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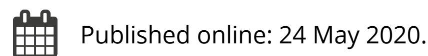
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Identification of the putative *N*-acetylglucosaminidase CseA associated with daughter cell separation in *Tetragenococcus halophilus*

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ABSTRACT
The lactic acid bacterium *Tetragenococcus halophilus*, which is used as a starter to brew soy sauce, comprises both cluster-forming strains and dispersed strains. The cluster-forming strains are industrially useful for obtaining clear soy sauce, because the cell clusters are trapped by filter cloth when the soy sauce mash is pressed. However, the molecular mechanism underlying cell cluster formation is unknown. Whole genome sequence analysis and subsequent target sequence analysis revealed that the cluster-forming strains commonly have functional defects in *N*-acetylglucosaminidase CseA, a peptidoglycan hydrolase. CseA is a multimodular protein that harbors a GH73 domain and six peptidoglycan-binding LysM domains. Recombinant CseA hydrolyzed peptidoglycan and promoted cell separation. Functional analysis of truncated CseA derivatives revealed that the LysM domains play an important role in efficient peptidoglycan degradation and cell separation. Taken together, the results of this study identify CseA as a factor that greatly affects the cluster formation in *T. halophilus*.

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Daughter cell separation; *N*-acetylglucosaminidase; *Tetragenococcus halophilus*; soy sauce; lactic acid bacteria

Soy sauce is a traditional fermented seasoning in East Asia including Japan, and it is currently used worldwide. Japanese style soy sauce is primarily made from soybeans and wheat. First, *koji* mold is mixed with processed soybeans and wheat, which are then cultured in an appropriate environment to make soy sauce *koji*. Then, the soy sauce *koji* is transferred into a tank and mixed with brine to make soy sauce mash, in which the proteins and polysaccharides of the soybeans and wheat are hydrolyzed by enzymes produced by *koji* mold. Subsequently, complex flavors and aromas are created through the successive microbial fermentation. Then, the aged soy sauce mash is pressed through a filter cloth to obtain the soy sauce. The soy sauce mash contains a high concentration of NaCl, which inhibits the growth of microorganisms except for the halotolerant yeast *Zygosaccharomyces rouxii* and the halophilic lactic acid bacterium *Tetragenococcus halophilus*, which are used as a starter for soy sauce fermentation [1].

T. halophilus, a homofermentative lactic acid bacterium, is a dominant bacterium in various salted and fermented foods, including soy and fish sauces [2–4]. In the first stage of soy sauce mash fermentation, *T. halophilus* decreases the pH by producing lactic acid, preparing an appropriate environment for the growth of *Z. rouxii* [5]. It is essential to select appropriate strains of *T. halophilus* used as a starter for soy sauce fermentation, because the quality of the resulting soy sauce depends on the strain of this species used, which has diverse phenotypes with respect to amino acid degradation, carbohydrate utilization, the reduction of redox potentials, and cell cluster formation, which appears similar to cell aggregation [6–13].

The mechanisms of aggregation in various species of lactic acid bacteria have been studied. Aggregation-promoting factors are secreted proteins that are involved in cell adhesion and aggregation [14–16]. Pilus gene clusters are associated with pilus biogenesis in *Lactococcus lactis*, and their overexpression promotes a pili-mediated autoaggregation phenotype [17,18]. In addition to aggregation, another aggregation-like phenotype is also observed due to the incomplete degradation of the septum peptidoglycan (PG) between daughter cells that is caused by a defect in peptidoglycan hydrolase (PGH). PGHs are involved in various bacterial physiological functions, such as autolysis, biofilm formation, sporulation, and cell separation during/after cell division [19]. In *Streptococcus thermophiles*, Cse has been identified as the major enzyme involved in cell separation [20]. Cse contains a putative LysM domain that is involved in cell wall attachment and a putative CHAP domain, which is supposed to be *N*-acetylmuramoyl-L-alanine amidase [20,21]. Indeed, the depletion of Cse protein results in the formation of extremely long chains of cells [21]. Similarly, the putative *N*-acetylglucosaminidase has been reported to play an important role in cell separation in *S. pneumoniae* [22], *S. gordonii* [23], and *Enterococcus faecalis* [24], performing the same function as an *N*-acetylmuramidase in *S. mutans* [25] and an endopeptidase in *Bacillus subtilis* [26–30].

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In 2000, Ueki et al. isolated a small number of cluster-forming strains and a large number of dispersed strains of *T. halophilus* from soy sauce mash [10]. Dispersed strains primarily grow as planktonic pairs or tetrads of cells, whereas those of cluster-forming strains form clusters and show sedimentary growth when cells are cultivated in a static liquid medium. Furthermore, Ueki et al. showed that dispersed strains pass through the filter cloth when soy sauce mash is pressed and cause an increase in turbidity, which increases the burden on the subsequent purification process. In contrast, cluster-forming strains are trapped by the filter cloth and do not increase the soy sauce turbidity [10,11]. Therefore, it is desirable to use a cluster-forming strain as a starter for soy sauce fermentation to improve productivity. However, the mechanism of *T. halophilus* cell cluster formation has not been elucidated, preventing the efficient acquisition and development of cluster-forming strains.

In this study, we attempted to identify the responsible genes for cell cluster formation in *T. halophilus* through genome analysis of dispersed mutants obtained from cluster-forming strains. Our data may contribute to the efficient selection and development of starter strains for soy sauce fermentation and to a better understanding of the molecular basis for the final step of cell division in *T. halophilus*.

Materials and methods

Strains, culture conditions and mutant isolation

The strains used in this study are listed in Table 1. *T. halophilus* was cultured statically at 30°C in De Man, Rogosa and Sharpe (MRS) medium (Becton Dickinson) or GM17 medium (M17 medium (Oxoid) containing 10% glucose instead of lactose) supplemented with or without 10% NaCl. The isolation of cluster-forming strains was performed as described by Ueki et al. [10]. Briefly, soy sauce mash was diluted and plated onto the LA13 agar medium (31). The colonies were cultivated in LA13 medium for 1 week and then the culture was filtered by No. 2 filter paper (Advantec). When the OD_{600} value of the filtrate was ten times less than that of the culture solution, the strain was considered to be a cluster-forming strain. All isolated strains were identified as *T. halophilus* by PCR amplification using a species-specific primer set [31]. To obtain spontaneous mutants that have lost the ability to form cell clusters, all cluster-forming strains were individually subcultured three times in the MRS medium supplemented with 10% NaCl. The emergence of the dispersed mutants was visually detected by a notable increase in culture supernatant turbidity. The cell forms were observed with a phase-contrast microscope BX53 (Olympus). The diameters of the cell clusters were measured using ImageJ (https://imagej.nih.gov/ij/) [32]. The longest chord was recorded as the diameter of each cell cluster. *Escherichia coli* strains were cultured in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) at 37°C, with ampicillin added to a final concentration of 50 μg/mL when necessary.

Sequence analysis

Chromosomal DNA was isolated following standard protocols [33]. The genome sequence of *T. halophilus* YA197 and DM1 was performed by a commercial genome sequence service (Filgen). The genomic libraries (inserts with 300-bp) were sequenced using NovaSeq 6000 (Illumina) with a 2 × 150-bp paired-end sequencing strategy. Approximately 1 Gb of reads was obtained per strain, where the genome coverage was approximately 400-fold for a *T. halophilus* genome size of 2.5 Mb. Preprocessing, assembly, and mutation detection was conducted using Maser [34]. Briefly, short reads of *T. halophilus* YA197 were assembled

Table 1. Strains used in this study.

Strain	Description	Source
Tetragenococcus halophilus		
NBRC 12172	Dispersed strain	National Bio Resource Center
NBRC 100498^T	Dispersed strain	National Bio Resource Center
YA163	Dispersed strain	Isolated from soy sauce mash.
JCM 19268	Dispersed strain	RIKEN BioResource Research Center
JCM 19269	Dispersed strain	RIKEN BioResource Research Center
S1	Dispersed strain	Isolated from soy sauce mash.
YA197	Cluster-forming strain	Isolated from soy sauce mash.
YA5	Cluster-forming strain	Wakinaka et al. [31]
WJ7	Cluster-forming strain	Isolated from fish *nukazuke* (salted and fermented fish with rice bran).
TL1	Cluster-forming strain	Isolated from soy sauce mash.
YG2	Cluster-forming strain	Isolated from soy sauce mash.
YG3	Cluster-forming strain	Isolated from soy sauce mash.
Y4C	Cluster-forming strain	Isolated from soy sauce mash.
DM1	Dispersed strain	YA197 derivative acquired in this study.
DM2	Dispersed strain	YA197 derivative acquired in this study.
Escherichia coli		
DH5α	For plasmid construction	
BL21(DE3)	For protein expression	

using Platanus 1.2.1 [35]. Then, YA197 and DM1 short reads were mapped to the YA197 scaffold using BWA [36], after which mutation detection was performed using GATK Haplotype Caller [37]. The mutation list of YA197 was subtracted from that of DM1 to identify unique mutations in DM1. Potential mutations were manually confirmed using Tablet [38]. The accession numbers of the raw reads are DRR203147 (YA197) and DRR203148 (DM1).

Sanger sequencing was performed by a commercial DNA sequence service (Fasmac). A putative *N*-acetylglucosaminidase gene (TEH_01050; for convenience, this gene and its translated product are referred to as *cseA* and CseA, respectively) was PCR amplified from *T. halophilus* genomic DNA using the primers cseAf and cseAr (Table 2). DNA polymerase KOD FX Neo (Toyobo) was used under the optimal conditions recommended by the manufacturer. The PCR products were separated on a 0.9% agarose gel and purified using the Wizard SV gel and PCR clean-up system (Promega), and the resulting DNA fragment was used as a sequence template. The accession numbers of the *cseA* genes are LC516470 to LC516483.

Plasmid construction

The *cseA* gene was PCR amplified from *T. halophilus* NBRC 12172 and DM1 genomic DNA using the primers cseA_NdeIf and cseA_EcoRIr, which append the restriction sites *Nde*I and *Eco*RI at opposing ends of the PCR products (Table 2). To construct pCD-6× LysM, the PCR products were digested with *Nde*I and *Eco*RI and ligated into the corresponding sites of pColdI (Takara). To construct truncated derivatives of CseA, a portion of the *cseA* gene was PCR amplified from pCD-6× LysM using the primers cseA_NdeIf and cseA_CD_EcoRIr, cseA_NdeIf and cseA_CD-LysM*1 _EcoRIr, cseA_NdeIf and cseA_CD-LysM*2-4_EcoRIr, and cseA_LysM*6_NdeIf and cseA_EcoRIr (Table 2). Each PCR product was digested with *Nde*I and *Eco*RI and ligated into the corresponding sites of pColdI.

Recombinant protein expression and purification

The aforementioned plasmids were introduced into *E. coli* BL21 (DE3) competent cells following standard protocols [33]. To produce recombinant protein, the plasmid-harboring *E. coli* cells were grown in LB medium containing ampicillin at 37°C with shaking. Isopropyl-β-D-thiogalactopyranoside was added to the culture at a final concentration of 1 mM when the OD_{600} of the culture reached approximately 0.5–1.0. Subsequently, the cells were cultivated an additional 20 h at 15°C and then harvested by centrifugation for 1 min at 20,000 × *g*. The cell pellets were washed twice with 100 mM Tris-HCl buffer (pH 7.5) containing 300 mM NaCl and then resuspended in the same buffer. Subsequently, the cells were disrupted by sonication (5 min, on ice), and the lysate was centrifuged for 10 min at 20,000 × *g* at 4°C to remove cellular debris. The recombinant proteins were then purified by His-tag affinity chromatography using Ni sepharose (GE), and the resulting proteins were filtrated with Amicon Ultra filters (Merk Millipore, 10 k MWCO). Subsequently, the purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of PG

PG was prepared as described by Navarre et al. [39] and Yamamoto et al. [40]. *T. halophilus* cells were harvested from a stationary-phase culture by centrifugation for 1 min at 20,000 × *g*, after which the cell pellets were washed with 10% NaCl, resuspended in 4% SDS, and boiled for 30 min before being incubated at room temperature overnight. The suspension was then boiled again for 10 min, and the SDS-insoluble cell wall was collected by centrifugation for 15 min at 20,000 × *g*. The pellet containing the cell wall PG was washed four times with water to obtain PG. Subsequently, purified PG was prepared by treating the PG with 10% trichloroacetic acid at room temperature for 1 day to remove anionic polymers such as wall teichoic acid. Then, the purified PG was collected by centrifugation, washed four times with water, and finally resuspended in water.

Enzyme and protein assays

The turbidimetric assay was conducted essentially as described by Hash [41]. Purified PG was resuspended in 25 mM citric acid buffer (pH 6.0) containing 2 M NaCl at an OD_{595} value of 1.0 and sonicated to

Table 2. Primers used in this study.

Primer name	Sequence (5′→3′)
cseA_NdeIf	AAAACATATGGAGGAAGCAGATAGTAGCGT
cseA_EcoRIr	AAAAGAATTCTTAGTTTACTTTTAAATTTT
cseA_CD_EcoRIr	AAAAGAATTCTTAATTTTCGTCGGTGTTTTCGT
cseA_CD-LysM*1_EcoRIr	AAAAGAATTCTTAAGAATTTCCGGTACCATTTGAG
cseA_CD-LysM*2-4_EcoRIr	AAAAGAATTCTTAACCTGAAGAATTATTAGAATTACTTG
cseA_LysM*6_ NdeIf	AAAACATATGACTGGTGAAGATAATAACTCTGC
cseAf	CTAGCGGCAAACTGCATATA
cseAr	TCACAAATCTCTCCTCCTTG

produce homogenous suspensions. The purified CseA (or water, as a control) was added to 400 μL of PG suspension at a final concentration of 1 μM, and the decrease in turbidity was continuously monitored at OD_{595} at 30-sec intervals for 30 min at 30°C.

The purified protein concentration was estimated by measuring the UV absorbance at 280 nm. The molar absorption coefficients were ε(CD) = 30370, ε(CD + LysM*1) = 44350, ε(CD + LysM*2) = 58330, ε(CD + LysM*3) = 72310, ε(CD + LysM*4) = 86290, ε(CD + LysM*6) = 107260, ε(LysM*6) = 76890, which were calculated based on the deduced amino acid compositions of these proteins [42].

Binding assay

The PG-binding assay was conducted as described by Meanage et al. [43]. The amount of PG was calculated by determining the OD_{540} value, assuming that 1 OD_{540} is equal to 6.45 mg/mL PG. Seven thousand five-hundred micrograms of purified PG or xylan (Sigma) in 30 μL of 25 mM citric acid buffer (pH 6.0) containing 2 M NaCl was incubated with 10 μg of recombinant protein on ice for 15 min. Then, the mixture was centrifuged to separate the pellet (bound fraction) and the supernatant (unbound fraction). Subsequently, the pellet was washed once with 100 μL of citric acid buffer, and the supernatant was concentrated using a centrifugal concentrator (Thermo Fisher). Both fractions (the washed pellet and concentrated supernatant) were resuspended in 50 μL of 10% trichloroacetic acid and incubated on ice for 30 min. After centrifuging, the trichloroacetic acid containing supernatant was discarded, and the insoluble fraction was washed twice with 300 μL of acetone. The acetone was then completely volatilized, and the bound and unbound fractions were resuspended in Laemmli buffer and then heated for 30 min at 70°C. Finally, the samples were centrifuged, and the supernatants loaded onto SDS-PAGE gels, electrophoresed, and then stained with Coomassie brilliant blue.

Results

Cell morphology of *T. halophilus*

Wild strains of *T. halophilus* isolated from soy sauce mash have been shown to include both dispersed and cluster-forming strains [10,11]. To elucidate the molecular mechanism of cluster formation in *T. halophilus*, we first attempted to isolate cluster-forming strains from fermented foods. Isolated strains and strains purchased from culture collection centers were cultured in liquid medium and then the cell forms were assessed using an optical microscope. The NBRC 12172, NBRC 100498^T, YA163, JCM 19268, JCM 19269, and S1 strains generally grew as planktonic cells, most of which were diplococci or tetracocci. The OD_{600} ratio of these cultures after and before filtration was approximately 0.7–0.9, suggesting that they are dispersed strains. In contrast, the YA197, YA5, WJ7, TL1, YG2, YG3, and Y4C strains showed sedimentary growth and formed cell clusters exceeding 10 μm in diameter. The OD_{600} ratio of these cultures after and before filtration was less than 0.1, suggesting that they are cluster-forming strains (Figure 1, Figure S1).

Isolation of dispersed mutants

The identification of the gene (or genes) responsible for cluster-formation in *T. halophilus* may help promote a better understanding of the molecular mechanism of cell separation and aggregation. Because the functional cloning strategies cannot be used with *T. halophilus* due to the absence of an efficient transformation system, we attempted to generate loss- or gain-of-function mutants, which resulted in the isolation of two of dispersed mutants (DM1 and DM2) from the cluster-forming strain YA197. Since dispersed mutants make the supernatant of the medium turbid, the emergence of dispersed mutants in the cultures of cluster-forming strains was easily detected. The obtained mutant strains grew as planktonic cells, most of which were diplococcic or tetracocci, identical to the dispersed strain (Figure 2(a,b)).

To investigate the mechanism underlying the cluster formation, whole-genome sequence analysis on YA197 and DM1 using an Illumina sequencing approach was performed. The short reads obtained for YA197 were assembled, and the resulting 227 scaffolds were used as the reference sequence. Subsequently, the short reads of YA197 and DM1 were aligned to the reference sequence, and the mutations between YA197 and DM1 were identified. This analysis resulted in the identification of only one mutation, a 168-bp deletion in the ORF of the *cseA* gene. CseA comprises a single N-terminal domain and six C-terminal repeat domains. Amino acid sequence homology comparisons revealed that the N- and C-terminal domains represent a putative *N*-acetylglucosaminidase belonging to the glycoside hydrolase (GH) 73 family and a LysM domain belonging to the carbohydrate binding module (CBM) 50, respectively. This gene corresponds to the TEH_01050 *N*-acetylglucosaminidase from the *T. halophilus* NBRC 12172 genome database (https://www.ncbi.nlm.nih.gov/).

We also observed that YA197 harbors a nonsense mutation in the *cseA* gene that results in a truncated protein due to the generation of a stop codon in the N-terminal region of the *N*-acetylglucosaminidase domain, apparently causing CseA to be nonfunctional. Interestingly, the deletion region in strain DM1 spans this nonsense mutation, resulting in a slightly shorter version of CseA that appears to be functional; that is,

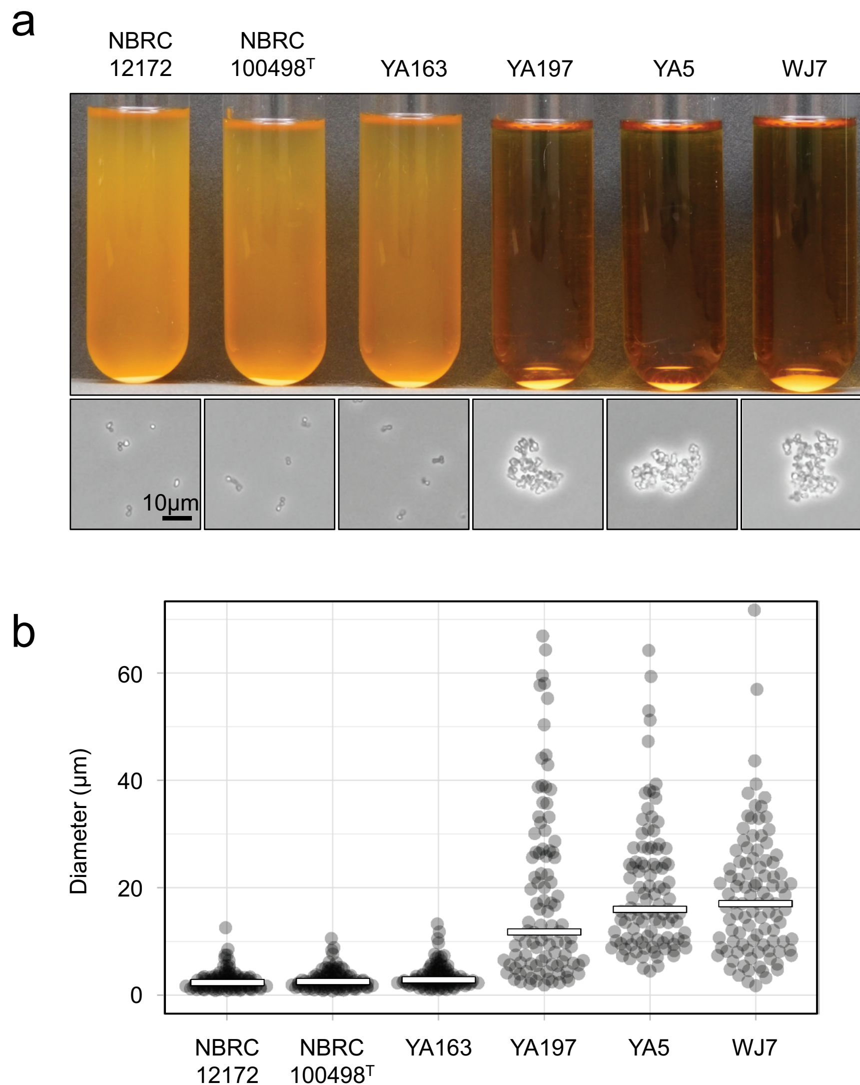

Figure 1. Cell morphology of cluster-forming and dispersed strains. (a) *T. halophilus* NBRC 12172, NBRC 100498^T, YA163 (dispersed strains), and YA197, YA5, WJ7 (cluster-forming strains) were cultured for 2 days in MRS medium supplemented with 10% NaCl, after which images were taken. The cell forms were observed with an optical microscope. The scale bar indicates 10 μm. (b) The diameters of the cell clusters were measured. White bar indicates median, n = 100. The color version of this figure is available online.

DM1 could be a revertant (Figure 2(c)). Sanger sequencing results revealed that the DM2 is also a similar revertant to DM1 (Figure 2(c), Fig. S2). These results suggest that cell cluster formation in *T. halophilus* is attributable to a lack of CseA activity.

Sequence analysis of cseA in cluster-forming and dispersed strains

To determine whether other cluster-forming strains also lack a functional *cseA* gene, we performed a sequence analysis of *cseA* gene in various strains. As expected, the *cseA* gene in other cluster-forming strains contain either a nonsense mutation (strains WJ7, YG2, YG3, and Y4 C), a frameshift mutation (strain TL1), or a transposon insertion (strain YA5), which probably represent loss-of-function mutations (Figure 3). Interestingly, a number of *cseA* genes of dispersed strains contain silent and missense mutations, but these mutations did not appear to significantly reduce protein function. These results suggest that cell cluster formation in *T. halophilus* depends on the presence or absence of a functional CseA.

Cell separation by recombinant CseA

The lack of PGH-encoding genes has been reported to cause incomplete separation of daughter cells [19,20,22,44]. To gain further insight into the molecular function of CseA in cell separation and cluster formation, the *cseA* gene was cloned from the dispersed strain NBRC 12172 and DM1, and heterologously expressed as His-tag fusion protein in *E. coli* BL21(DE3). The recombinant protein was purified by His-tag affinity chromatography and ultrafiltration, and the purified protein was analyzed by SDS-PAGE (Figure 4(a)). To

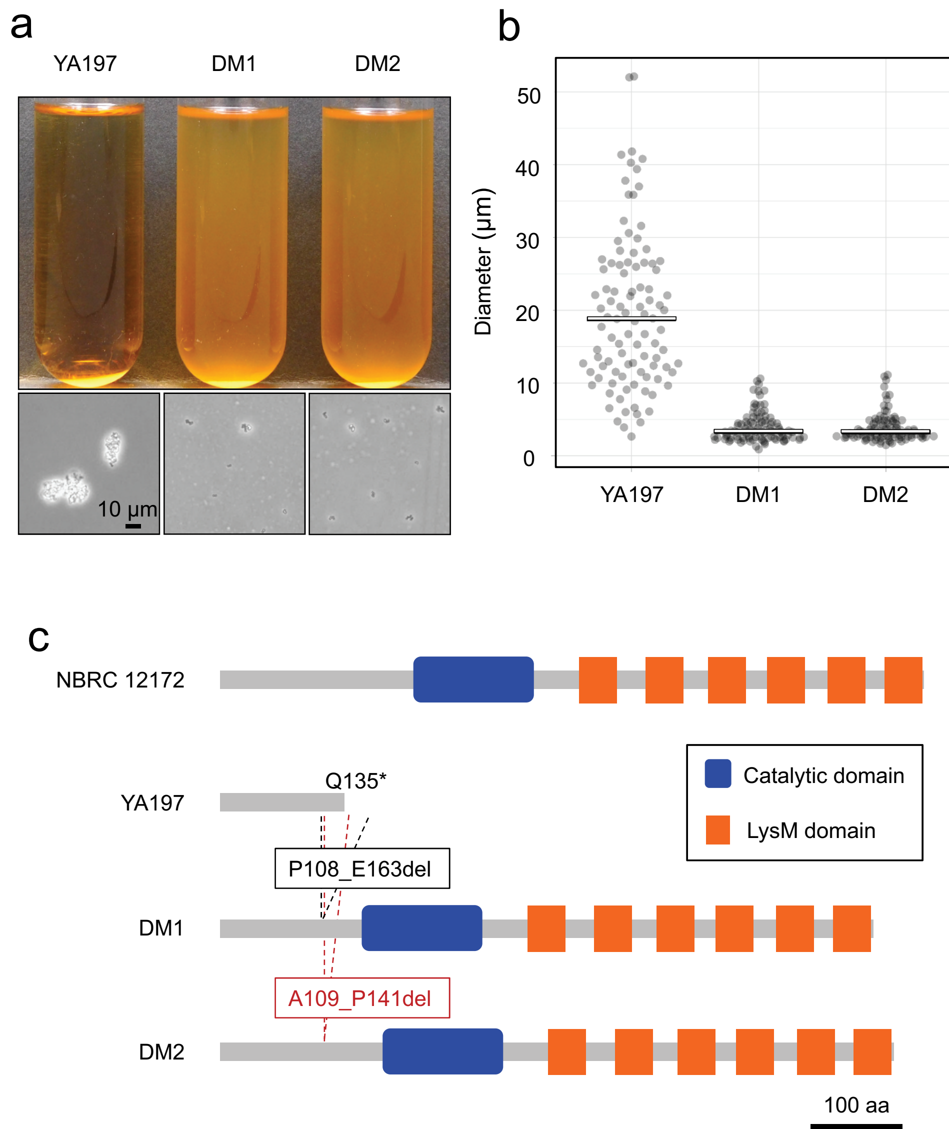

Figure 2. Isolation of dispersed mutants and identification of mutations. (a) *T. halophiluis* YA197 (cluster-forming strain) and DM1 and DM2 (dispersed mutants) were cultured for 2 days in the MRS medium supplemented with 10% NaCl, after which images were taken. The cell forms were observed with an optical microscope. The scale bar indicates 10 μm. (b) The diameters of the cell clusters were measured. White bar indicates median, n = 100. (c) Domains and amino acid positions were assigned by Pfam (https://pfam.xfam.org/). The color version of this figure is available online.

investigate the function of CseA with respect to cell separation, YA197 was cultured in MRS medium with the recombinant CseA. The results showed that YA197 cell clusters were not observed in CseA-containing medium, whereas in medium supplemented with heat-inactivated CseA, cell clusters were observed (Figure 4 (b,c)). Similarly, no cell clusters were observed in the presence of recombinant CseA derived from the dispersed mutant DM1, indicating that CseA of DM1 also maintained cell dispersing activity (Figure S3). Next, we assessed whether the recombinant CseA had PGH activity. PG was prepared from *T. halophilus* and cell wall teichoic acids were chemically removed. As PG degraded, the OD_{595} value decreased to <75% within 30 min (Figure 4(d)). These results suggest that recombinant CseA contributes to daughter cell separation through the degradation of PG in *T. halophilus*, identical to that observed in other Firmicutes [19,20,22,44].

Functional analysis of domains of CseA

The C-terminus of CseA harbors six tandem repeats of the LysM motif, which are general peptidoglycan-binding modules. To further examine the contribution of the LysM domains to cell separation, we constructed six truncated derivatives of CseA (Figure 5(a,b)) that were heterologously expressed and purified from *E. coli* BL21(DE3). The purified proteins were added to MRS medium in which the cluster-forming strain YA197 was cultured. The catalytic domain (CD), CD+LysM*1 and LysM*6 had no or little effect on cell separation. In contrast, the addition of CD+LysM*2, CD+LysM*3, and CD+LysM*4 tended to inhibit the formation of cell clusters depending on the number of LysM domains present (Figure 5(c,d)). In addition, we performed an *in vitro* PG-binding assay using the CD and LysM*6 proteins. Interestingly, the LysM*6 protein specifically

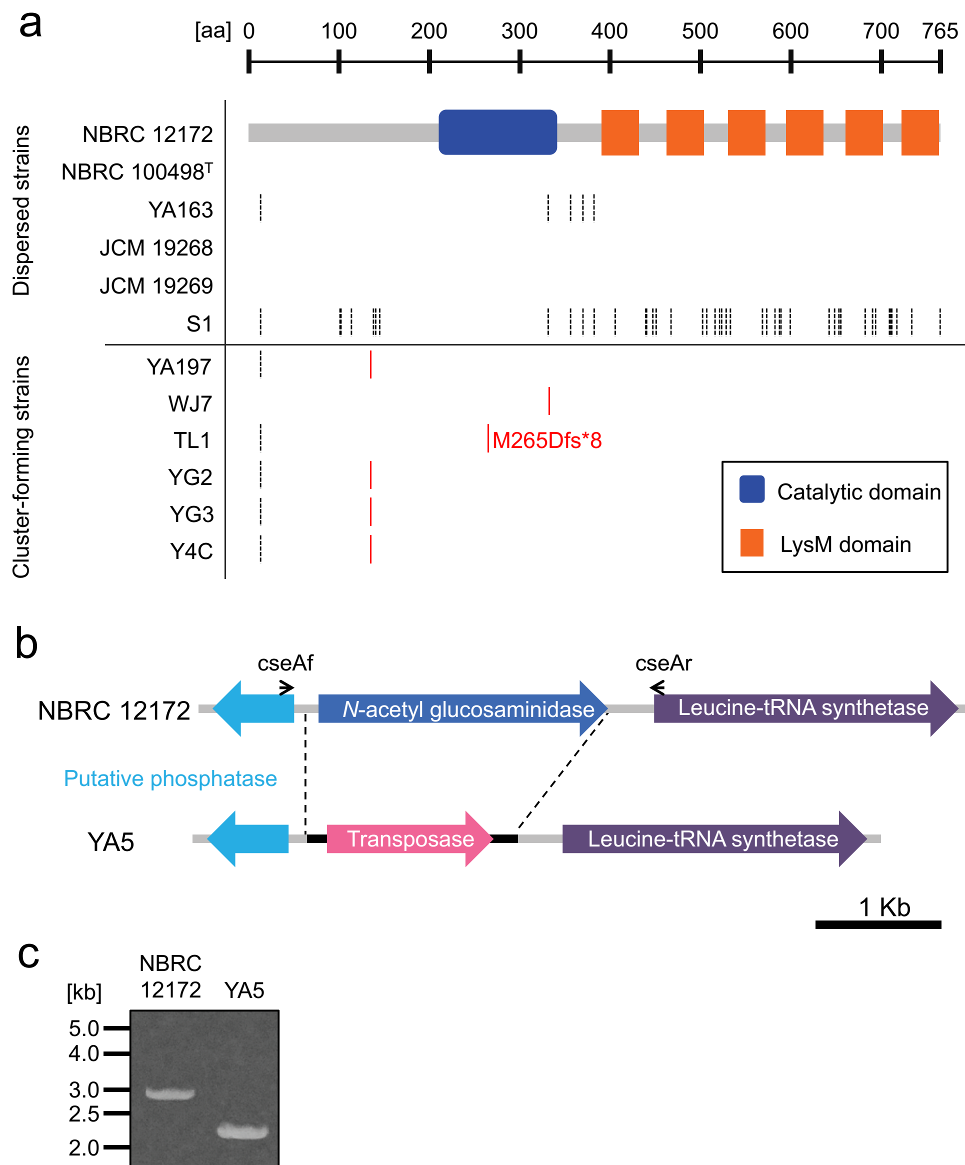

Figure 3. Comparison of CseA between cluster-forming and dispersed strains. (a) The CseA structure of each cluster-forming and the dispersed strain was compared with that of NBRC 12172. The dashed lines indicate a missense mutation. The solid lines indicate nonsense mutations except for TL1, which has frameshift mutation. (b) Schematic representation of the locus of the genes around *cseA* in NBRC 12172 and YA5 are shown. The arrows indicate the primers used in panel (c). (c) The results of a PCR analysis of the region surrounding the *cseA* locus in NBRC 12172 and YA5 is shown. The color version of this figure is available online.

bound to PG, not xylan, whereas the CD protein did not bind to both PG and xylan (Figure 5(e)). The amount of LysM*6 protein in the unbound fraction was decreased by removing teichoic acid from PG via trichloroacetic acid treatment. This result is consistent with that of a previous report in which the PG-binding ability of the LysM domain was shown to be prevented by anionic polymers [40].

NaCl requirement for PGH activity in CseA

During the CseA enzyme assay described above, we observed that the enzymatic activity of CseA is affected by NaCl concentration. To investigate the optimum NaCl concentration for CseA activity, we performed enzyme assays using various concentration of NaCl. The maximum activity of CseA was exhibited in a buffer containing 2 M NaCl, whereas CseA barely hydrolyzed peptidoglycan in a buffer without NaCl (Figure 6(a)). This NaCl requirement of CseA lead us to examine whether the dispersed strains form cell clusters in the absence of NaCl. Since the growth of *T. halophilus* was remarkably poor in MRS medium without NaCl, we used GM17 medium as an NaCl-free medium. The results showed that the dispersed strain NBRC 12172 formed cell clusters in NaCl-free GM17 medium (Figure 6(b,c)), supporting the idea that CseA requires a certain amount of NaCl and that *T. halophilus* cell separation would be dependent on CseA.

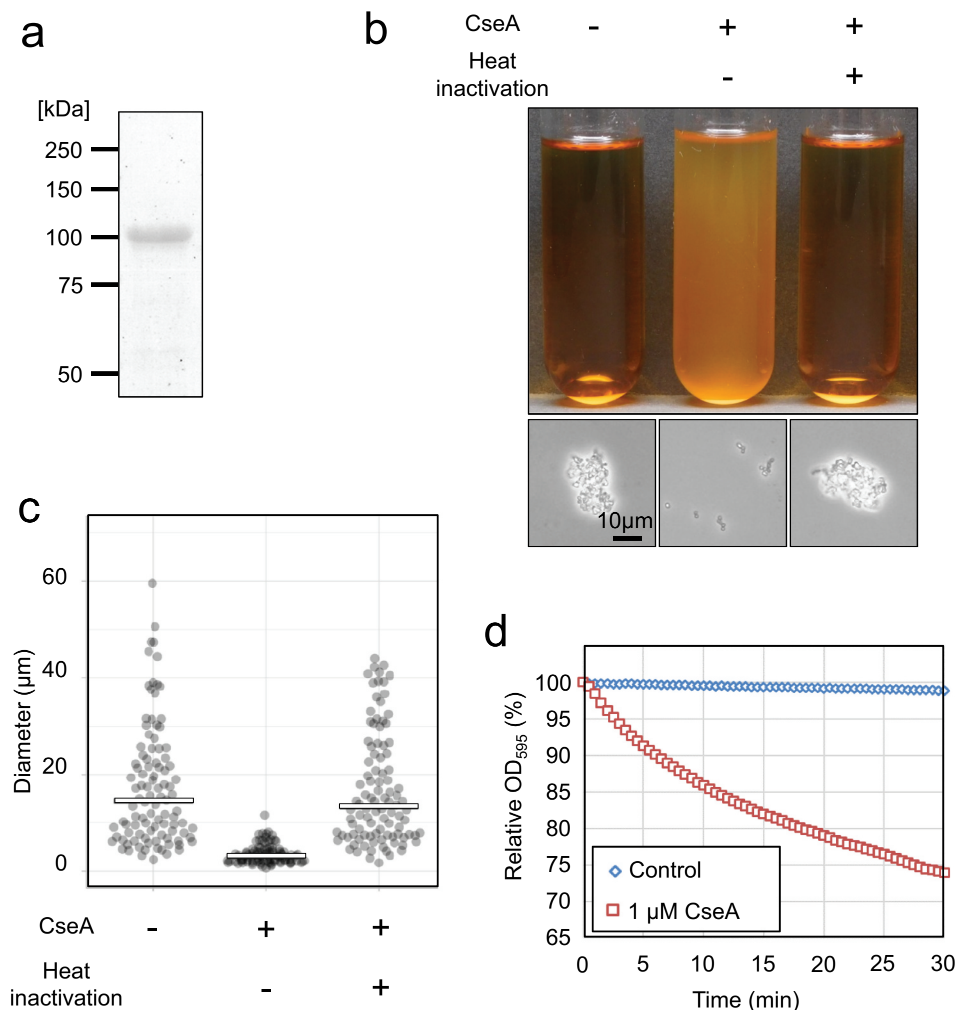

Figure 4. Effect of recombinant CseA addition on cell separation. Heterologous expression and purification of recombinant CseA as a His-tag fusion protein derived from the dispersed strain NBRC 12172 was performed. (a) The purified protein was analyzed by SDS-PAGE. (b) *T. halophilus* YA197 was cultured for 2 days in MRS medium supplemented with 10% NaCl with or without 50 nM recombinant CseA, after which images were taken. The heat inactivation step was performed in an autoclave (121°C, 15 min). The cell forms were observed with an optical microscope. The scale bar indicates 10 µm. (c) The diameters of the cell clusters were measured. White bar indicates median, n = 100. (d) The enzyme assay was performed using PG derived from *T. halophilus*. The decrease in turbidity was continuously monitored at OD_{595} for 30-sec intervals over 30 min at 30°C. The color version of this figure is available online.

Discussion

In this study, *T. halophilus* cluster formation was shown to be caused by a failure of daughter cell separation due to a nonfunctional CseA, one of the multiple PGHs in *T. halophilus*. The results of numerous studies indicate that a lack of one or several PGHs related to cell separation (such as *N*-acetyl-β-D-muramidase, *N*-acetyl-β-D-glucosaminidase, *N*-acetylmuramyl-L-alanine amidase, and endopeptidase) leads to the formation of clusters of unseparated cells [19–23,25,44]. Our experimental data in *T. halophilus* show that CseA-deficient strains form cell clusters that can be dispersed by the addition of purified CseA enzyme (Figure 4(b,c)). Although the current absence of an efficient gene knock-out system in this species prevents further confirmation of the *cseA* function in cell separation, it is reasonable to suggest that CseA plays a major role in *T. halophilus* cell separation on the basis of our data and the previous findings obtained for various bacteria.

Most Firmicutes PGHs are characterized by their modular structure and are composed of at least two domains, one having catalytic activity and the other containing a recognition/cell wall binding domain [45]. The gene *cseA* is responsible for cell separation in *T. halophilus*, and encodes a multimodular protein comprising a GH73 domain and six C-terminal peptidoglycan-binding LysM domains. Most GH73 family members exhibit *N*-acetylglucosaminidase activity in which the GlcNAc-β1, 4-MurNAc linkages of PG are cleaved to release GlcNAc reducing ends. The GH73 family is divided into 5 subgroups based on their sequence similarity [46], with CseA belonging to subgroup 2, members of which have two conserved carboxylate residues involved in the classical inverting acid-base catalytic mechanism [46]. Subgroup 2 also includes the

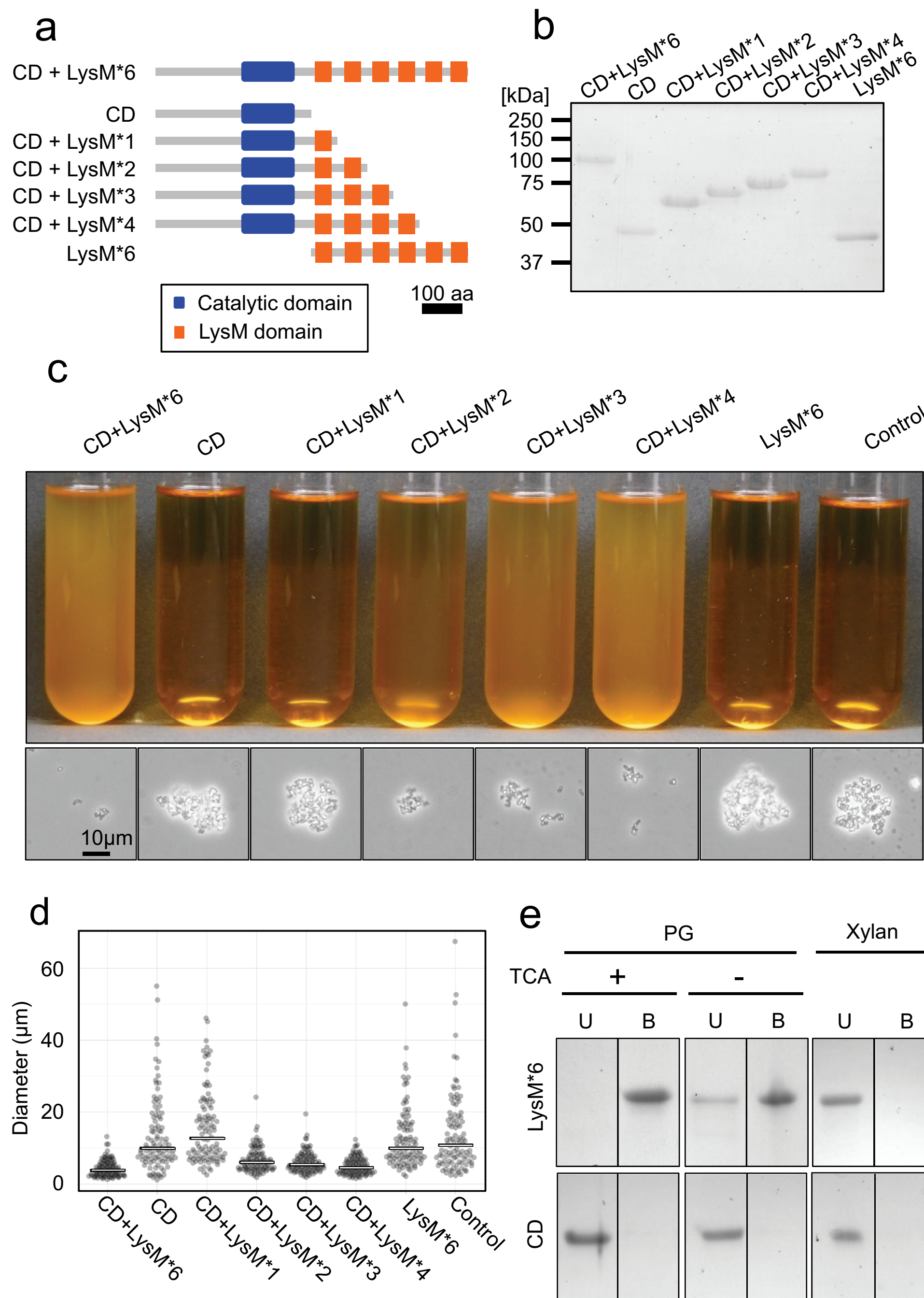

Figure 5. Heterologous expression of CseA mutant proteins and effect on cell separation. (a) Schematic representation of the structure of each CseA mutant protein is shown. (b) The heterologously expressed and purified proteins were analyzed by SDS-PAGE. (c) *T. halophilus* YA197 was cultured for 2 days in MRS medium supplemented with 10% NaCl with 50 nM each mutant protein except for control, in which no CseA mutant proteins were used. The cell forms were observed with an optical microscope. The scale bar indicates 10 μm. (d) The diameters of the cell clusters were measured. White bar indicates median, n = 100. (e) Binding assays were performed using PG, PG without cell wall teichoic acid, or xylan as substrates. U and B indicate the unbound and bound fractions, respectively. The color version of this figure is available online.

Enterococcus faecalis enzyme AtlA, which is structurally and functionally similar to CseA and comprises an N-terminal GH73 *N*-acetylglucosaminidase domain and six C-terminal of LysM domains. Furthermore, AtlA is an important enzyme during cell separation, having the identical function as CseA [24].

In this study, we showed that LysM domains are necessary to allow CseA to bind PG and that multiple LysM domains increase the efficiency of cell separation. This phenomenon may be due to individual LysM modules binding in a cooperative manner to long PG molecules [43], where multiple LysM domains enable efficient enrichment of the enzyme on PG. The *in vitro* binding assay results showed that the binding capacity of multiple LysM domains toward peptidoglycan was increased by removing cell wall teichoic acid. The subcellular localization of gram-positive PGHs appears to be controlled by teichoic acid components of the cell wall layer [47], and cell wall-associated teichoic acid is not present or it is

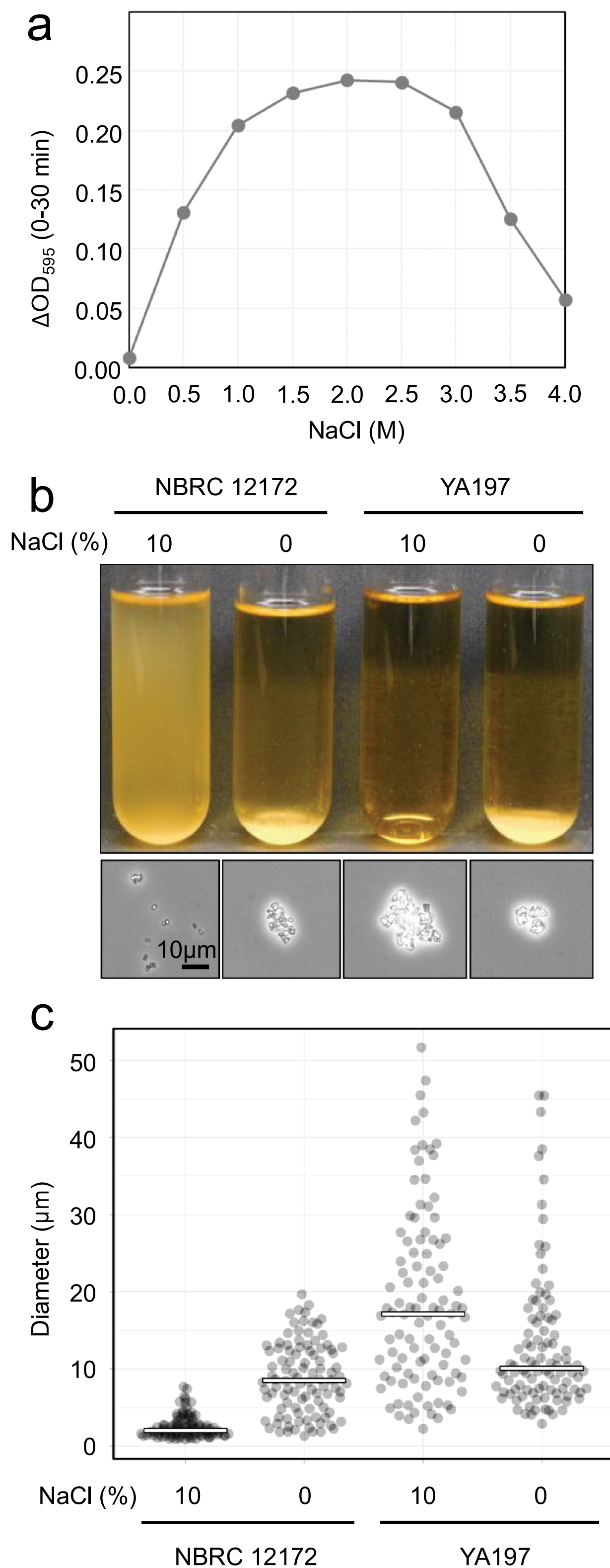

Figure 6. NaCl requirement of CseA. (a) The correlation between NaCl concentration in citrate buffer and CseA enzymatic activity was measured. (b) *T. halophilus* NBRC 12172 and YA197 were cultured for 2 days in GM17 medium containing 10 or 0% NaCl, after which images were taken. The cell forms were observed with an optical microscope. The scale bar indicates 10 μm. (c) The diameters of the cell clusters were measured. White bar indicates median, n = 100. The color version of this figure is available online.

not yet fully polymerized in septal PG [48]. The CseA properties that favor its binding to unmodified peptidoglycan will allow the efficient degradation of septal PG and efficient cell separation. Thus, multiple LysM domains may be essential for the biological function of CseA.

Interestingly, we observed that CseA required a high concentration of NaCl to exert sufficient PGH activity and that the dispersed strain NBRC 12172 also formed a cell cluster at low NaCl concentrations (Figure 6). The latter phenomenon was also observed by Ueki et al. [10]. Thus, the NaCl requirement of CseA, which is responsible for cell separation, accounts for cell cluster formation by dispersed strains under low NaCl conditions. Does NaCl-dependent cell separation in halophilic lactic acid bacteria correlate with some physiological role or environmental adaptation? Bacterial self-aggregation is considered to be a means of protection from environmental stress [49,50]. Therefore, cell cluster formation under low NaCl conditions in *T. halophilus* may be a potential strategy to survive in low salt environments that contain many competing microorganisms. To our knowledge, no PGHs belonging to the GH73 family that exhibit NaCl requirements have been reported so far. Further structural and functional studies of the CseA and comparative analysis of CseA with PGH of other bacteria will be required to elucidate this unique property.

T. halophilus harbors several putative PGH-encoding genes besides *cseA*. For example, TEH_13460 encodes an *N*-acetylglucosaminidase with a LysM domain, similar to CseA, while TEH_08900 and TEH_17920 encode *N*-acetylmuramoyl-L-alanine amidases with a SH3 domain, which is reported to possess PG-binding activity like that observed for LysM [45,51]. The possibility remains that these enzymes contribute to cell separation with CseA in a cooperative manner. Thus, additional studies are needed to elucidate the detailed molecular mechanisms of cell separation in *T. halophilus*.

In terms of practical application, the spontaneous emergence of dispersed mutants from cluster-forming strains had cast doubt on the stability of the cluster-forming phenotype. However, dispersed mutants have not yet been obtained except for YA197 derivatives, despite of repeated subcultivation. Therefore, the cluster-forming phenotype is generally stable and is unlikely to cause any practical problems as soy sauce brewing starter.

Author contribution

Designed the research: DS JW. Performed the experiments DS JW. Analyzed the data DS JW. Wrote the paper DS TW JW.

Disclosure statement

No potential conflict of interest was reported by the authors.

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