Glycoside Hydrolase DisH from *Desulfovibrio vulgaris* Degrades the *N*-Acetylgalactosamine Component of Diverse Biofilms

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SIGNIFICANCE

The global costs of corrosion are more than $2.5 trillion every year (3.4% of the global gross domestic product), and a large part of corrosion (30%) is microbiologically influenced corrosion (MIC), which affects oil production, drinking water systems, and pipelines. MIC is commonly caused by sulfate-reducing bacteria (SRB) biofilms, and *Desulfovibrio vulgaris* is the model organism. The biofilm matrix of *D. vulgaris* consists of proteins and polysaccharides of mannose, fucose, and *N*-acetylgalactosamine (GalNAc). However, little is known about how to control its biofilm formation. Since bacteria must degrade their biofilm to disperse, in this work, we identified and then studied predicted secreted glycoside hydrolases from *D. vulgaris.* We show here that DisH (DVU2239, dispersal hexosaminidase), a previously uncharacterized protein, is an *N*-acetyl-β-*D*-hexosaminidase that disperses the *D. vulgaris* biofilm and inhibits biofilm formation. DisH is the first glycoside hydrolase that has both GalNAc-ase/GlcNAc-ase degradation activities for diverse biofilms. Hence, we have found a key factor controlling *D. vulgaris* biofilm formation.

ABSTRACT

Biofilms of sulfate-reducing bacteria (SRB) produce H_2S, which contributes to corrosion. Although bacterial cells in biofilms are cemented together, they often dissolve their own biofilm to allow the cells to disperse. Using *Desulfovibrio vulgaris* as a model SRB, we sought polysaccharide-degrading enzymes that disperse its biofilm. Using a whole-genome approach, we identified eight enzymes as putative extracellular glycoside hydrolases including DisH (DVU2239, dispersal hexosaminidase), an enzyme that we demonstrated here, by utilizing various *p*-nitrooligosaccharide substrates, to be an *N*-acetyl-β-*D*-hexosaminidase. For *N*-acetyl-β-*D*-galactosamine (GalNAc), V_{max} was 3.6 μmol of *p*-nitrophenyl/min/(mg protein) and K_m was 0.8 mM; the specific activity for *N*-acetyl β-*D*-glucosamine was 7.8 μmol of *p*-nitrophenyl/min/(mg protein). Since GalNAc is one of the three exopolysaccharide matrix components of *D. vulgaris,* purified DisH was found to disperse 63 ± 2% biofilm as well as inhibit biofilm formation up to 47 ± 4%. The temperature and pH optima are 60°C and pH 6, respectively; DisH is also inhibited by

copper and is secreted. In addition, since polymers of GalNAc and GlcNAc are found in the matrix of diverse bacteria, DisH dispersed biofilms of *Pseudomonas* aeruginosa, *Escherichia coli*, and *Bacillus subtilis*. Therefore, DisH has the potential to inhibit and disperse a wide-range of biofilms.

INTRODUCTION

Sulfate-reducing bacteria (SRB) are bacteria that obtain energy by reducing sulfate to hydrogen sulfide (H_2S) (Heidelberg *et al.*, 2004). This reaction by SRB can cause microbially induced corrosion (MIC) in anaerobic environments (Enning and Garrelfs, 2014). Annual global corrosion costs are around 3.4% of the global gross domestic product ($2.5 trillion (Koch *et al.*, 2016)), and biocorrosion is responsible for up to 30% of all corrosion losses, especially those in oil production, drinking water systems, pipelines, steel piling in quays, harbors and jetties (Beech and Sunner, 2007; Koch *et al.*, 2016). For example, SRB corrode buried gas transmission pipelines as well as cause reservoir souring by their H_2S production (Priha *et al.*, 2013; Enning and Garrelfs, 2014). As the MIC is commonly caused by SRB biofilms (Lee *et al.*, 1995), inhibiting and dispersing their biofilms is imperative.

Bacterial biofilms are cells embedded in a dense extracellular matrix; the intensively-studied *Pseudomonas aeruginosa* and *Escherichia coli* biofilms indicate that these matrixes are usually composed of exopolysaccharides (EPS) (Franklin *et al.*, 2011), extracellular DNA (Whitchurch *et al.*, 2002), and proteins (Fong and Yildiz, 2015). *Desulfovibrio vulgaris* Hildenborough is a Gram-negative model SRB whose biofilm consists primarily of proteins (Clark *et al.*, 2007), while the biofilm of another *Desulfovibrio* species, *D. desulfuricans*, consists of EPS that includes glucose (major carbohydrate), mannose, and galactose (Beech *et al.*, 1991). Our work has determined that the biofilm of *D. vulgaris* Hildenborough includes mannose, fucose, and *N*-acetyl β-*D*-galactosamine (GalNac) (Poosarla *et al.*, 2017).

Due to changing environmental conditions (e.g., shortage or abundance of nutrients) (Karatan and Watnick, 2009; Wood, 2014), bacteria frequently degrade their own biofilms so they may disperse (Karatan and Watnick, 2009; Kaplan, 2010); this requires secreting enzymes. Mimicking this natural

event, EPS-degrading enzymes (Itoh *et al.*, 2005; Yu *et al.*, 2015), proteases (Clark *et al.*, 2007), DNase (Whitchurch *et al.*, 2002) and some small molecular inhibitors (Sambanthamoorthy *et al.*, 2014) have been used successfully to degrade the biofilm matrix and cause dispersal. For example, the matrix of the model *P. aeruginosa* biofilm consists of alginate (Gacesa and Russell, 1990), Pel polysaccharide (Jennings *et al.*, 2015), Psl polysaccharide (Jackson *et al.*, 2004), and extracellular DNA (Whitchurch *et al.*, 2002; Jennings *et al.*, 2015). Hence, the deacetylase PelA and glycoside hydrolase PslG disperse *P. aeruginosa* biofilms (Yu *et al.*, 2015; Baker *et al.*, 2016); Pel EPS consists of *N*-acetyl β-*D*-glucosamine (GlcNAc) and GalNAc (Jennings *et al.*, 2015) and PelA removes acetyl groups (Colvin *et al.*, 2013), whereas Psl EPS consists of mannose, rhamnose, and glucose (Byrd *et al.*, 2009), and PslG binds to mannose suggesting it degrades the mannose glycosidic linkage in the EPS (Baker *et al.*, 2015). Similarly, DNase disperses early *P. aeruginosa* biofilms (Whitchurch *et al.*, 2002). Dispersin B from *Actinobacillus actinomycetemcomitans*, an *N*-acetyl β-*D*-glucosaminidase, degrades its matrix (Kaplan *et al.*, 2003; Ramasubbu *et al.*, 2005) as well as the matrix *Staphylococcus epidermidis*, *Escherichia coli*, *Yersinia pestis* and *P. fluorescens* (Itoh *et al.*, 2005).

However, enzymes from SRB capable of dispersing its biofilm by degrading the polysaccharide components of its biofilm have not been investigated; biofilm dispersal as a result of protease addition has been shown (Clark *et al.*, 2007). In this work, based on a genomic analysis, we identified eight putative enzymes secreted by *D. vulgaris* Hildenborough capable of dispersing its biofilm by degrading the polysaccharide component of its matrix. Specifically, we demonstrated DVU2239 is an *N*-acetyl β-*D*-hexosaminidase, which has the novel feature that it hydrolyzes the linkages of both GalNAc and GlcNAc; we suggest the name DisH for dispersal hexosaminidase for DVU2239. Critically, DisH inhibits biofilm formation as well as disperses a pre-formed biofilm of *D. vulgaris* when supplied exogenously. We also determined the temperature and pH optima of this enzyme as well as its thermostability and pH stability. Since the biofilm matrix of *E. coli* (Wang *et al.*, 2004), *Bacillus subtilis* (Roux *et al.*, 2015), and *Staphylococcus aureus* (Cramton *et al.*, 1999) include GlcNAc polymers, the ability of DisH to disperse existing biofilms of these strains (along with biofilms of *P. aeruginosa* which has utilizes both GalNAc

and GlcNAc polymers as indicated above) was investigated.

RESULTS

Bioinformatics to identify putative glycoside hydrolases. Since EPS-degrading enzymes are sometimes encoded by EPS biosynthesis loci (Schiller *et al.*, 1993; Friedman and Kolter, 2004; Jackson *et al.*, 2004), we first investigated the *D. vulgaris* genome to identify EPS biosynthesis gene loci by gene annotation analysis and found nine putative EPS operons (**Table S1**). These operons include genes that likely contain enzymes for utilizing fucose (e.g., *dvu0090* and *dvu0074*), for utilizing GalNAc (e.g., *dvu0319*), and for utilizing mannose (e.g., *dvu0685* and *dvu0697*); however, we did not find any glycoside hydrolase genes in these loci. Recognizing that that these EPS-degrading enzymes must be secreted, we focused on those genes that encode proteins with signal sequences by utilizing SignalP 4.1 and found 394 putative secreted proteins. By using whole genome protein domain analysis with the NCBI Conserved Domain Search Service, we determined that *D. vulgaris* has 19 putative glycoside hydrolase family proteins. By combining the results of both searches, eight genes which possibly encode extracellular EPS degradation enzymes were identified: *dvu0041*, *dvu0677*, *dvu1128*, *dvu1536*, *dvu2202*, *dvu2239* (*disH*), *dvu2699*, and *dvu3205*.

Protein purification. According to the signal peptide prediction result, the scores of proteins DVU0041 and DVU1536 were lower (data not shown), so the DNA fragments of the remaining six candidate genes (*dvu0677*, *dvu1128*, *dvu2202*, *disH*, *dvu2699*, and *dvu3205*), were amplified from genomic DNA of *D. vulgaris* for expression in *E. coli* without the signal sequence and with an N-terminal His tag; these proteins were named $DisH_{48\text{-}481}$, $DVU3205_{36\text{-}481}$, $DVU0677_{28\text{-}349}$, $DVU1128_{32\text{-}221}$, $DVU2202_{35\text{-}505}$, and $DVU2699_{31\text{-}215}$ (**Table 1** and **Table 2**). Of these six, $DisH_{48\text{-}481}$ was successfully purified as a soluble protein and studied further ($DVU3205_{36\text{-}481}$ and $DVU0677_{28\text{-}349}$ were also soluble, whereas $DVU1128_{32\text{-}221}$, $DVU2202_{35\text{-}505}$, and $DVU2699_{31\text{-}215}$ were insoluble). $DisH_{48\text{-}481}$ was 47 kDa as expected (total 441 aa with His tag) and the purity was 90% (**Fig. S1A**). To confirm correct protein folding, circular dichroism was performed with bovine serum albumin (BSA) as the positive control and denatured protein as a negative

control; this analysis showed $DisH_{48-481}$ has good secondary structure indicating that it was folded correctly (**Fig. S1B**).

DisH is a family 3 glycoside hydrolase (**Fig. 1A**) and shares the highest amino acid identity (36%) with glycoside hydrolase NagZ from *Bacillus subtilis* (PDB number 4GYK). Notably, the homolog of NagZ in *Neisseria gonorrhoeae* (35% amino acid identify with DisH) triggers biofilm dispersal (Bhoopalan *et al.*, 2016). Furthermore, DisH has a conserved histidine (His^{248}) and aspartic acid (Asp^{246}) residue with NagZ as the catalytic acid/base residues (**Fig. 1B**). In addition, the protein sequence of DisH is highly conserved in the genomes of *D. vulgaris* strains (100% amino acid identity among the strains Hildenborough, DP4, and RCH1 and 59% identity in strain Miyazaki F). Furthermore, DisH is also conserved in other *Desulfovibrio* species; for example, there is 52%-54% amino acid identity in *Desulfovibrio africanus* strains, 59% identity in *Desulfovibrio termitidis*, and 53% identity in *Desulfovibrio* sp. A2. These bioinformatics results suggest that there may be a general biofilm dispersal mechanism in *Desulfovibrio* spp.

Enzyme activities of DisH. To determine the substrate of DisH, enzyme activity assays were performed with six different nitrooligosaccharide substrates: 4-nitrophenyl β-*D*-glucopyranoside, 4-nitrophenyl *N*-acetyl-β-*D*-galactosaminide, 4-nitrophenyl *N*-acetyl-β-*D*-glucosaminide, 4-nitrophenyl β-*D*-mannopyranoside, 4-nitrophenyl β-*D*-fucopyranoside, and 2-nitrophenyl β-*D*-galactopyranoside. In the assay, the enzyme hydrolyzes the *p*-nitrophenyl group from the substrate which results in the formation of a colorimetric (400 nm) product that is proportional to the activity present. $DisH_{48-481}$ hydrolyzed the glycosidic linkages of GlcNAc (7.8 ± 0.4 μmol of *p*-nitrophenyl (pNP)/min/(mg protein)), GalNAc (3.7 ± 0.4 μmol of pNP/min/(mg protein)), and β-*D*-glucose (0.051 ± 0.001 μmol of pNP/min/(mg protein)), while it does not have activity on mannose, fucose and galactose (**Table 3**). The positive control was commercial β-glucosidase (6 U/mg, Sigma #49290, diluted to 0.01 μmol of pNP/min/mg), which hydrolyzes the glycosidic linkages of β-*D*-glucose and β-*D*-fucose (e.g., 4-nitrophenyl β-*D*-glucopyranoside and 4-nitrophenyl β-*D*-fucopyranoside), and the negative control was reaction mixture without enzymes but with buffer. Hence, DisH is an *N*-acetyl β-*D*-hexosaminidase (Hex, E.C. 3.2.1.52).

For 4-nitrophenyl *N*-acetyl-β-*D*-galactosaminide, the V_{max} was 3.6 µmol *p*-nitrophenyl/min/(mg protein), and the K_m was 0.8 mM (**Fig. 2A**).

Since $DisH_{48-481}$ showed glycosidase activity, the effect of metals on the enzyme activity was investigated. Among Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} ions, Cu^{2+} inhibited the enzyme activity of $DisH_{48-481}$, and the half maximal inhibitory concentration was 0.6 mM (**Table 4** and **Fig. 2B**).

The optimal temperature for $DisH_{48-481}$ was 60°C, and the activity increased 4.5 times higher above that at 37°C (**Fig. 2C**). For thermostability, $DisH_{48-481}$ maintained 80% activity when incubated at 50°C for one hour (**Fig. 2D**). The optimal pH of $DisH_{48-481}$ was 6 (**Fig. 2E**). The enzyme was stable for about one hour at pH 6 to 9 in the absence of the substrate (**Fig. 2F**).

SRB biofilm inhibition by $DisH_{48-481}$. Purified $DisH_{48-481}$ was evaluated for its ability to inhibit *D. vulgaris* biofilm formed in modified Baar's medium at 30°C for 24 hours. Protease from *Bacillus* sp. (savinase, Sigma Cat #P3111, stock conc. 16 U/mL) was used as a positive control (Clark *et al.*, 2007), and both inactivated $DisH_{48-481}$ and PBS buffer (pH 7.4) were used as negative controls. After testing a series of concentration of $DisH_{48-481}$ for biofilm dispersal and inhibition, the optimum working concentration was found to be 50 ng/µL (data not shown) which is the same as the concentration of protease used previously (Clark *et al.*, 2007). $DisH_{48-481}$ at 50 ng/µL inhibited 47 ± 4 % of the biofilm formation and was as effective as the protease control (**Fig. 3**).

SRB biofilm dispersal by $DisH_{48-481}$. Since biofilm of *D. vulgaris* consists partially of GalNAc (Poosarla *et al.*, 2017), $DisH_{48-481}$ was investigated for its ability to disperse *D. vulgaris* biofilms. For 24-h biofilms formed at 30°C in modified Baar's medium, planktonic cells were removed and 50 ng/µL (Itoh *et al.*, 2005) of $DisH_{48-481}$ was added in non-nutritive buffer to the biofilm and incubated at 30°C for 2 to 18 h. Also, protease (savinase) was used as a positive control and both inactivated $DisH_{48-481}$ and PBS buffer (pH 7.4) were used as negative controls. $DisH_{48-481}$ dispersed 32 ± 5% of a 24 hours old biofilm in two h, and 63 ± 2% in 18 hours (**Fig. 4A** and **4B**) whereas the protease control dispersed 71 ± 3 % of a 24 hours old biofilm in two hours. Therefore, overall, $DisH_{48-481}$ removed comparable levels of SRB biofilm.

Diverse biofilm inhibition by $DisH_{48-481}$. Purified $DisH_{48-481}$ was also evaluated for its ability to inhibit

biofilms of *P. aeruginosa, E. coli, B. subtilis*, and *S. aureus* formed in LB and M9G medium at different temperatures and ages since the biofilms of these strains include GalNAc and GlcNAc polymers. Protease (savinase) was used again as a positive control as well as heat-inactivated $DisH_{48\text{-}481}$ and PBS buffer as negative controls.

When final 50 ng/μL $DisH_{48\text{-}481}$ was added at the beginning of the biofilm formation of *P. aeruginosa* PA14, after incubating at 26°C for 10 h (Itoh *et al.*, 2005), DisH inhibited 85 ± 1% of the biofilm formation (**Fig. 4A**). For *E. coli* TG1, after incubating at 37°C for 24 h, DisH inhibited 35 ± 4% of the biofilm formation (**Fig. 4B**). For *B. subtilis* and *S. aureus*, after incubating at 37°C and 30°C for 24 h, $DisH_{48\text{-}481}$ inhibited 42 ± 4% and 79 ± 7%, respectively, of biofilm formation (**Fig. 4C** and **4D**).

Diverse biofilm dispersal by $DisH_{48\text{-}481}$. Since $DisH_{48\text{-}481}$ inhibited the biofilm formation of non-SRB bacteria, biofilm dispersal was investigated with the same biofilm formation conditions. After the biofilms were formed, planktonic cells were removed and $DisH_{48\text{-}481}$ was added at 50 ng/μL in non-nutritive PBS buffer and incubated at the same temperature for 2 h. Two hours was chosen for the enzyme reaction since longer dispersal time's result in artifacts related to biofilm growth. In addition, protease (savinase), inactivated $DisH_{48\text{-}481}$ and PBS buffer (pH 7.4) were used as controls.

$DisH_{48\text{-}481}$ dispersed 52 ± 6% of the pre-formed biofilm of *P. aeruginosa* PA14 (**Fig. 5A**), 40 ± 7% of the pre-formed biofilm of *E. coli* TG1 in 2 h (**Fig. 5B**), and 42 ± 8% of the pre-formed biofilm of *B. subtilis* (**Fig. 5C**). Although $DisH_{48\text{-}481}$ inhibited the biofilm formation of *S. aureus*, it did not disperse its pre-formed biofilm (**Fig. 5D**).

DisH is secreted. To investigate whether DisH is secreted, we cloned *disH* with its native signal sequence, via vector pET-27b(+)-DisH (full-length), then performed both a Western and enzyme assays. We utilized *E. coli* since it is Gram negative like *D. vulgaris.* For the Western blot, both full-length and leaderless DisH were found in whole cells, while only leaderless DisH was present in the 10× concentrated supernatant (**Fig. S2B**). No DisH was found in the negative control (empty plasmid 10× concentrated supernatants, lane 5).

Corroborating these results, the same samples that were used for the Western were assayed for DisH

enzyme activity. Cells that utilize the plasmid that produces $DisH_{48-481}$ that lacks a signal sequence, pET-27b(+)-His-$DisH_{48-481}$, and the corresponding empty plasmid, pET-27b(+)), were also utilized. Using empty plasmid strains as negative controls, supernatants of *E. coli* producing DisH with its signal sequence had an activity of 0.75 ± 0.06 nmol of PNP/min/mL, which indicates that full-length DisH protein is secreted. Based on the enzyme activity, DisH in the supernatant was 23 ± 2% of the activity in found from whole-cell lysates. Furthermore, the supernatant of *E. coli* producing $DisH_{48-481}$ (DisH without a signal sequence) had no activity, which shows the leader sequence is required for secretion (**Table S2**). Moreover, since there was no enzyme activity detected in supernatants of the leaderless DisH but robust activity was detected with the same construct for the cell lysates, the DisH that was detected in the supernatants using the full-length construct are not from cell lysis but instead arise by secretion (after cleavage of the signal sequence).

DISCUSSION

In this work, we identified, through bioinformatics for secreted proteins, a putative glycoside hydrolase, DisH, of the representative SRB *D. vulgaris.* Unlike the alginate lyase gene *algL* (Schiller *et al.*, 1993), the glycoside hydrolases *pslG* (Jackson *et al.*, 2004), and *pelA* (Friedman and Kolter, 2004) of *P. aeruginosa,* no EPS-degrading genes were found in the nine putative EPS biosynthesis loci we identified in *D. vulgaris* (**Table S1**). We then purified $DisH_{48-481}$ and determined it is an *N*-acetyl β-*D*-hexosaminidase that both inhibits and disperses *D. vulgaris* biofilms. Since over 60% (18 h incubation) of the *D. vulgaris* biofilm was dispersed by DisH, GalNAc is a major building block of the biofilm matrix. Hence, we identified a potent, novel means to disperse corrosion-causing biofilms by mimicking the way the bacterium controls its own biofilm formation.

DisH has significant activity for both GalNAc and GlcNAc like most of *N*-acetyl β-*D*-hexosaminidases from fungi and bacteria where the ratio of activities is commonly in the range of 1.5 to 4.0 (Horsch *et al.*, 1997) (DisH is 2.1). Hence, DisH holds much promise for dispersing biofilms from other non-SRB bacteria. To demonstrate the utility of DisH for altering biofilm formation, we evaluated

both biofilm inhibition and dispersal with four diverse bacterial species. We found DisH disperses well the biofilm of *P. aeruginosa* PA14, which has a biofilm that includes a GalNAc/GlcNAc polymer matrix (Jennings *et al.*, 2015) and that DisH nearly completely inhibited the biofilm formation of this strain. For the biofilms made with GlcNAc (*E. coli, B. subtilis*, and *S. aureus*), DisH also inhibited and dispersed these biofilms. Therefore, DisH inhibits/disperses biofilms containing GlcNAc or GalNAc polymers of both Gram-negative and Gram-positive bacteria, and it has activity with biofilms with varying structure. The different working efficiencies of DisH with the various strains might be due to the different polymers (e.g., different crosslinking) used in the various biofilms and the presence of other components, like cellulose (Zogaj *et al.*, 2001), curli (Kikuchi *et al.*, 2005), and colanic acid (Kostakioti *et al.*, 2013) in *E. coli*, which may cause interference. Surprisingly, DisH did not disperse the biofilm of *S. aureus*. This may be due to modification of the GlcNAc polymer as the biofilm matrix matures, like deacetylation (Vuong *et al.*, 2004).

For comparison of DisH with other dispersal enzymes, dispersin B from *A. actinomycetemcomitans* only works on some biofilms that include GlcNAc, like *E. coli* MG1655, *S. epidermidis* 1457, and *P. fluorescens* WCS365, but it increases the biofilm formation of *P. aeruginosa* PAO1 (containing Pel and Psl EPS) (Itoh *et al.*, 2005). In addition, β-*N*-acetylglucosaminidase NagZ, from *Neisseria gonorrhoeae*, which only has activity with polymers of GlcNAc, dispersed gonococcal biofilms while dispersin B could not (Bhoopalan *et al.*, 2016). Hence, the primary advantage of DisH is that it is a β-*N*-acetylglucosaminidase with activities on GlcNAc and GalNAc and disperses biofilms made with these two sugar polymers; therefore, DisH is the first secreted glycoside hydrolase that has both GalNAc-ase/GlcNAc-ase degradation activities and disperses/inhibits biofilms well.

To date, the mechanisms by which SRB form and disperse their biofilms are not clear compared to the better-studied biofilm reference bacteria *E. coli* (Kaplan, 2010), *P. aeruginosa* (Baker *et al.*, 2016), *Vibrio cholerae* (Teschler *et al.*, 2015), and *Bacillus subtilis* (Driks, 2011). Remaining questions for SRB include determining (i) the role of the nine identified EPS loci in biofilm formation, (ii) whether polysaccharides beyond mannose, GalNAc, and fucose play a role in SRB biofilm formation, (iii) the

structure of the matrix polysaccharides, (iv) the role of protein in SRB biofilm formation, and (v) how biofilm formation and dispersal are regulated. However, we have demonstrated clearly that SRB biofilms may be dispersed by their own secreted glycoside hydrolases.

EXPERIMENTAL PROCEDURES

Bacterial strains, medium and growth conditions. *Desulfovibrio vulgaris* Hildenborough (ATCC 29579) was grown anaerobically in 25 mL screwcap tubes containing 10 mL of modified Baar's medium (ATCC medium no. 1249) with 0.025% sodium sulfide (as an oxygen scavenger). Initial cultures were grown from glycerol stocks stored at -80°C; all subcultures were grown from a 5% inoculum from the initial culture and were incubated at 30°C without shaking. *E. coli* strains were cultured at 37°C with shaking at 250 rpm using LB medium with 30 ng/μL chloramphenicol to maintain plasmid pLacI, and 50 ng/μL kanamycin to maintain plasmid pET-27b(+) carrying the six enzyme genes *dvu0677, dvu1128, dvu2202, dvu2239 (disH), dvu2699,* and *dvu3205*. *P. aeruginosa* PA14, *E. coli* TG1, *B. subtilis,* and *S. aureus* were routinely grown in LB medium. The strains and plasmids used in this study are listed in **Table 1**.

Purification of DisH. DNA restriction, agarose gel electrophoresis, cloning, and transformation of *E. coli* were performed following established protocols (Sambrook *et al.*, 1989). Genomic DNA was purified from *D. vulgaris* cultures using the UltraClean™ Microbial DNA Isolation Kit. The sequence encoding DisH (residues 48-481) was amplified using forward primer His-dvu2239F-*Nde*I and reverse primer dvu2239R-*Hin*dIII (**Table 2**), which introduce *Nde*I and *Hin*dIII sites, and cloned into pET-27b(+). The first 47 residues of DisH were removed because they were predicted to be a signal peptide by the SignalP 4.1 Server (Petersen *et al.*, 2011). Similarly, the other five genes without their signal peptide sequences were cloned into pET-27b(+) with the primers listed in **Table 2**.

To purify the putative glycoside hydrolase proteins, using DisH as an example, plasmid pET-27b(+)-His-DisH$_{48\text{-}481}$ was transformed into *E.coli* Rosetta (DE3)/pLacI and induced to express recombinant DisH$_{48\text{-}481}$ by adding isopropyl-β-*D*-thiogalactopyranoside (IPTG, 1 mM) at a turbidity of 600 nm of

about 0.5, and incubated further at 37°C for about 6 hours. Two liters of the recombinant cells carrying pET-27b(+)-His-Dis$H_{48\text{-}481}$ were harvested by centrifugation at 7,000 g for 10 min at 4°C, and resuspended in 50 mL of 20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole buffer (pH 8.0). The resuspensions were lysed by adding lysozyme (100 μg/mL) with 100 μL of protease inhibitor cocktail (Sigma cat no. P8849) and incubated on ice for 30 min. The lysed cells were broken by sonication (3× amplitude 40 for 1 min, Q700 sonicator, Qsonica, USA) and supernatants were harvested by centrifuge (20,000 g, 4°C, and 45 min). The resulting supernatants were filtered (0.22 μm) and loaded onto an HisTrap FF crude column (GE Healthcare Life Sciences, USA), washed with 20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole buffer (pH 8.0) and eluted with 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole buffer, pH 8. Protein purity was determined using a 10% SDS-PAGE gel. Protein concentrations were determined by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) using BSA as a standard.

Circular dichroism. Proteins Dis$H_{48\text{-}481}$ and BSA (positive control) were diluted to 0.15 mg/mL by 0.1 M PBS buffer (pH7.4) for circular dichroism analysis. Denatured protein samples (100 mM, negative controls) were made by SDS treatment at 37°C for 30 min (Bhuyan, 2010). Circular dichroism spectra of these samples were recorded with a J-1500 High performance circular dichroism spectrometer (Jasco, Germany) from 180 to 260 nm at 25°C in a 0.1 cm path length cell.

Protein domain analysis and sequence alignment. Whole genome protein domain analysis was performed using the Conserved Domain Search Service of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). Database searching and protein sequence alignment was performed via Basic Local Alignment Search Tool at NCBI and against the Carbohydrate-active enzymes database (CAZy, www.cazy.org). The protein 3D structure prediction was performed using the SWISS-MODEL workspace (Biasini *et al.*, 2014). Signal peptide analysis was performed by SignalP 4.1 Server (Petersen *et al.*, 2011), and the multiple protein sequence alignment was analyzed using Clustal X2.0 (Larkin *et al.*, 2007).

Enzyme activity and biochemical assays. To determine the substrate of Dis$H_{48\text{-}481}$, six different

nitrooligosaccharide substrates (final concentration 5 mM), 4-nitrophenyl *N*-acetyl-β-*D*-galactosaminide, 4-nitrophenyl *N*-acetyl-β-*D*-glucosaminide, 4-nitrophenyl β-*D*-glucopyranoside, 4-nitrophenyl β-*D*-mannopyranoside, 4-nitrophenyl β-*D*-fucopyranoside and 2-nitrophenyl β-*D*-galactopyranoside were utilized in a reaction mixture containing final concentration 0.05 M acetate buffer (pH 5.0). The reaction mix (375 μL) was pre-incubated for 5 minutes at 37°C, then 125 μL of 3 ng/μL to 1 μg/μL of DisH or 0.01 U/mg of commercial β-glucosidase (positive control, Sigma cat no. 49290) was added to the mixtures and incubated for 15 minutes at 60°C. The reaction was stopped by adding 500 μL of 0.2 M Na_2CO_3. The activity was determined by reading the increase in absorbance at 400 nm as a result of *p*-nitrophenyl (pNP) release. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol pNP per min under the assay conditions described here. The kinetic constants K_m and V_{max} were determined with substrate 4-nitrophenyl *N*-acetyl-β-*D*-galactosaminide (from 5 to 5000 μM) using Lineweaver-Burk plots (Lineweaver and Burk, 1934).

For metal ion inhibition of DisH, 1 μg/μL of DisH or 0.01 U/mg of commercial β-glucosidase was pre-incubated with final concentration 10 mM metal ions, including Na^+, Ca^{2+}, K^+, Mg^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+}, for 30 min at 37°C, and then the mixtures were used for the enzyme activity assay with substrate 4-nitrophenyl β-*D*-glucopyranoside (final concentration 5 mM) at pH 5 and 37°C as indicated above. For copper inhibition, final concentrations 0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1.0 mM, 2.0 mM, 5.0 mM, and 10.0 mM of Cu^{2+} were used in the pre-incubation step with 1 μg/μL of DisH.

The optimum temperature was determined by measuring the DisH activity with 4-nitrophenyl β-*D*-glucopyranoside (5 mM) at 30°C, 40°C, 50°C, 60°C, 70°C and 80°C for 15 minutes (pH 5.0). Temperature stability was determined by pre-incubating DisH in the absence of substrate at these temperatures for 60 minutes, then the assayed was performed with 4-nitrophenyl β-*D*-glucopyranoside (pH 5.0, 37°C, and 15 min). Similarly, the optimum pH was determined by measuring DisH activity with 4-nitrophenyl β-*D*-glucopyranoside (5 mM, 37°C, 15 min) at various pH conditions (pH 4 to 10) by using sodium acetate buffer (0.1 M, pH 3-5), phosphate buffer (0.1 M, pH 6-7), Tris-HCl buffer (0.1 M, pH 7-9) and glycine-NaOH buffer (0.1 M, pH 9-12). The pH stability was determined by pre-incubating the

enzyme in the absence of the substrate at these pH conditions for 60 minutes followed by measuring DisH activity with 4-nitrophenyl β-*D*-glucopyranoside (5 mM, pH 5.0, 37°C, and 15 minutes).

Biofilm formation, dispersal, and inhibition assays. Biofilms of *D. vulgaris* were developed in modified Baar's medium (300 µL) in 96-well polystyrene plates (Fisher Scientific, cat no. 07-200-656) by incubating in an anaerobic glove box at 30°C for 24 hours without shaking. Biofilms of *P. aeruginosa* PA14 were formed at 26°C for 10 h in LB medium, *E. coli* TG1 biofilms were formed at 37°C for 24 h in LB medium, *B. subtilis* biofilms were formed at 37°C for 24 h in M9 minimal medium with 0.4% glucose (M9G) (Harwood and Cutting, 1990), and *S. aureus* biofilms were formed at 30°C for 24 h in M9G medium. The planktonic cell turbidity was measured at 620 nm using a Sunrise microplate reader (Tecan, Austria Gesellschaft, Salzburg, Austria). For the biofilm dispersal assay, using a multi-channel pipette, planktonic cells were removed gently from each well in the 96-well plate, and the wells were washed once with PBS buffer (pH 7.4) once. Then enzymes or buffer (negative control) were added to the wells; the plates was then incubated at 30°C for 2 hours and 18 hours anaerobically (for *D. vulgaris*) or aerobically (for the other strains). Protease from *Bacillus* sp. (Savinase$^{@}$, Sigma cat no. P3111) was used as positive control (final concentration 0.2 U/mL).). $DisH_{48\text{-}481}$ was used at 50 ng/µL, and as a negative control, $DisH_{48\text{-}481}$ was inactivated by heat treating at 98°C for 30 min. For biofilm inhibition assay, these treatments were added to the wells when the bacterial culture was inoculated. The plates were then incubated at 30°C for 24 hours anaerobically or aerobically. After incubation, the cultures were discarded, the wells were washed three times with distilled water by dipping the plates into a 1 L of distilled water, and the plates were dried using a piece of paper towel. Crystal violet (0.1% in 300 µL) was added to each well (Fletcher, 1977), the plates were incubated for 20 minutes at room temperature, and the staining solution was discarded. The plates were washed three times with distilled water by dipping the plates into a 1 L solution of distilled water, 300 µL of 95% ethanol was added to each well, and the plates were soaked for 5 min to dissolve the crystal violet. Total biofilm was measured spectrophotometrically at 540 nm using the Sunrise microplate reader.

Western blot. As for cloning $disH_{48\text{-}481}$, with the exception of placing the His tag at the caroboxy

terminus, full-length *disH* was amplified from the *D. vulgaris* genomic DNA using forward primer full-disHF-*Eco*RI and reverse primer full-disHR-His-*Hin*dIII (**Table 2**), which introduce *Eco*RI and *Hin*dIII sites, and cloned into pET-27b(+) (**Table 2**). Plasmid pET-27b(+)-DisH (full-length) and empty plasmid pET-27b(+) were transformed into *E.coli* BL21 (DE3); full-length DisH production was induced by adding 1 mM IPTG at a turbidity of 600 nm ~0.5, and cells were incubated at 18°C for 24 hours. The cells and supernatants of these two strains were harvested at 10,000 g for 10 min at 4°C. The cell pellets were resuspended with PBS buffer (pH 7.4) to normalize the turbidity to 2, lysed by sonicating at level 3, 20 sec on ice using a Sonic Dismembrator 60 (Fisher Scientific). The supernatants were filtered (0.22 μm) to remove planktonic cells, and concentrated 10× via a speed vacuum (Vacufuge Plus, Eppendorf) in V-AQ mode at 30 °C. For SDS-PAGE, purified $DisH_{48-481}$ protein was used as a positive control. Proteins were transferred by a Mini Trans-Blot® Cell (Bio-Rad) to polyvinylidene difluoride membranes (Immun-Blot® PVDF Membrane, Bio-Rad cat#162-0177); a His-tag antibody was used to detect DisH (6×-His Tag Antibody, HRP conjugate (His.H8), Invitrogrn, cat#MA1-21315-HRP). The antibody of the blot was detected using the SuperSignal®West Pico Chemiluminescent substrate (Thermo Scientific cat#34077) and CL-XPosure™ Film (Thermo Scientific cat#34090) in 10 sec within a Autoradiography Cassette (Fisher Scientific cat#FBCS 57).

Statistical analysis. All experiments were conducted for at least three biologically independent replicates. Technical replicates were averaged to produce replicate means that were subsequently used for analysis. For comparisons, differences were evaluated by a one-way ANOVA test of mean replicate measurements, which were considered statistically significant if $P < 0.05$ (Sigma plot, version 12, Systat Software, Inc., San Jose, CA).

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REFERENCES

Baker, P., Whitfield, G.B., Hill, P.J., Little, D.J., Pestrak, M.J., Robinson, H. *et al.* (2015) Characterization of the *Pseudomonas aeruginosa* glycoside hydrolase PslG reveals that its levels are critical for Psl polysaccharide biosynthesis and biofilm formation. *J Biol Chem* **290**: 28374-28387.

Baker, P., Hill, P.J., Snarr, B.D., Alnabelseya, N., Pestrak, M.J., Lee, M.J. *et al.* (2016) Exopolysaccharide biosynthetic glycoside hydrolases can be utilized to disrupt and prevent *Pseudomonas aeruginosa* biofilms. *Sci Adv* **2**: e1501632.

Beech, I.B., and Sunner, J.A. (2007) Sulphate-reducing bacteria and their role in corrosion of ferrous materials. *Sulphate-Reducing Bacteria: Environmental and Engineered Systems*: 459-482.

Beech, I.B., Gaylarde, C.C., Smith, J.J., and Geesey, G.G. (1991) Extracellular polysaccharides from *Desulfovibrio desulfuricans* and *Pseudomonas fluorescens* in the presence of mild and stainless steel. *Appl Microbiol Biotechnol* **35**: 65-71.

Bhoopalan, S.V., Piekarowicz, A., Lenz, J.D., Dillard, J.P., and Stein, D.C. (2016) *nagZ* triggers gonococcal biofilm disassembly. *Sci Rep* **6**: 22372.

Bhuyan, A.K. (2010) On the mechanism of SDS-induced protein denaturation. *Biopolymers* **93**: 186-199.

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T. *et al.* (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* **42**: W252-258.

Byrd, M.S., Sadovskaya, I., Vinogradov, E., Lu, H.P., Sprinkle, A.B., Richardson, S.H. *et al.* (2009) Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. *Mol Microbiol* **73**: 622-638.

Clark, M.E., Edelmann, R.E., Duley, M.L., Wall, J.D., and Fields, M.W. (2007) Biofilm formation in *Desulfovibrio vulgaris* Hildenborough is dependent upon protein filaments. *Environ Microbiol* **9**: 2844-2854.

Colvin, K.M., Alnabelseya, N., Baker, P., Whitney, J.C., Howell, P.L., and Parsek, M.R. (2013) PelA deacetylase activity is required for Pel polysaccharide synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **195**: 2329-2339.

Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W., and Gotz, F. (1999) The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect Immun* **67**: 5427-5433.

Driks, A. (2011) Tapping into the biofilm: insights into assembly and disassembly of a novel amyloid fibre in *Bacillus subtilis*. *Mol Microbiol* **80**: 1133-1136.

Enning, D., and Garrelfs, J. (2014) Corrosion of iron by sulfate-reducing bacteria: new views of an old problem. *Appl Environ Microbiol* **80**: 1226-1236.

Fletcher, M. (1977) The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. *Can J Microbiol* **23**: 1-6.

Fong, J.N., and Yildiz, F.H. (2015) Biofilm matrix proteins. *Microbiol Spectr* **3**.

Franklin, M.J., Nivens, D.E., Weadge, J.T., and Howell, P.L. (2011) Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. *Front Microbiol* **2**: 167.

Friedman, L., and Kolter, R. (2004) Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Mol Microbiol* **51**: 675-690.

Gacesa, P., and Russell, N.J. (1990) *Pseudomonas infection and alginates : biochemistry, genetics, and pathology*. London: Chapman and Hall.

Harwood, C.R., and Cutting, S.M. (1990) Chemically defined growth media and supplements. In *Molecular Biological Methods for Bacillus*. Harwood, C.R., and Cutting, S.M. (eds): Chichester, UK: Wiley, p. 548.

Heidelberg, J.F., Seshadri, R., Haveman, S.A., Hemme, C.L., Paulsen, I.T., Kolonay, J.F. *et al.* (2004) The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Nat Biotechnol* **22**: 554-559.

Horsch, M., Mayer, C., Sennhauser, U., and Rast, D.M. (1997) β-*N*-acetylhexosaminidase: a target for the design of antifungal agents. *Pharmacol Ther* **76**: 187-218.

Itoh, Y., Wang, X., Hinnebusch, B.J., Preston, J.F., 3rd, and Romeo, T. (2005) Depolymerization of β-1,6-*N*-acetyl-*D*-glucosamine disrupts the integrity of diverse bacterial biofilms. *J Bacteriol* **187**: 382-387.

Jackson, K.D., Starkey, M., Kremer, S., Parsek, M.R., and Wozniak, D.J. (2004) Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. *J Bacteriol* **186**: 4466-4475.

Jennings, L.K., Storek, K.M., Ledvina, H.E., Coulon, C., Marmont, L.S., Sadovskaya, I. *et al.* (2015) Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix. *Proc Natl Acad Sci U S A* **112**: 11353-11358.

Kaplan, J.B. (2010) Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *J Dent Res* **89**: 205-218.

Kaplan, J.B., Ragunath, C., Ramasubbu, N., and Fine, D.H. (2003) Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous beta-hexosaminidase activity. *J Bacteriol* **185**: 4693-4698.

Karatan, E., and Watnick, P. (2009) Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol Rev* **73**: 310-347.

Kikuchi, T., Mizunoe, Y., Takade, A., Naito, S., and Yoshida, S. (2005) Curli fibers are required for development of biofilm architecture in *Escherichia coli* K-12 and enhance bacterial adherence to human uroepithelial cells. *Microbiol Immunol* **49**: 875-884.

Koch, G., Varney, J., Thompson, N., Moghissi, O., Gould, M., and Payer, J. (2016) International measures of prevention, application, and economics of corrosion technologies study. *NACE International*.

Kostakioti, M., Hadjifrangiskou, M., and Hultgren, S.J. (2013) Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harb Perspect Med* **3**: a010306.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H. *et al.* (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947-2948.

Lee, W., Lewandowski, Z., Nielsen, P.H., and Hamilton, W.A. (1995) Role of sulfate-reducing bacteria in corrosion of mild-steel - a review. *Biofouling* **8**: 165-194.

Lineweaver, H., and Burk, D. (1934) The determination of enzyme dissociation constants. *J Am Chem Soc* **56**: 658-666.

Petersen, T.N., Brunak, S., von Heijne, G., and Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* **8**: 785-786.

Poosarla, V.G., Wood, T.L., Zhu, L., Miller, D.S., Yin, B., and Wood, T.K. (2017) Dispersal and inhibitory roles of mannose, 2-deoxy-*D*-glucose, and *N*-acetylgalactosaminidase on the biofilm of *Desulfovibrio vulgaris*. *Environ Microbiol Rep* **9**: 779-787.

Priha, O., Nyyssonen, M., Bomberg, M., Laitila, A., Simell, J., Kapanen, A., and Juvonen, R. (2013) Application of denaturing high-performance liquid chromatography for monitoring sulfate-reducing bacteria in oil fields. *Appl Environ Microbiol* **79**: 5186-5196.

Ramasubbu, N., Thomas, L.M., Ragunath, C., and Kaplan, J.B. (2005) Structural analysis of dispersin B, a biofilm-releasing glycoside hydrolase from the periodontopathogen *Actinobacillus actinomycetemcomitans*. *J Mol Biol* **349**: 475-486.

Roux, D., Cywes-Bentley, C., Zhang, Y.F., Pons, S., Konkol, M., Kearns, D.B. *et al.* (2015) Identification of poly-*N*-acetylglucosamine as a major polysaccharide component of the *Bacillus subtilis* biofilm matrix. *J Biol Chem* **290**: 19261-19272.

Sambanthamoorthy, K., Luo, C., Pattabiraman, N., Feng, X., Koestler, B., Waters, C.M., and Palys, T.J. (2014) Identification of small molecules inhibiting diguanylate cyclases to control bacterial biofilm development. *Biofouling* **30**: 17-28.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning : a Laboratory Manual, 2nd edn*. New York: Cold Spring Harbor Laboratory, Cold Spring Harbor.

Schiller, N.L., Monday, S.R., Boyd, C.M., Keen, N.T., and Ohman, D.E. (1993) Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (*algL*): cloning, sequencing, and expression in Escherichia coli. *J Bacteriol* **175**: 4780-4789.

Teschler, J.K., Zamorano-Sanchez, D., Utada, A.S., Warner, C.J., Wong, G.C., Linington, R.G., and Yildiz, F.H. (2015) Living in the matrix: assembly and control of *Vibrio cholerae* biofilms. *Nat Rev Microbiol* **13**: 255-268.

Vuong, C., Kocianova, S., Voyich, J.M., Yao, Y., Fischer, E.R., DeLeo, F.R., and Otto, M. (2004) A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J Biol Chem* **279**: 54881-54886.

Wang, X., Preston, J.F., 3rd, and Romeo, T. (2004) The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J Bacteriol* **186**: 2724-2734.

Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C., and Mattick, J.S. (2002) Extracellular DNA required for bacterial biofilm formation. *Science* **295**: 1487.

Wood, T.K. (2014) Biofilm dispersal: deciding when it is better to travel. *Mol Microbiol* **94**: 747-750.

Yu, S., Su, T., Wu, H., Liu, S., Wang, D., Zhao, T. *et al.* (2015) PslG, a self-produced glycosyl hydrolase, triggers biofilm disassembly by disrupting exopolysaccharide matrix. *Cell Res* **25**: 1352-1367.

Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W., and Romling, U. (2001) The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Mol Microbiol* **39**: 1452-1463.

Table 1. Bacterial strains and plasmids used in this study. Cm^R and Km^R denote chloramphenicol and kanamycin resistance, respectively.

Strains & plasmids	Features	Source
Strains		
D. vulgaris Hildenborough	Wild-type, ATCC 29579	ATCC
E. coli HST08	F- *endA1 supE44 thi-1 recA*1 *relA*1 *gyrA*96 *phoA* Φ80d *lacZ*ΔM15 Δ(*lacZYA* - argF) U169, Δ (*mrr - hsdRMS* – *mcrBC*) Δ *mcrA* λ-	TAKARA®
E. coli Rosetta (DE3)/pLacI	F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3) pLacIRARE (Cm^R)	Novagen®
E. coli BL21 (DE3)	F^- *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3)	M. Nomura
P. aeruginosa PA14	wild-type	F. Ausubel
E. coli TG1	K-12 *supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)*5, ($r_K^- m_K^-$)	J. Minshull
B. subtilis	ATCC 6633, wild-type	ATCC
S. aureus	ATCC 29213, wild-type	K. Urish
Plasmids		
pET-27b(+)	P_{T7}, pBR322 ori,, Km^R	Novagen®
pLacI	p15a ori, Cm^R; supplies tRNAs for 7 rare codones (AGA, AGG, AUA, CUA, GGA, CCC, and CGG)	Novagen®
pET-27b(+)-His-DisH$_{48-481}$	P_{T7}::*His*$_6$-*dvu2239*, pBR322 ori,, Km^R	This work
pET-27b(+)-DisH (full-length)	P_{T7}::*dvu2239-His*$_6$, pBR322 ori,, Km^R	This work
pET-27b(+)-His-DVU0677$_{28-349}$	P_{T7}::*His*$_6$-*dvu0677*, pBR322 ori,, Km^R	This work
pET-27b(+)-His-DVU1128$_{32-221}$	P_{T7}::*His*$_6$-*dvu1128*, pBR322 ori,, Km^R	This work
pET-27b(+)-His-DVU2202$_{35-505}$	P_{T7}::*His*$_6$-*dvu2202*, pBR322 ori,, Km^R	This work
pET-27b(+)-His-DVU2699$_{31-215}$	P_{T7}::*His*$_6$-*dvu2699*, pBR322 ori,, Km^R	This work
pET-27b(+)-His-DVU3205$_{36-481}$	P_{T7}::*His*$_6$-*dvu3205*, pBR322 ori,, Km^R	This work

Table 2. Primers used in this study.

His-dvu2239F-*Nde*I	5’-GGAATTCCATATGCACCATCACCATCACCATCAATGCGCCTCTGCCAACGCCACGG-3’
dvu2239R-*Hin*dIII	5’-CCCAAGCTTTCACTGCCCGATGCCGATGCCAGTG-3’
full-disHF-*Eco*RI	5’-CCGGAATTCCATACAAACCAAGGAGCCCGTCATGACTGTTCCTGTGGTGCCG-3’
full-disHR-His-*Hin*dIII	5’- CCCAAGCTTTCAATGGTGGTGGTGATGATGCTGCCCGATGCCGATGCC -3’
His-dvu1128F-*Nhe*I	5’-CTAGCTAGCATGCACCATCACCATCACCATGCCCCATCCATCCCCGCGCAAG-3’
dvu1128R-*Hin*dIII	5’-CCCAAGCTTCTAGTCATGCCGACCCCCTTCACTG-3’
His-dvu2202F-*Nde*I	5’-GGAATTCCATATGCACCATCACCATCACCATATGGTCGCAGCCCAGGAGGATGAC-3’
dvu2202R-*Hin*dIII	5’-CCCAAGCTTTCACTTCTGCAGTGCCTCCAGCACC-3’
His-dvu2699F-*Nde*I	5’-GGAATTCCATATGCACCATCACCATCACCATGCCCCGGCTATCCCCATGCAG-3’
dvu2699R-*Hin*dIII	5’-CCCAAGCTTCTACTCATGGCACATGCCCCCGCCCCAG-3’
His-dvu3205F-*Nde*I	5’-GGAATTCCATATGCACCATCACCATCACCATTCTCTTCCGCACCACCACGACAACG-3’
dvu3205R-*Hin*dIII	5’-CCCAAGCTTCTAGATGAAGGGCGCGACGGGCGTC-3’

Table 3. Enzyme activities of purified $DisH_{48-481}$.

Subatrates (5 mM)	Specific activity (μmol of pNP/min/mg)
4-nitrophenyl *N*-acetyl-β-*D*-galactosaminide	3.7 ± 0.4
4-nitrophenyl *N*-acetyl-β-*D*-glucosaminide	7.8 ± 0.4
4-nitrophenyl β-*D*-glucopyranoside	0.051 ± 0.001
4-nitrophenyl β-*D*-mannopyranoside	0.0063 ± 0.0003
4-nitrophenyl β-*D*-fucopyranoside	0.00727 ± 0.00002
2-nitrophenyl β-*D*-galactopyranoside	0.0065 ± 0.0003

Table 4. Effect of metal ions on the activity of purified DisH.

Metal ions (10 mM)	Residual activity (%)
Ca^{2+}	95.9 ± 0.5
K^{+}	93 ± 9
Mg^{2+}	101 ± 9
Mn^{2+}	107 ± 13
Cu^{2+}	4.7 ± 0.3
Co^{2+}	101 ± 2
Ni^{2+}	77 ± 3

FIGURE LEGENDS

Fig. 1 Domain organization and alignment of DisH with GH3 family members. (A) The glycoside hydrolase family 3 domain of DisH is shown in blue, and the signal peptide is highlighted in green. **(B)** Protein sequence alignment (**.**, **:**, or ***** on the top of the alignment indicate conserved sites in "weak" amino acid groups, "strong" groups, and identical amino acids, respectively). Catalytic residues are indicated by red arrows. *D. v*: *Desulfovibrio vulgaris* Hildenborough (YP_011452), *B. sp*: *Bilophila* sp. 4_1_30 (WP_009733653), *D. e*: *Desulfomicrobium escambiense* (WP_084435603), *C. a*: *Chrysiogenes arsenatis* (WP_027389719), *H. a*: *Halodesulfovibrio aestuarii* (WP_027361073), and *B. s*: *Bacillus subtilis* subsp. *subtilis* str. 168 (NP_388047).

Fig. 2 Enzyme activities of $DisH_{48\text{-}481}$. (A) The kinetic constants K_m and V_{max} of $DisH_{48\text{-}481}$ as determined by the Lineweaver-Burk method (Lineweaver and Burk, 1934). 4-Nitrophenyl *N*-acetyl-β-*D*-galactosaminide was used as the substrate at 60°C and pH 5.0. **(B)** Cu^{2+} inhibition of $DisH_{48\text{-}481}$ activity of at 37°C and pH 5.0. **(C)** Temperature optimum of $DisH_{48\text{-}481}$ (at pH 5.0, 15 minutes for each temperature). **(D)** Thermostability of $DisH_{48\text{-}481}$ (at pH 5.0, 15 minutes for each temperature). **(E)** pH optimum of $DisH_{48\text{-}481}$ (at 37°C, 15 minutes for each pH). **(F)** pH stability of DisH (at 37°C, 15 minutes for each pH). For panels **(B)** to **(F),** 4-nitrophenyl β-*D*-glucopyranoside was used at 5 mM as the substrate. The values shown represent means of two replicate experiments and the error bar indicates one standard deviation.

Fig. 3 *D. vulgaris* biofilm inhibition and dispersal by $DisH_{48\text{-}481}$. (A) Normalized biofilm formation ($OD_{540\ nm}/OD_{620\ nm}$) of *D. vulgaris* after 24 h at 30°C in modified Baar's medium in the presence of $DisH_{48\text{-}481}$ at 50 ng/μL. **(B)** Remaining biofilm ($OD_{540\ nm}$) of *D. vulgaris* formed for 24 h at 30°C in modified Baar's medium after contact with $DisH_{48\text{-}481}$ (50 ng/μL) in non-nutritive buffer for 2 h at 30°C. **(C)** Remaining biofilm ($OD_{540\ nm}$) of *D. vulgaris* formed for 24 h at 30°C in modified Baar's medium after contact with $DisH_{48\text{-}481}$ (50 ng/μL) in non-nutritive buffer for 18 h at 30°C**.** Savinase was used as the positive control at 0.0005 U/μL (one unit hydrolyzes casein to release one μmole of tyrosine per min at pH 7.5), and both inactivated $DisH_{48\text{-}481}$ and PBS buffer (pH 7.4) were used as negative controls. The error bar indicates one standard deviation. The symbols * ($P < 0.05$) and ** ($P < 0.01$) indicate significant differences versus the PBS buffer control group via one-way ANOVA.

Fig. 4 Non-SRB biofilm inhibition by $DisH_{48\text{-}481}$. (A) Normalized biofilm formation ($OD_{540\ nm}/OD_{620\ nm}$) of *P. aeruginosa* PA14 after 10 h at 26°C in LB medium in the presence of $DisH_{48\text{-}481}$. **(B)**

Normalized biofilm formation of *E. coli* TG1 after 24 h at 37°C in LB medium in the presence of $DisH_{48\text{-}481}$. (C) Normalized biofilm formation of *B. subtilis* ATCC6633 after 24 h at 37°C in M9G medium in the presence of $DisH_{48\text{-}481}$. (D) Normalized biofilm formation ($OD_{540\ nm}/OD_{620\ nm}$) of *S. aureus* strain ATCC29213 after 24 h at 30°C in M9G medium in the presence of $DisH_{48\text{-}481}$. $DisH_{48\text{-}481}$ was used at 50 ng/μL. The error bar indicates one standard deviation. The symbols * ($P < 0.05$) and ** ($P < 0.01$) indicate significant differences versus the PBS buffer control group via one-way ANOVA.

Fig. 5 Non-SRB biofilm dispersal by $DisH_{48\text{-}481}$. (A) Remaining biofilm ($OD_{540\ nm}$) of *P. aeruginosa* PA14 after 10 h at 26°C in LB medium in the presence of $DisH_{48\text{-}481}$. **(B)** Remaining biofilm of *E. coli* TG1 after 24 h at 37°C in LB medium in the presence of $DisH_{48\text{-}481}$. **(C)** Remaining biofilm of *B. subtilis* after 24 h at 37°C in M9G medium in the presence of $DisH_{48\text{-}481}$. **(D)** Remaining biofilm of *S. aureus* after 24 h at 30°C in M9G medium in the presence of $DisH_{48\text{-}481}$ at 50 ng/μL. $DisH_{48\text{-}481}$ was used at 50 ng/μL. The error bar indicates one standard deviation. The symbols * ($P < 0.05$) and ** ($P < 0.01$) indicate significant differences versus the PBS buffer control group via one-way ANOVA.

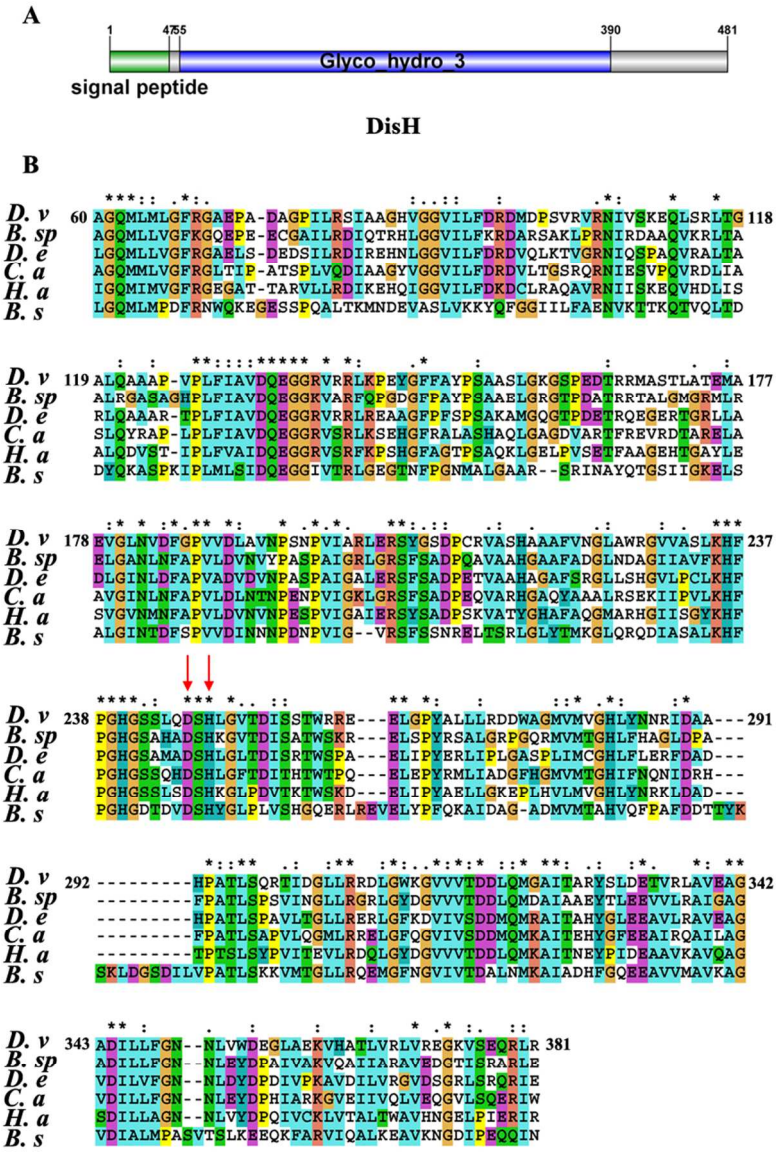

Fig. 1. Domain organization and alignment of DisH with GH3 family members. (A) The glycoside hydrolase family 3 domain of DisH is shown in blue, and the signal peptide is highlighted in green. (B) Protein sequence alignment (., :, or * on the top of the alignment indicate conserved sites in "weak" amino acid groups, "strong" groups, and identical amino acids, respectively). Catalytic residues are indicated by red arrows. D. v: Desulfovibrio vulgaris Hildenborough (YP_011452), B. sp: Bilophila sp. 4_1_30 (WP_009733653), D. e: Desulfomicrobium escambiense (WP_084435603), C. a: Chrysiogenes arsenatis (WP_027389719), H. a: Halodesulfovibrio aestuarii (WP_027361073), and B. s: Bacillus subtilis subsp. subtilis str. 168 (NP_388047).

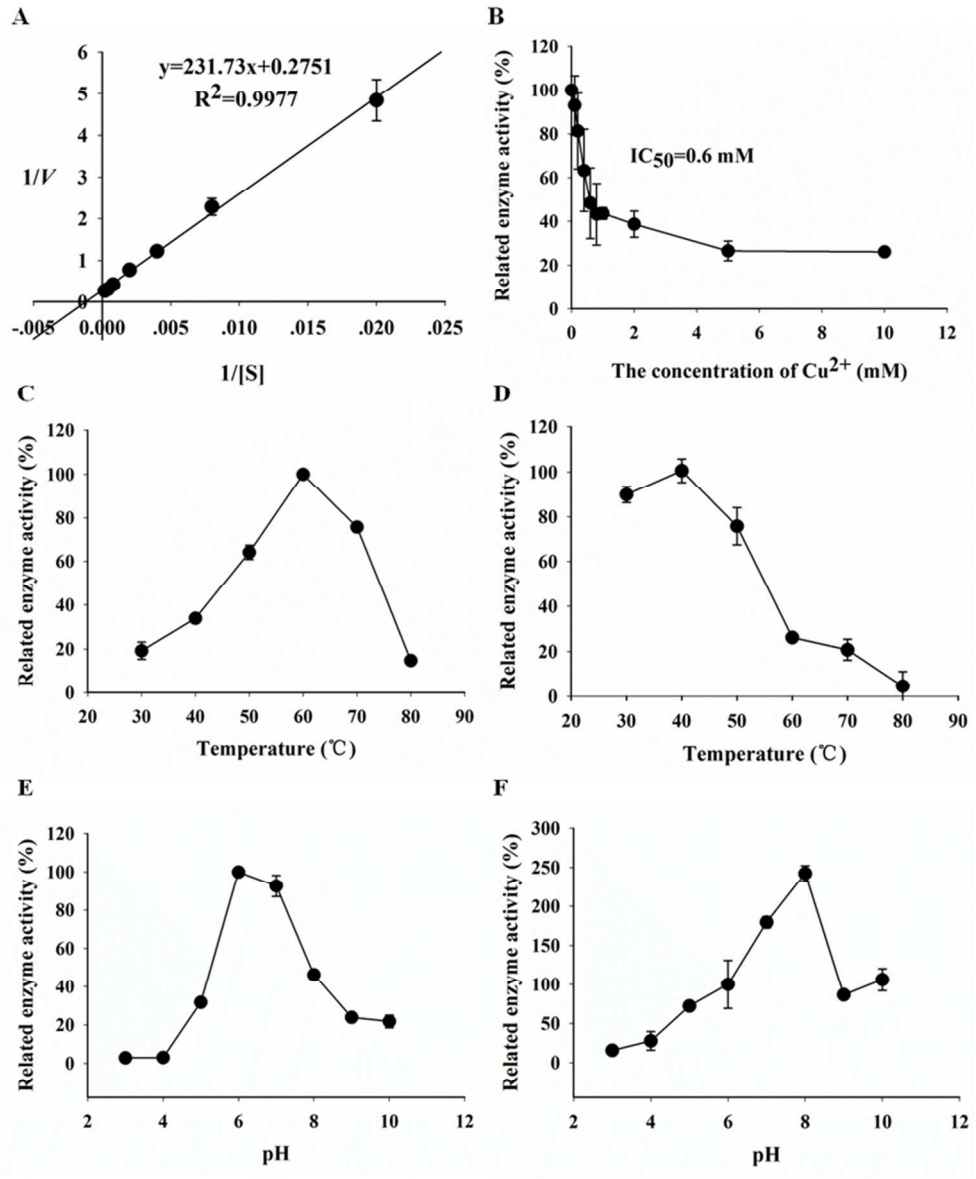

Fig. 2. Enzyme activities of DisH48-481. (A) The kinetic constants Km and Vmax of DisH48-481 as determined by the Lineweaver-Burk method (Lineweaver and Burk, 1934). 4-Nitrophenyl N-acetyl-β-D-galactosaminide was used as the substrate at 60°C and pH 5.0. (B) Cu2+ inhibition of DisH48-481 activity of at 37°C and pH 5.0. (C) Temperature optimum of DisH48-481 (at pH 5.0, 15 minutes for each temperature). (D) Thermostability of DisH48-481 (at pH 5.0, 15 minutes for each temperature). (E) pH optimum of DisH48-481 (at 37°C, 15 minutes for each pH). (F) pH stability of DisH (at 37°C, 15 minutes for each pH). For panels (B) to (F), 4-nitrophenyl β-D-glucopyranoside was used at 5 mM as the substrate. The values shown represent means of two replicate experiments and the error bar indicates one standard deviation.

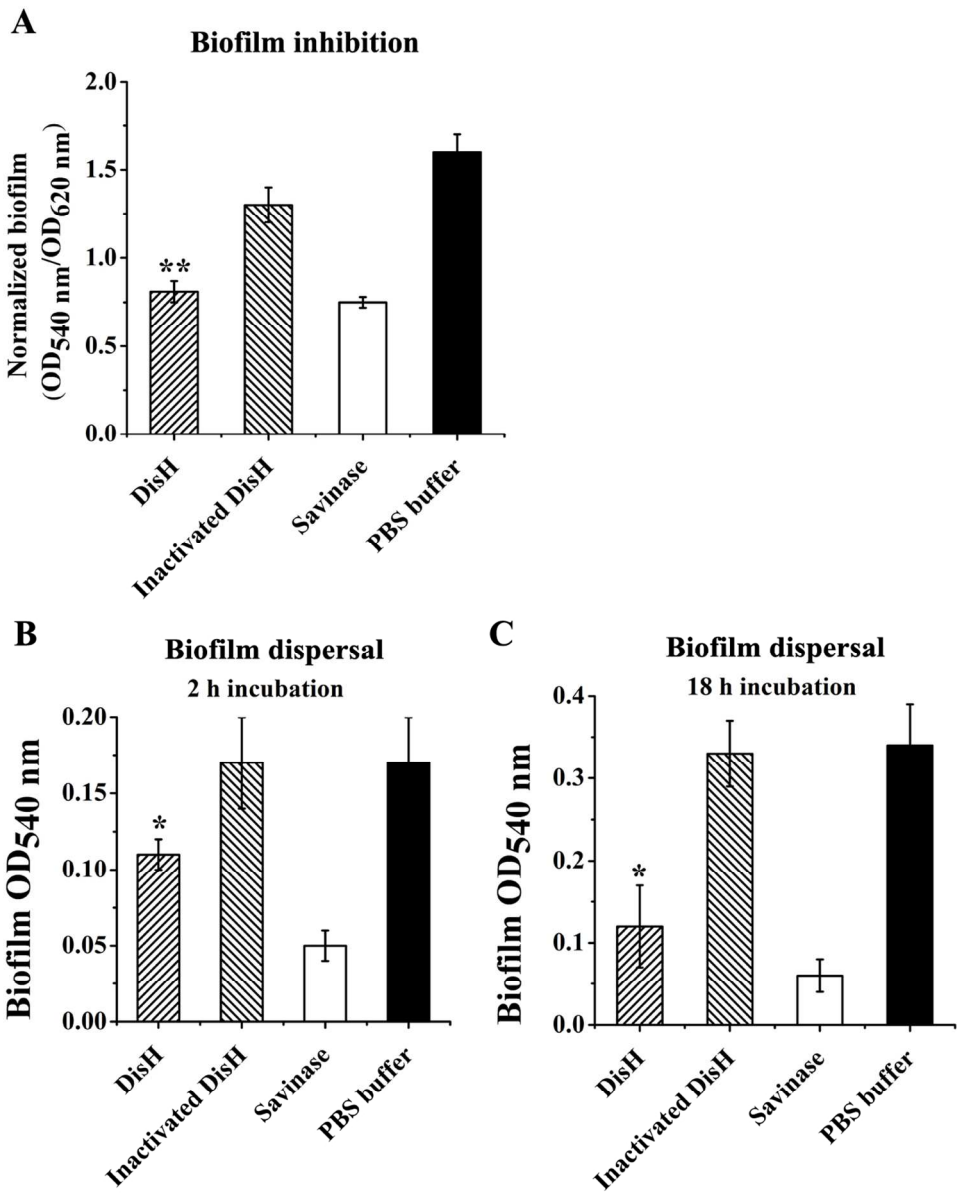

Fig. 3. D. vulgaris biofilm inhibition and dispersal by DisH48-481. (A) Normalized biofilm formation (OD540 nm/OD620 nm) of D. vulgaris after 24 h at 30°C in modified Baar's medium in the presence of DisH48-481 at 50 ng/μL. (B) Remaining biofilm (OD540 nm) of D. vulgaris formed for 24 h at 30°C in modified Baar's medium after contact with DisH48-481 (50 ng/μL) in non-nutritive buffer for 2 h at 30°C. (C) Remaining biofilm (OD540 nm) of D. vulgaris formed for 24 h at 30°C in modified Baar's medium after contact with DisH48-481 (50 ng/μL) in non-nutritive buffer for 18 h at 30°C. Savinase was used as the positive control at 0.0005 U/μL (one unit hydrolyzes casein to release one μmole of tyrosine per min at pH 7.5), and both inactivated DisH48-481 and PBS buffer (pH 7.4) were used as negative controls. The error bar indicates one standard deviation. The symbols * ($P < 0.05$) and ** ($P < 0.01$) indicate significant differences versus the PBS buffer control group via one-way ANOVA.

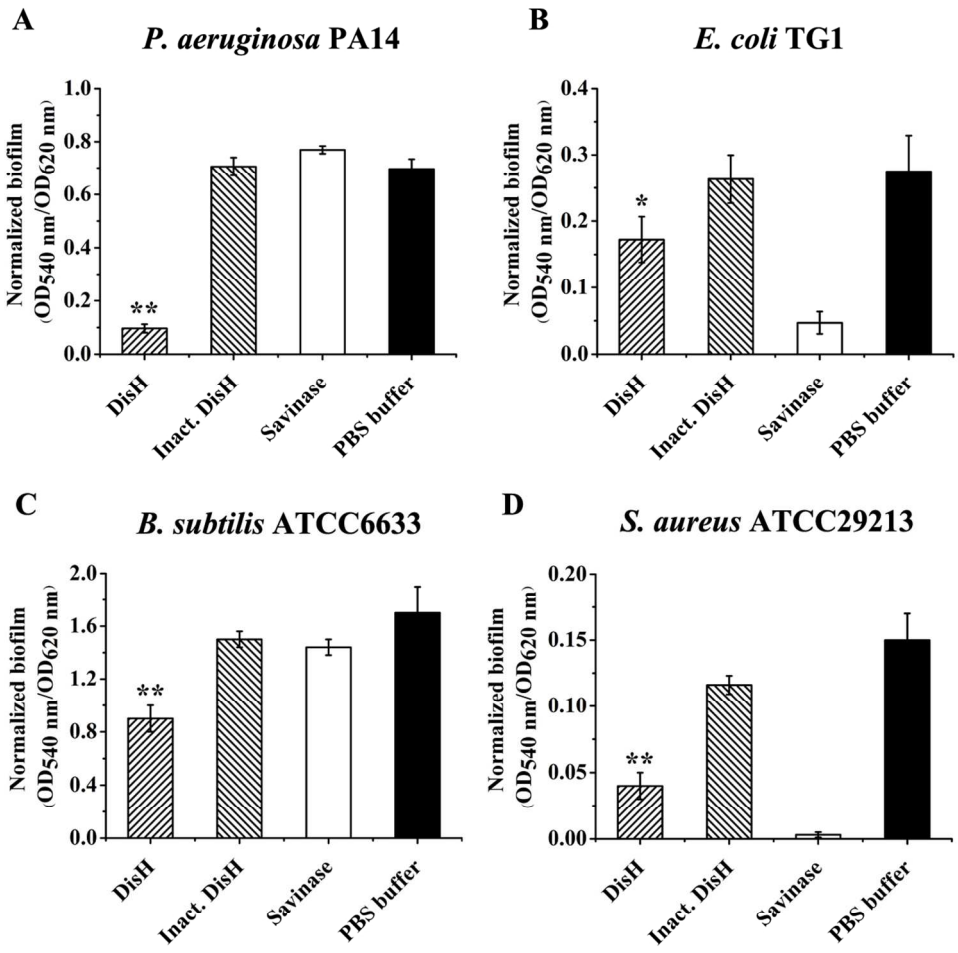

Fig. 4. Non-SRB biofilm inhibition by DisH48-481. (A) Normalized biofilm formation (OD540 nm/OD620 nm) of P. aeruginosa PA14 after 10 h at 26°C in LB medium in the presence of DisH48-481. (B) Normalized biofilm formation of E. coli TG1 after 24 h at 37°C in LB medium in the presence of DisH48-481. (C) Normalized biofilm formation of B. subtilis ATCC6633 after 24 h at 37°C in M9G medium in the presence of DisH48-481. (D) Normalized biofilm formation (OD540 nm/OD620 nm) of S. aureus strain ATCC29213 after 24 h at 30°C in M9G medium in the presence of DisH48-481. DisH48-481 was used at 50 ng/μL. The error bar indicates one standard deviation. The symbols * ($P < 0.05$) and ** ($P < 0.01$) indicate significant differences versus the PBS buffer control group via one-way ANOVA.

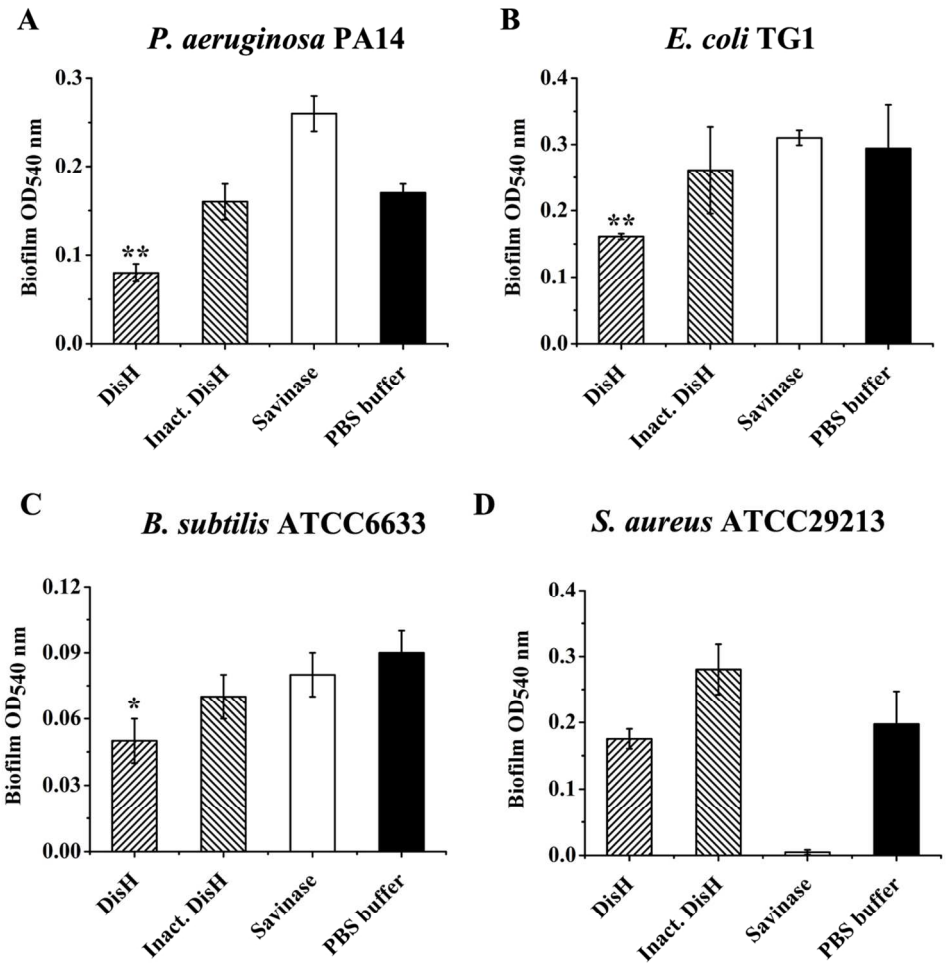

Fig. 5. Non-SRB biofilm dispersal by DisH48-481. (A) Remaining biofilm (OD540 nm) of P. aeruginosa PA14 after 10 h at 26°C in LB medium in the presence of DisH48-481. (B) Remaining biofilm of E. coli TG1 after 24 h at 37°C in LB medium in the presence of DisH48-481. (C) Remaining biofilm of B. subtilis after 24 h at 37°C in M9G medium in the presence of DisH48-481. (D) Remaining biofilm of S. aureus after 24 h at 30°C in M9G medium in the presence of DisH48-481 at 50 ng/μL. DisH48-481 was used at 50 ng/μL. The error bar indicates one standard deviation. The symbols * ($P < 0.05$) and ** ($P < 0.01$) indicate significant differences versus the PBS buffer control group via one-way ANOVA.