-CTERM protein is probably secreted into periplasm via Type II secretion system and may be further sorted and translocated out of cells and become part of extracellular polymeric substances required for floc formation. The so called PEP-CTERM/exosortase system, found in many Gram-negative bacteria including the

floc-forming *Aquincola* and *Zoogloea* strains, seems to be involved in the production of the extracellular polymeric substance composed of exopolysaccharides and mature PEP-CTERM proteins [10].

Previously it had been revealed that most of the secreted polysaccharides were released and dissolved in the culture broth rather flocculating the bacterial cells when *rpoN1* gene was inactivated [3]. Fiber-like exopolysaccharides could no longer be precipitated and extracted from the cell-free supernatants of bacterial cultures of RN12T4 mutant when the *pepA* gene was overexpressed (Figs. 6A, B, C, and D). Quantitative measurements

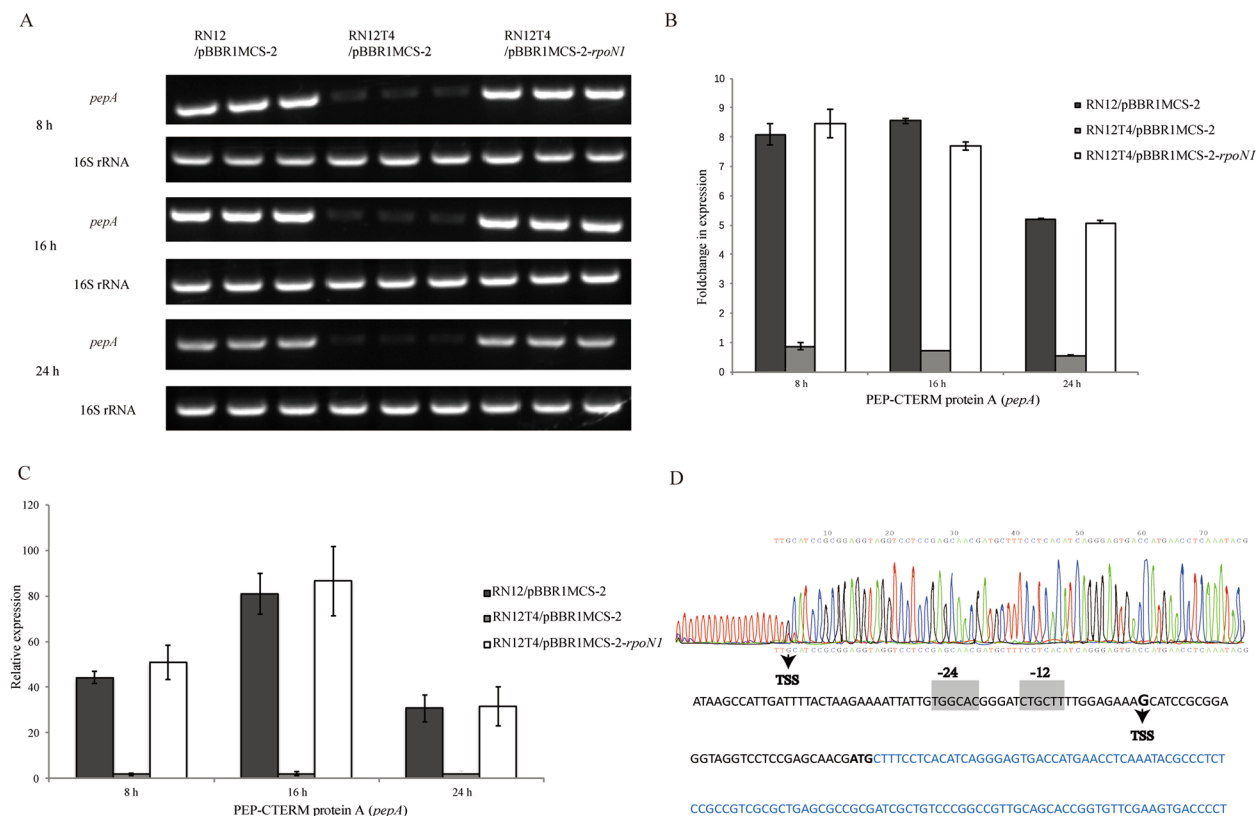


Fig. 4 Transcriptional analyses showing that the PEP-CTERM gene *pepA* was regulated by the RpoN1 sigma factor. Bacteria were cultivated at 28°C in the R2A medium with shaking (200 rpm). The data represent the samples collected at 8 h, 16 h and 24 h. **A** Transcription of the *pepA* gene was examined by semi-quantitative RT-PCR with 16S rRNA gene as the loading control. **B** Trace quantity plotting using 'Quantity One' software. The experiments were performed in triplicate. **C** Transcription of the gene was examined by real-time PCR, relative transcript levels were calculated according to the $2^{-\Delta\Delta C_t}$ method, 16S rRNA was analyzed and used as an internal control gene. The experiments were performed in triplicate. Error bars represent the standard deviation. **D** Determination of the transcriptional start site (TSS) of the *pepA* gene in *A. tertiarycarbonis* RN12 strain. The blue sequence is the open reading frame of *pepA* and the arrow-pointed nucleotide G is the determined transcriptional start site (+1). The shadowed sequences TGGCAC and CTGCTT are the putative -24 and -12 motifs of the promoter recognized by the sigma factor RpoN1.

also demonstrated that the bound exopolysaccharides increased, whereas the soluble exopolysaccharides decreased markedly when the floc-forming phenotype was rescued by the plasmid borne *pepA* gene ($p < 0.001$) (Fig. 6E). These results suggest that the released exopolysaccharides dissolved in the supernatants of culture broth decreased markedly and could no longer be extracted when PepA protein was overexpressed. In the meantime, the bound exopolysaccharides increased and floc-forming phenotype was recovered in the RN12T4 mutant. These facts indicate that *the* exopolysaccharides became to be tightly bound to bulks of bacterial cells to form the flocs via a PEP-CTERM protein mediated process. The secreted PEP-CTERM proteins may interact with extracellular polysaccharide chains to form bound EPS via glycosylation [10], although the underlying mechanism remains to be further investigated.

Interestingly, there are more than 60 PEP-CTERM domain protein encoding genes identified in the genome

of *A. tertiarycarbonis* RN12. We chose and tested several other genes, encoding the typical PEP-CTERM domain protein which harbors both N-terminal secretion signal and C-terminal PEP-CTERM domain as well as a high percentage of asparagine residues and nontypical ones. Results show that only the *pepA* gene have the ability to rescue the foc-forming of RN12T4 strain (Supplemental Fig. S5). We suppose that the floc forming of *A. tertiarycarbonis* RN12 require many proteins (including PEP-CTERM domain proteins and some other proteins), and this trait was likely to be maintained or partially maintained just in the absence of *pepA* gene. However, our criterion was absolutely no microbial suspensions in liquids in the process of screening mutants, so no such gene was found. Plasmid borne *pepA* can rescue as a result of the over expression.

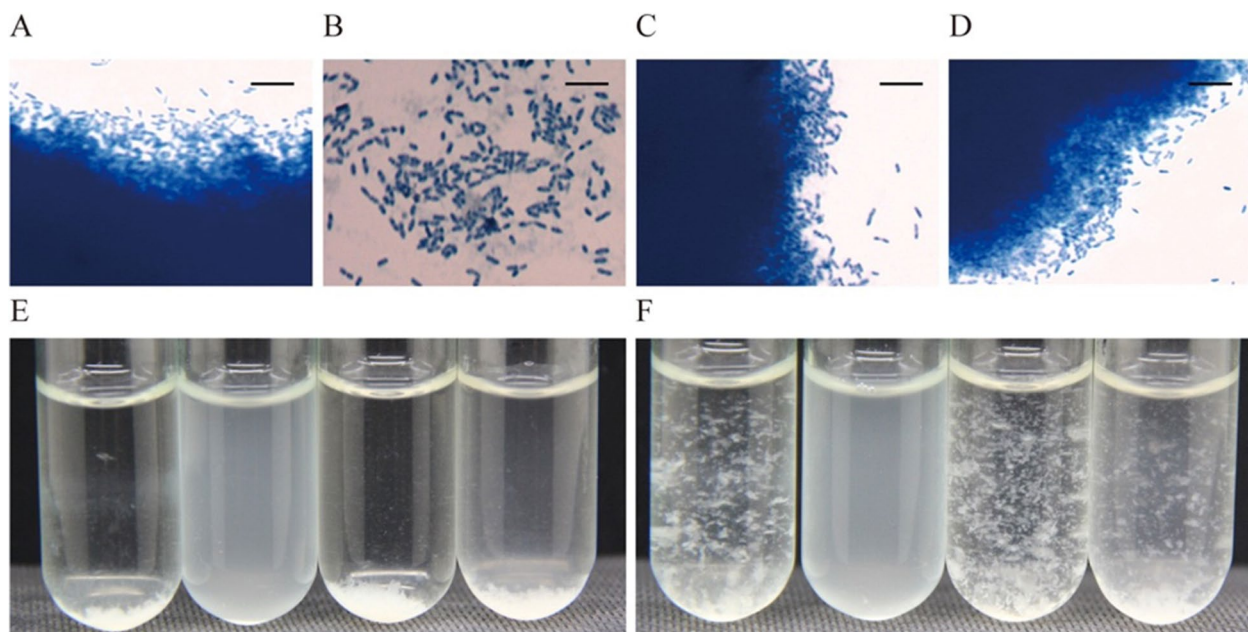


Fig. 5 Overexpression of the PEP-CTERM gene *pepA* circumvented the requirement of RpoN1 for floc formation in the RN12T4 mutant. Plasmid borne wild type *pepA* gene recovered the floc-forming phenotype of the RN12T4 mutant similar to that of the wild type strain RN12 with the empty vector. **A** Wild type RN12 strain carrying the pBBR1MCS-2 empty vector. **B** RN12T4 mutant with pBBR1MCS-2 empty vector. **C** RN12T4 mutant with pBBR1MCS-2-*rpoN1* construct. **D** RN12T4 mutant with pBBR1MCS-2-*pepA* construct. **E** Agitated bacterial cultures from panels A to D, from left to right. **F** Settled bacterial cultures from panels (A) to (D), from left to right. **A** to **D** were visualized by light microscope after staining with methylene blue ($\times 1000$ magnification); Bars: 10 μ m

Western blot analysis on the recombinant PepA protein

The PEP-CTERM sorting domain proteins usually contain an amino terminal (N-terminal) signal peptide (Supplemental Fig. S6A) and therefore these putative proteins may be secreted into the periplasmic space by the Type II secretion system [10]. The function of the PepA signal peptide was further confirmed by phosphatase A-(PhoA) fusion assay (Supplemental Fig. S6B). To monitor the expression of the recombinant PepA protein in the *A. tertiaricarbonis* mutants, we inserted a polyhistidine (His) tag between the P33 and V34 residues after the intact signal peptide by using DNA recombination technology. This construct still could rescue the floc formation phenotype of RN12T4 mutant, indicating that the His-tag did not affect the cellular function of the recombinant PepA protein. Three bands were detected in the Western blot analyses using anti-His-tag monoclonal antibodies (Fig. 7A). The Histidine-tagged recombinant proteins were purified and subjected them to the UPLC coupled LTQ-Orbitrap Elite ETD Mass Spectrometry analyses. All three bands contains the polyhistidine sequence tag and the downstream PepA peptide sequences, indicating the presence of the recombinant PepA proteins in the targeted bands and a post-translational processing and modification of such a PEP-CTERM protein (Fig. 7B,

C and D). The N-terminal signal peptide of the PEP-CTERM protein PepA seemed to be cleaved as computationally predicted while the C-terminus might also be further cleaved, which needs to be further investigated and the role of the VPEP motif also needs to be defined.

Overexpression of *pepA* did not rescue biofilm formation and swarming ability

RpoN sigma factor is a global regulator [15–18] and RpoN1 regulates a series of cellular functions including floc formation, swarming motility and biofilm formation in *A. tertiaricarbonis* RN12 [3]. The swarming motility and biofilm formation were monitored in various strains to further reveal the cellular role of PEP-CTERM protein PepA. Neither swarming motility nor biofilm formation was rescued by plasmid-mediated overexpressing *pepA* gene in the *rpoN1* mutant RN12T4 (Fig. 8). It is suggested that *pepA* and/or other PEP-CTERM genes are only involved in floc formation but not biofilm formation or swarming motility.

Taken together, our results demonstrated that the RpoN1-regulated *pepA* gene and probably other PEP-CTERM genes were involved in the floc formation of *A. tertiaricarbonis* RN12 strain. Furthermore, our results also indicated that EPS components involved in the

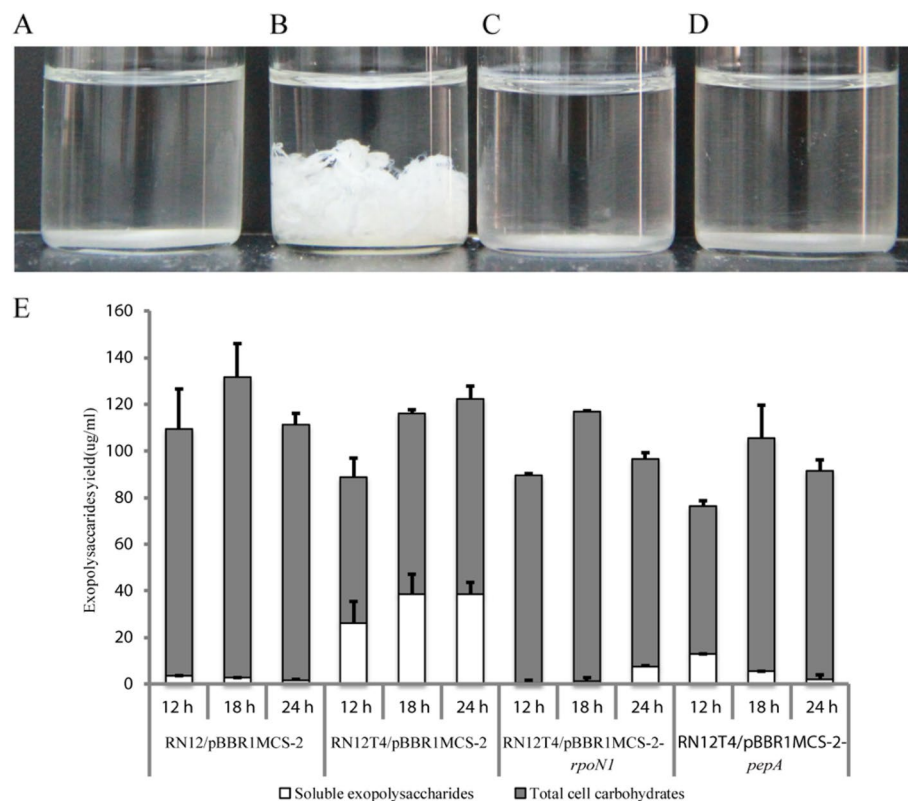


Fig. 6 Qualitative and quantitative analyses of the extracellular polysaccharides of *A. tertiarycarbonis* wild type strain and mutants demonstrating that PepA overexpression decreased the release of soluble exopolysaccharides produced by the *rpoN1* mutant. Upper panel: Ethanol-precipitated exopolysaccharides extracted from the cell free supernatants of bacterial cultures of wild type strain RN12 with the pBBR1MCS-2 empty vector (A), RN12T4 mutant with the empty vector (B), RN12T4 mutant with pBBR1MCS-2-*rpoN1* (C) and RN12T4 mutant with pBBR1MCS-2-*pepA* (D). Lower panel: Average yields of the total cell carbohydrates (grey bars) and released soluble exopolysaccharides (S-EPS, white bars) levels of bacterial cultures of A, B, C and D were measured using the phenol-sulfuric acid method at 12 h, 18 h and 24 h (E). Values were shown as micrograms per milliliter of bacterial suspension. All experiments were performed in triplicate; Error bars represent the standard deviation

formation of flocs, the microbial suspensions in liquids, and the formation of biofilm, attached to surfaces, seemed to be different, despite similar extracellular polymeric matrices participated in the formation of these two important bacterial aggregates. Further structural analyses on the extracellular polymeric substances, including exopolysaccharides and PEP-CTERM proteins, are needed to reveal the mechanisms underlying the floc formation of *Aquicola*, *Zoogloea* and other bacteria.

Conclusions

In this study we have identified several genes involved in floc formation of *Aquicola tertiarycarbonis* RN12 strain, in addition to the previously identified large gene cluster for exopolysaccharide biosynthesis and an RpoN1 sigma factor. The RpoN1 sigma factor regulates the transcription of a PEP-CTERM gene, whose overexpression could prevent the release of exopolysaccharides and rescue the floc formation of the *rpoN1* mutant. These results

indicate that floc formation is a tightly regulated process and the RpoN1-dependent PEP-CTERM proteins are involved in self-flocculation of bacterial cells of *Aquicola* and probably other bacteria.

Methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 2. Bacterial strains were cultured in the Luria-Bertani broth (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0), R2A medium [19], and *Zoogloea* medium (ZM) [19] (supplemented with 15 µg/ml of gentamycin or 50 µg/ml of kanamycin, 50 µg/ml of diaminopimelic acid, and 1.5 g/L Biosharp agar when necessary). The *A. tertiarycarbonis* RN12 strains were grown at 28 °C.

Bioinformatics analysis

Multiple sequence alignments were performed by the Clustal X alignment program [20]. Hidden Markov

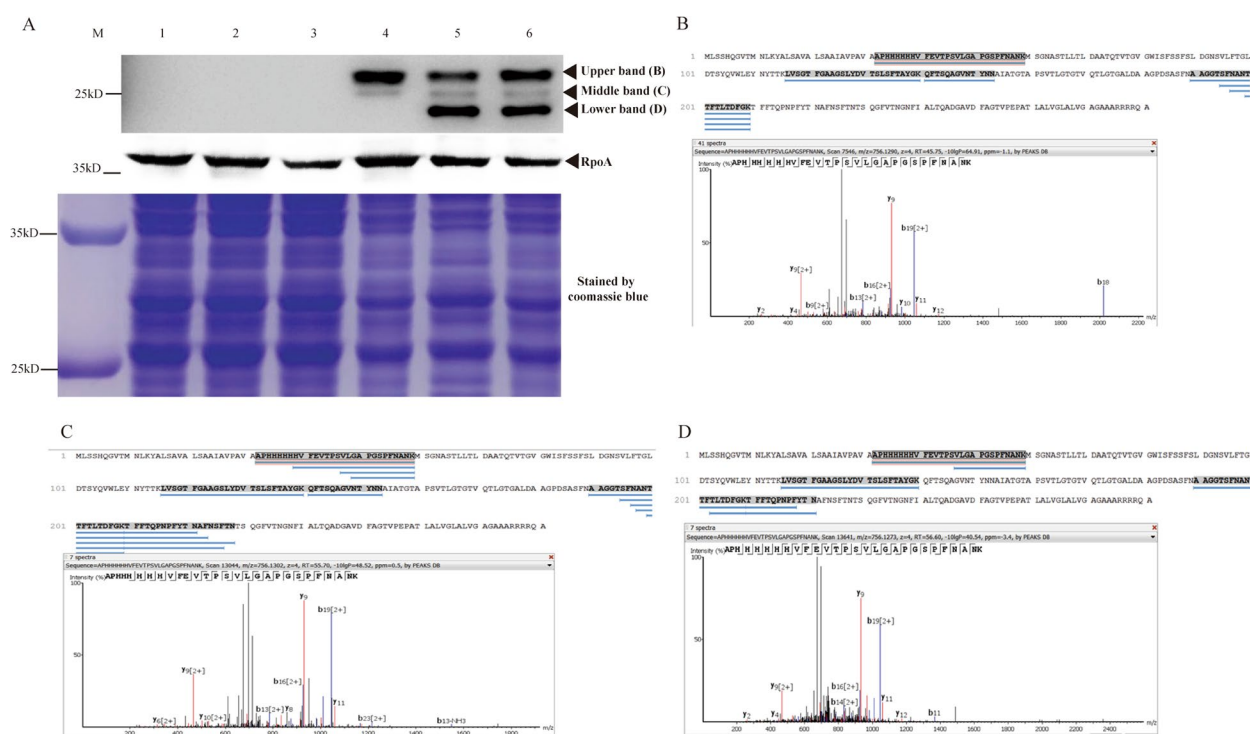


Fig. 7 Western blot and tandem mass spectrometry (MS/MS) analyses of His-tagged recombinant PEP-CTERM protein of PepA in the *A. tertiarycarbonis* strains. The samples were taken at 12 h, 18 h and 24 h. Immunoblotting was performed with His-specific monoclonal mouse antibody followed by a goat anti-mouse IgG conjugated to HRP. **A** PVDF membrane incubated with His-tag-specific antibody Western ECL (Upper panel) and three bands, upper, middle and lower, appeared; a PVDF membrane incubated with anti-RpoA antibody (Generated using the RpoA recombinant protein of closely related *Zoogloea resiniphila* MMB strain as the antigen) Western ECL as a loading control (Middle panel) and also a Coomassie Brilliant Blue stain of the SDS-PAGE gel as a loading control (Lower panel). Lane M: The size marker; Lane 1–Lane 3: The proteins extracted from the RN12T4 mutant carrying the empty vector pBBR1MCS-2 over the time course; Lane 4–Lane 6: The proteins extracted from RN12T4 mutant carrying pBBR1MCS-2-*pepA* over the time course. The molecular weight of the marker bands is indicated in kilo-Daltons (KD). Mass spectrometry identification of the upper (**B**), middle band (**C**) and lower band (**D**) detected by the anti-His tag antibody in the Western blots. The MS/MS sequence coverage of proteins is 31, 37 and 30%, respectively. MS/MS spectra of the tryptic peptide APHHHHHVFEVTPSVLGAPGSPFNANK ($m/z = 756.129$) confirmed the presence of the hexahistidine tag in all three bands. The western blots used in figures were from two regions of one membrane (The RpoA was about 35KD and the PepA was lower than 29KD)

models (HMMs) previously constructed [10] were used to search for PEP-CTERM proteins. The two-tailed independent-sample t-test was conducted to test the significance level in this manuscript.

Transposon mutagenesis and genetic complementation

The *mariner* transposon mutant libraries were generated as previously described [12, 21]. *Escherichia coli* WM3064 strain carrying the transposon delivery suicide plasmid, pminiHmar RB1 (courtesy by Dr. Daad Saffarini) as the donor strain and the *A. tertiarycarbonis* RN12 strain as the recipient strain for biparental conjugation. After 4–6 h of mating on LB agar plates supplemented with diaminopimelic acid, the bacterial cells were diluted and plated on R2A agar plates supplemented with kanamycin (50 µg/ml) and the mutants deficient in floc formation were screened out. The transposon insertion site in each mutant was mapped as previously described [22].

The chromosomal DNA of these non-floc forming strains were digested with *SphI* and selfligated by T4 DNA ligase (Takara, Dalian, China). Then, the circular closed DNA was introduced into the EC100D *pir*⁺ Electrocompetent *E. coli* through electroporation [23, 24]. Finally, the plasmids were extracted and sequenced to identify the insertion site. For genetic complementation analyses, the target genes were PCR amplified and cloned into the pBBR1MCS-5 vector [25] (primers and restriction sites were shown in Table S1). The recombinants and empty vector were transferred into the *A. tertiarycarbonis* RN12 wild type strain and mutant strains via conjugation using WM3064 as the donor strain.

Exopolysaccharide quantification

Due to the fact that the exopolysaccharides were released into the medium in the RN12T4 mutant, we divided exopolysaccharides of cells into two sections:

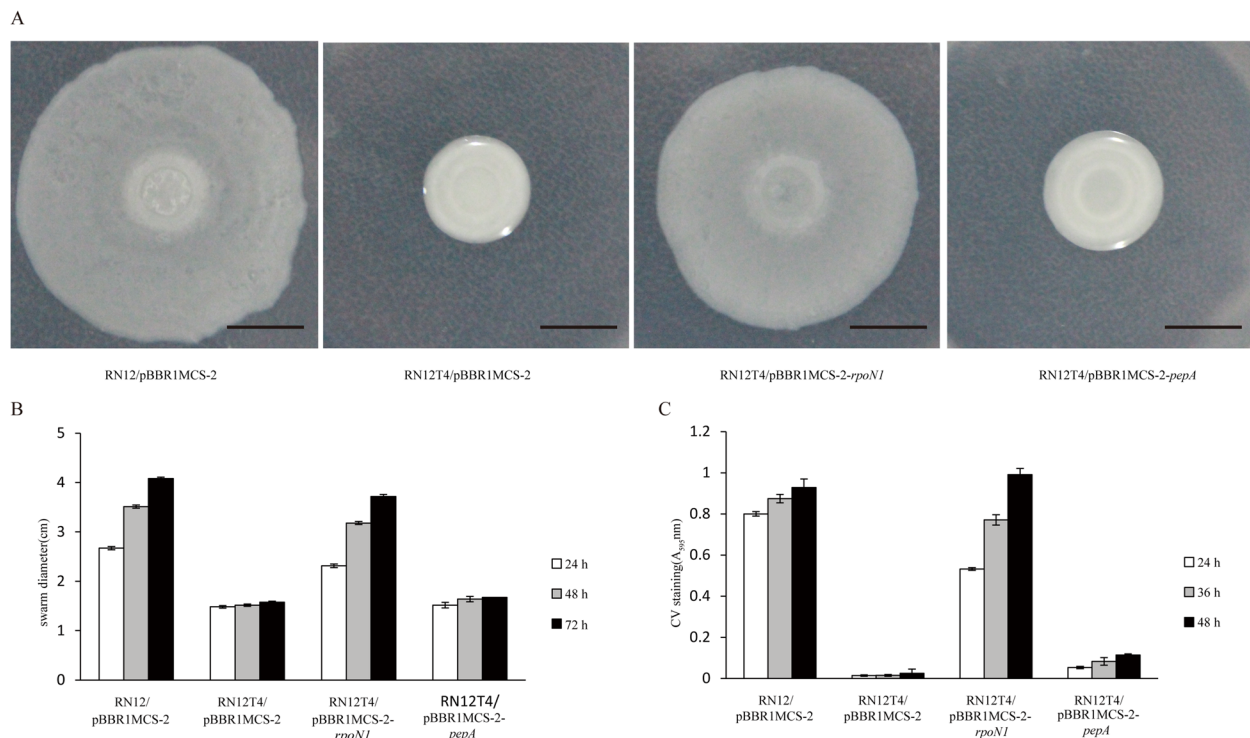


Fig. 8 The overexpression of PEP-CTERM gene *pepA* did not rescue the swarming motility and biofilm formation of the *rpoN1* disruptant of *A. tertiaryaricarbonis*. **A** Colony morphology of each strain after three day incubation. Scale bar: 1 cm; **B** Swarming motility expressed as colony diameters at 24 h, 48 h and 72 h; **C** Biofilm levels measured after 24 h, 36 h and 48 h incubation at 28 °C in the R2A media. Each sample was plated in triplicate. Biofilms were stained with 0.1% crystal violet (CV). CV was dissolved with 30% acetic acid and quantified by measuring the optical density at 595 nm; Error bars represent the standard deviation

soluble exopolysaccharides and total cell carbohydrates as previously described [3]. The soluble exopolysaccharides

Table 2 Bacterial strains and plasmids used in this study

Strains or plasmids	Description	Source or reference
Strains		
<i>E. coli</i> DH5α	<i>F</i> [−] <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoRnupG</i> Φ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169, <i>hsdR17</i> (<i>r</i> _K) [−] <i>m</i> _K ⁺ <i>phoA</i> λ [−]	Takara
<i>Escherichia coli</i> WM3064	<i>thrB1004 pro thirpsLhsdS lacZDM15 RP4–1360 (araBAD)567dapA1341::[ermpir(wt)]</i>	W. Metcalf
<i>E. coli</i> EC100D ⁺	<i>F</i> [−] <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74 recA1 endA1 araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU-galk</i> λ [−] <i>rpsL</i> (<i>Str</i> ^R) <i>nupGpir</i> ⁺ (<i>DHFR</i>)	Epicentre Technologies
RN12WT	Floc-forming strain	(3)
RN12T4 mutant	mariner transposon insertion in <i>rpoN1</i> at nt 1274	(3)
RN12M35 mutant	mariner transposon insertion in <i>epsB2</i> at nt 1295	This study
RN12M51 mutant	mariner transposon insertion in <i>prfT</i> at nt 2097	This study
plasmids		
pminiHmarRB1	The mariner transposon delivery plasmid R6K	(15)
pBBR1MCS-2	Broad-host-range shuttle vector, Km ^r	(20)
pBBR1MCS-5	Broad-host-range cloning vector, Gm ^r	(20)
pBBR1MCS2- <i>rpoN1</i>	<i>rpoN1</i> gene cloned in pBBR1MCS-2, Km ^r	(3)
pBBR1MCS2- <i>pepA</i>	<i>pepA</i> gene cloned in pBBR1MCS-2, Km ^r	This study
pBBR1MCS-5- <i>prfT</i>	<i>prfT</i> gene cloned in pBBR1MCS-5, Gm ^r	This study
pBBR1MCS-5- <i>epsB2</i>	<i>epsB2</i> gene cloned in pBBR1MCS-5, Gm ^r	This study

Tandem mass spectrometry analyses of his-tagged proteins

Three coomassie blue stained SDS-PAGE gel bands corresponding to those targeted by the anti-His-tag antibody in the immunoblots were excised and destained in 25 mM ammonium bicarbonate-50% acetonitrile at least 3 times and dehydrated with acetonitrile, then reduced with 25 mM DTT in 50 mM ammonium bicarbonate at 56 °C for 25 min, and alkylated with 55 mM iodoacetamide at room temperature in the dark for 30 min. After washing and dehydration in 25 mM ammonium bicarbonate-50% acetonitrile and absolute acetonitrile, gel pieces were digested with 10 ng/μL mass spectrometry grade trypsin gold (Promega, V5280) or chymotrypsin (Promega, V1062) in 25 mM ammonium bicarbonate at 37 °C for 16 hrs. Peptides were extracted sequentially with 20 mM ammonium bicarbonate, 50% acetonitrile and 5% formic acid, and then pooled peptides were evaporated to dryness in acid-resistant CentriVap centrifugal vacuum concentrator (Labconco Kansas City, MO), and re-suspended with 20 μL of 2% methanol-1% formic acid. NanoLC-MSMS analysis of in-gel digested peptide was performed on Waters® nanoACQUITY UPLC® coupled LTQ-Orbitrap Elite ETD Mass Spectrometer (Thermo Fisher Scientific) as previously described [35].

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12866-022-02745-1>.

Additional file 1: Table S1. Primers used in this study. Table S2.

Identification of the insertional sites of transposon mutants of *Aquicola tertiarycarbonis* RN12 defective in the floc formation. **Table S3.** The gene products of the two gene clusters identified in *A. tertiarycarbonis* RN12 strain and the predicted orthologues in the closely related proteobacterial genomes of *Rubrivivax gelatinosus* IL144 and *Leptothrix cholodnii* SP-6 (the polypeptide sequence identity was shown). **Supplemental Fig. S1.** The glycosyltransferase EpsB2 is a putative cytoplasmic membrane bound protein with five transmembrane domains as computationally predicted by using Protter software. Five transmembrane domains have been predicted, but the actual topological traits of this glycosyltransferase remains to be characterized experimentally. **Supplemental Fig. S2.** The genetic analysis of the glycosyl transferase gene *epsB2* in the RN12M35 transposon insertional mutant deficient in floc formation. **Supplemental Fig. S3.** The genetic analysis of the *prfR* gene in the RN12M47 transposon insertional mutant deficient in floc formation. **Supplemental Fig. S4.** Transcriptional analyses of the *prfT* gene downstream of *pepA* showed RpoN1-independent expression. **Supplemental Fig. S5.** The genetic analysis of PEP-CTERM genes in the RN12T4 transposon insertional mutant deficient in floc formation. **Supplemental Fig. S6.** The alkaline phosphatase A (PhoA)-fusion assay demonstrated that the PEP-CTERM protein PepA is secreted into the periplasm as computationally predicted. **Supplemental Fig. S7.** Transcription of the gene was examined by semi-quantitative RT-PCR with 16S rRNA gene as the loading control. **Supplemental Fig. S8.** The full gels and blots.

Acknowledgements

Not applicable.

Authors' contributions

D.Q. and M.X. designed the experiments; M.X., D.Y., H.C., J. D., N.G. and S.L. did the data curation and formal analysis; M.X. and D.Q. wrote the original manuscript; X.B. and D.Q. reviewed and edited the manuscript. All authors have read and approved the manuscript.

Funding

This work was supported by the grants from National Natural Science Foundation of China (31900095) to X.M., National Key R&D Program of China (2019YFA0905500) to D. Q., Innovative Research Team Foundation of the Department of Education of Hubei Province, China (T2020034) to X.M., Key Research and Development Program of Anhui Province (2022107020008) to N.G., and National Natural Science Foundation of China (32102769) to J.D.

Availability of data and materials

The complete genome of *Aquicola tertiarycarbonis* RN12 strain has been submitted in the GenBank under accession number CP097635 (chromosome 1), CP097636 (chromosome 2), CP097637 (plasmid A), and CP097638 (plasmid B).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

All authors declare having no conflicts of interest.

Received: 18 January 2022 Accepted: 21 December 2022

Published online: 19 January 2023

References

- Allen MS, Welch KT, Prebyl BS, Baker DC, Meyers AJ, Saylor GS. Analysis and glycosyl composition of the exopolysaccharide isolated from the floc-forming wastewater bacterium *Thauera* sp. MZ1T. *Environ Microbiol.* 2004;6(8):780–90.
- An W, Guo F, Song Y, Gao N, Bai S, Dai J, et al. Comparative genomics analyses on EPS biosynthesis genes required for floc formation of *Zoogloea resiniphila* and other activated sludge bacteria. *Water Res.* 2016;102:494–504.
- Yu D, Xia M, Zhang L, Song Y, Duan Y, Yuan T, et al. RpoN (sigma54) is required for Floc formation but not for extracellular polysaccharide biosynthesis in a Floc-forming *Aquicola tertiarycarbonis* strain. *Appl Environ Microbiol.* 2017;83(14):1–17.
- Muller RH, Rohwerder T, Harms H. Degradation of fuel oxygenates and their main intermediates by *Aquicola tertiarycarbonis* L108. *Microbiol.* 2008;154(Pt 5):1414–21.
- Schafer F, Muzica L, Schuster J, Treuter N, Rosell M, Harms H, et al. Formation of alkenes via degradation of tert-alkyl ethers and alcohols by *Aquicola tertiarycarbonis* L108 and *Methylobium* spp. *Appl Environ Microbiol.* 2011;77(17):5981–7.
- Schuster J, Schafer F, Hubler N, Brandt A, Rosell M, Hartig C, et al. Bacterial degradation of tert-amyl alcohol proceeds via hemiterpene 2-methyl-3-buten-2-ol by employing the tertiary alcohol desaturase function of the Rieske nonheme mononuclear iron oxygenase MdpJ. *J Bacteriol.* 2012;194(5):972–81.
- Schuster J, Purswani J, Breuer U, Pozo C, Harms H, Muller RH, et al. Constitutive expression of the cytochrome P450 EthABCD monooxygenase system enables degradation of synthetic dialkyl ethers in *Aquicola tertiarycarbonis* L108. *Appl Environ Microbiol.* 2013;79(7):2321–7.
- Xia S, Jia R, Feng F, Xie K, Li H, Jing D, et al. Effect of solids retention time on antibiotics removal performance and microbial communities in an a/O-MBR process. *Bioresour Technol.* 2012;106:36–43.
- Guo J, Peng Y, Yang X, Wang Z, Zhu A. Changes in the microbial community structure of filaments and floc formers in response to various

- carbon sources and feeding patterns. *Appl Microbiol Biotechnol*. 2014;98(17):7633–44.
10. Haft DH, Paulsen IT, Ward N, Selengut JD. Exopolysaccharide-associated protein sorting in environmental organisms: the PEP-CTERM/EpsH system. Application of a novel phylogenetic profiling heuristic. *BMC Biol*. 2006;4:29.
 11. Rohwerder T, Muller RH, Weichler MT, Schuster J, Hubschmann T, Muller S, et al. Cultivation of *Aquicola tertiicarbonis* L108 on the fuel oxygenate intermediate tert-butyl alcohol induces aerobic anoxygenic photosynthesis at extremely low feeding rates. *Microbiology*. 2013;159(Pt 10):2180–90.
 12. Bouhenni R, Gehrke A, Saffarini D. Identification of genes involved in cytochrome c biogenesis in *Shewanella oneidensis*, using a modified mariner transposon. *Appl Environ Microbiol*. 2005;71(8):4935–7.
 13. Li R, Withers RT, Dai J, Ruan J, Li W, Dai Y, et al. Truncated type IV pilin PilA(108) activates the intramembrane protease AlgW to cleave MucA and PilA(108) itself in vitro. *Arch Microbiol*. 2016;198(9):885–92.
 14. Yoshida T, Ayabe Y, Yasunaga M, Usami Y, Habe H, Nojiri H, et al. Genes involved in the synthesis of the exopolysaccharide methanolan by the obligate methylotroph *Methylobacillus* sp strain 125. *Microbiology*. 2003;149(Pt 2):431–44.
 15. Yip ES, Grublesky BT, Hussa EA, Visick KL. A novel, conserved cluster of genes promotes symbiotic colonization and sigma-dependent biofilm formation by *Vibrio fischeri*. *Mol Microbiol*. 2005;57(5):1485–98.
 16. Saldias MS, Lamothe J, Wu R, Valvano MA. Burkholderia cenocepacia requires the RpoN sigma factor for biofilm formation and intracellular trafficking within macrophages. *Infect Immun*. 2008;76(3):1059–67.
 17. Hao B, Mo ZL, Xiao P, Pan HJ, Lan X, Li GY. Role of alternative sigma factor 54 (RpoN) from *Vibrio anguillarum* M3 in protease secretion, exopolysaccharide production, biofilm formation, and virulence. *Appl Microbiol Biotechnol*. 2013;97(6):2575–85.
 18. Zhao K, Liu M, Burgess RR. Promoter and regulon analysis of nitrogen assimilation factor, sigma54, reveal alternative strategy for *E. coli* MG1655 flagellar biosynthesis. *Nucleic Acids Res*. 2010;38(4):1273–83.
 19. Reasoner DJ, Geldreich EE. A new medium for the enumeration and subculture of bacteria from potable water. *Appl Environ Microbiol*. 1985;49(1):1–7.
 20. Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. Multiple sequence alignment with Clustal X. *Trends Biochem Sci*. 1998;23(10):403–5.
 21. Wong SM, Mekalanos JJ. Genetic footprinting with mariner-based transposition in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A*. 2000;97(18):10191–6.
 22. Qiu D, Eisinger VM, Head NE, Pier GB, Yu HD. ClpXP proteases positively regulate alginate overexpression and mucoid conversion in *Pseudomonas aeruginosa*. *Microbiology*. 2008;154(Pt 7):2119–30.
 23. Sugar IP, Neumann E. Stochastic-model for electric field-induced membrane pores electroporation. *Biophys Chem*. 1984;19(3):211–25.
 24. Neumann E, Schaefferidder M, Wang Y, Hofschneider PH. Gene-transfer into mouse lymphoma cells by electroporation in high electric-fields. *EMBO J*. 1982;1(7):841–5.
 25. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM 2nd, et al. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene*. 1995;166(1):175–6.
 26. Wingender J, Strathmann M, Rode A, Leis A, Flemming HC. Isolation and biochemical characterization of extracellular polymeric substances from *Pseudomonas aeruginosa*. *Methods Enzymol*. 2001;336:302–14.
 27. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem*. 1956;28(3):350–6.
 28. O'Toole GA. Microtiter dish biofilm formation assay. *J Visual Exper JoVE*. 2011;(47):1–3.
 29. Pehl MJ, Jamieson WD, Kong K, Forbester JL, Fredendall RJ, Gregory GA, et al. Genes that influence swarming motility and biofilm formation in *Variovorax paradoxus* EPS. *PLoS One*. 2012;7(2):1–12.
 30. Kelman A, Hruschka J. The role of motility and aerotaxis in the selective increase of avirulent bacteria in still broth cultures of *Pseudomonas solanacearum*. *J Gen Microbiol*. 1973;76(1):177–88.
 31. Tans-Kersten J, Brown D, Allen C. Swimming motility, a virulence trait of *Ralstonia solanacearum*, is regulated by FlhDC and the plant host environment. *Mol Plant-microbe Interact: MPMI*. 2004;17(6):686–95.
 32. Dai J, Wei H, Tian C, Damron FH, Zhou J, Qiu D. An extracytoplasmic function sigma factor-dependent periplasmic glutathione peroxidase is involved in oxidative stress response of *Shewanella oneidensis*. *BMC Microbiol*. 2015;15:34.
 33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(–Delta Delta C) method. *Methods*. 2001;25(4):402–8.
 34. Mendoza-Vargas A, Olvera L, Olvera M, Grande R, Vega-Alvarado L, Taboada B, et al. Genome-wide identification of transcription start sites, promoters and transcription factor binding sites in *E. coli*. *PLoS One*. 2009;4(10):e7526.
 35. Chng J, Wang T, Nian R, Lau A, Hoi KM, Ho SC, et al. Cleavage efficient 2A peptides for high level monoclonal antibody expression in CHO cells. *mAbs*. 2015;7(2):403–12.
 36. Fukui T, Yoshimoto A, Matsumoto M, Hosokawa S, Saito T. Enzymatic synthesis of poly-beta-hydroxybutyrate in *Zoogloea ramigera*. *Arch Microbiol*. 1976;110(23):149–56.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

