Identification and characterization of target genes of the GinI/GinR quorum-sensing system in *Gluconacetobacter intermedius*

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The GinI/GinR quorum-sensing system represses oxidative fermentation, including acetic acid and gluconic acid fermentation, as well as antifoam activity in *Gluconacetobacter intermedius* NCI1051. An 89 aa protein, GinA, whose production is induced by the quorum-sensing system, represses both oxidative fermentation and antifoam activity via a still unknown mechanism, although an OmpA family protein, GmpA, as a target of the GinI/GinR quorum-sensing system via GinA, has been found to repress oxidative fermentation. In this study, four novel GinA-inducible genes (*gltA*, *pdeA*, *pdeB* and *nagA*) were identified and their involvement in oxidative fermentation and antifoam activity was examined by gene disruption. Disruption of *nagA* (which encodes a putative *N*-acetylglucosamine-6-phosphate deacetylase) decreased the growth rate in the exponential growth phase, indicating that *nagA* was required for the rapid growth of the strain. This unexpected finding revealed a new aspect of the GinI/GinR quorum-sensing system: it accelerates exponential growth by induction of *nagA*. In contrast, *gltA* (a putative glycosyltransferase) and *pdeA* (a putative cyclic-di-GMP phosphodiesterase) were shown to repress oxidative fermentation, including acetic acid and gluconic acid fermentation. *gltA* was also shown to repress antifoam activity. Disruption of *pdeB* (a putative phosphodiesterase/diguanylate cyclase) caused no phenotypic changes. Taking our previous results into consideration, these results showed an apparently complex mechanism for repressing oxidative fermentation by the quorum-sensing system; at least three GinA-inducible genes, *gltA*, *pdeA* and *gmpA*, were involved in the repression of oxidative fermentation by the GinI/GinR quorum-sensing system, the most characteristic feature of the acetic acid bacteria.

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INTRODUCTION

Many Gram-negative bacteria use *N*-acylhomoserine lactone (AHL)-dependent quorum-sensing systems to regulate gene expression in concert with cell density (Fuqua *et al.*, 1994, 1996). For example, the phenotypes controlled by the quorum-sensing systems include secondary metabolite production, swimming and swarming motility, conjugal plasmid transfer, biofilm formation, and virulence (Waters & Bassler, 2005; Williams, 2006). AHL-dependent quorum sensing consists of a LuxR-type protein, which is an AHL-dependent transcriptional regulator, and a LuxI-type protein, which is an AHL

synthase. In general, LuxR-type proteins bind their cognate AHLs once the concentration of the AHL reaches a critical level, and the resulting complex activates the transcription of specific target genes by binding the promoter recognition sequence termed the *lux* box.

Acetic acid bacteria are Gram-negative obligatory aerobic bacteria with the ability to oxidize ethanol and sugars into their corresponding organic acids. *Acetobacter* and *Gluconacetobacter* are used to commercially produce vinegar because of their great ability to oxidize ethanol into acetic acid and their strong resistance to acetic acid and ethanol. Two membrane-bound enzymes, alcohol dehydrogenase and aldehyde dehydrogenase, catalyse the oxidation of ethanol. We have previously found that *Gluconacetobacter intermedius* NCI1051 contains a GinI/GinR quorum-sensing system (Iida *et al.*, 2008a). GinI synthesizes three AHLs with different acyl chains, and GinR serves as an AHL-dependent transcriptional regulator to control the *lux* box-containing promoter of *ginI*. *ginI* is co-transcribed with *ginA*, encoding a small protein consisting

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Abbreviations: AHL, *N*-acylhomoserine lactone; cyclic-di-GMP, cyclic-dimeric GMP; RACE, rapid amplification of cDNA ends; RT, reverse transcription.

The GenBank/EMBL/DDBJ accession numbers for the *gltA*, *pdeA*, *pdeB* and *nagA* sequences of *Gluconacetobacter intermedius* are AB481109, AB481110 and AB481111.

of 89 aa. The GinI/GinR quorum-sensing system controls the expression of GinA, which in turn represses oxidative fermentation, including acetic acid and gluconic acid fermentation, growth in medium containing ethanol and the antifoam activity of cells. Furthermore, by proteome analysis, we have identified an OmpA family protein, GmpA, as a target of the GinI/GinR quorum-sensing system via GinA and shown its negative effects on oxidative fermentation (Iida *et al.*, 2008b). However, the phenotypes of the *gmpA*::Km mutant were not completely the same as those of *ginA*::Km and *ginI*::Km mutants. These observations raised the possibility that some other target gene(s) of GinA are present and affect these phenotypes in some unknown way.

The purpose of this study was to find new target genes of GinA and analyse their function by gene disruption in *G. intermedius* NCI1051. Four GinA-inducible genes have been suggested by transcriptome analysis (T. Kaga, personal communication) using a DNA microarray designed for *Gluconacetobacter polyoxogenes* NCI1028, which is closely related to *G. intermedius* NCI1051. We cloned these four genes from *G. intermedius* NCI1051 and confirmed that their expression is dependent on GinA. We disrupted each of the four genes and found that two of them were involved in the repression of oxidative fermentation in *G. intermedius* NCI1051, one of the most characteristic features of the acetic acid bacteria.

METHODS

Bacterial strains and plasmids. *G. intermedius* NCI1051 and *G. polyoxogenes* NCI1028 were stock cultures from the Central Research Institute, Mizkan Group Corporation. The *Acetobacter/Gluconacetobacter-Escherichia coli* shuttle vector pMV24 has been described elsewhere (Fukaya *et al.*, 1989). The *ginI*-disrupted mutant (*ginI*::Km) and the *ginA*-disrupted mutant (*ginA*::Km) have also been described previously (Iida *et al.*, 2008a). pGinIA, which is a pMV24-derived plasmid, contains the intact *ginI* and *ginA* sequences (Iida *et al.*, 2008a). *E. coli* JM109 and plasmid pUC19, used for DNA manipulation, were purchased from Takara Biochemicals.

Media and cultural conditions. Yeast peptone glucose (YPG) medium (pH 6.5) consisted of 5 g yeast extract (Wako Pure Chemicals), 3 g polypeptone (Wako Pure Chemicals) and 30 g glucose in 1 l water. The *Gluconacetobacter* strains were grown at 30 °C in YPG medium with 1% (v/v) cellulase (Celluclast 1.5 L; Novozymes) with or without 2% (v/v) ethanol.

For acetic acid fermentation tests, the *Gluconacetobacter* strains were first cultured at 30 °C for 24 h in 5 ml YPG medium with 1% (v/v) cellulase in a test tube with shaking. A portion (5 ml) of this culture was inoculated into 1.5 l YPG medium supplemented with 3% (v/v) ethanol, 1% (v/v) cellulase and 0.001% (v/v) silicone (KM72; Shin-Etsu Chemical) in a 3 l mini-jar fermenter (Bioneer 300 3L, B. E. Marubishi) and cultured at 30 °C with agitation at 500 r.p.m. and aeration at a rate of 1.0 l min⁻¹. The ethanol concentration was automatically maintained at 2% (v/v) by adding ethanol during cultivation. The acetic acid and gluconic acid concentrations in the culture broths were determined by HPLC with a Shodex RSpak KC-811 column and a Shimadzu conductivity detector CDD-10A.

Bacterial growth was monitored by measuring the turbidity of the cultures at 660 nm using a photometer.

E. coli was cultured at 37 °C in LB medium. Ampicillin and kanamycin were used at final concentrations of 100 µg ml⁻¹ when necessary to maintain the plasmids.

DNA manipulation. Restriction enzymes, T4 DNA ligase and other DNA-modifying enzymes were purchased from Takara Biochemicals. All DNA manipulations in *E. coli* were performed as described elsewhere (Ausubel *et al.*, 1987; Maniatis *et al.*, 1982). The *Gluconacetobacter* strains were transformed by electroporation (Wong *et al.*, 1990). Chromosomal DNA from *Gluconacetobacter* strains was isolated with the GenomicPrep Cells and Tissue DNA Isolation kit (GE Healthcare). Chromosomal DNAs from *G. intermedius* NCI1051 and *G. polyoxogenes* NCI1028 were used as the templates for PCR. All nucleotide sequences were determined on a CEQ Dye Terminator Cycle Sequencer using a Quick Start kit (Beckman Coulter).

Microarray experiments. Microarray analysis was performed using a DNA microarray for *G. polyoxogenes* NCI1028, which is closely related to *G. intermedius* NCI1051 (T. Kaga, personal communication). The *G. polyoxogenes* microarray consisted of 60-mer oligonucleotides that were designed on the basis of ORFs of *G. polyoxogenes* NCI1028. Total RNA was isolated with the RNeasy kit (Qiagen) from cells grown to exponential and stationary phases at 30 °C in YPG medium containing 2% (v/v) ethanol and 1% (v/v) cellulase in a shaking flask and treated with the RNase-free DNase set (Qiagen). For each sample, RNA was extracted from two independently grown cultures. cDNA was synthesized from the total RNA and labelled. The labelled cDNA was hybridized to the *G. polyoxogenes* microarrays. Hybridization and scanning were done by using NimbleGen systems. Data were analysed using the robust multi-chip analysis and these normalized data were processed with the Nandemo Analysis 1.0.1 software (Genefrontier).

Cloning of *gltA*, *pdeA*, *pdeB* and *nagA*. For cloning of the GP0586 homologue named *gltA*, 1.3 and 1.9 kb fragments, both containing a portion of the GP0586 sequence in *G. polyoxogenes* NCI1028 (K. Kondo, personal communication), were amplified by PCR with primers gF and gR, and primers PgF and PgR (Table 1), respectively. The amplified fragment was used as the ³²P-labelled probe for Southern hybridization against the restriction enzyme-digested chromosomal DNA of *G. intermedius* NCI1051. The restriction enzymes used were *Pst*I, *Bal*I, *Sma*I and *Nae*I. Standard DNA manipulation, including colony hybridization using the 1.3 and 1.9 kb fragments as probes, gave three DNA fragments, a 2.2 kb *Pst*I fragment, a 2.8 kb *Bal*I fragment and a 2.2 kb *Bal*I fragment. Because the 0.2 kb intervening region between the two *Bal*I fragments was not cloned, a 0.3 kb DNA fragment containing the 0.2 kb region was amplified by PCR using the primers gBBF and gBBR (Table 1) and the chromosomal DNA of *G. intermedius* NCI1051 was used as a template. These fragments were cloned into pUC19.

Similarly, for cloning of the GP0611 homologue named *pdeA*, a 0.9 kb fragment containing a portion of the GP0611 sequence in *G. polyoxogenes* NCI1028 was amplified by PCR with primers pAF and pAR (Table 1). The amplified fragment was used as a probe for Southern hybridization. Standard DNA manipulation detected a 5.5 kb *Sma*I fragment, which contained a *pdeA* gene. The 5.5 kb *Sma*I fragment was cloned into pUC19. For cloning of the DV214 homologue named *pdeB*, a 1.0 kb fragment containing a portion of the DV214 sequence in *G. polyoxogenes* NCI1028 was amplified by PCR with primers pBF and pBR (Table 1). The amplified fragment was used as the probe for Southern hybridization. Standard DNA manipulation yielded a 3.0 kb *Nae*I fragment, which contained the *pdeB* gene. The 3.0 kb *Nae*I fragment was cloned into pUC19. For

Table 1. Primers used in this study

Primer	Sequence (5' to 3')*
gF	GCAGGCGTTCGACCCGGGAC
gR	GATCGTGACGTGGTAGCCGA
gBBF	<u>GGCGAGCTCCGCGCGCAGCGGGTGTTCG</u>
gBBR	<u>GCGTCTAGAGA</u> ACCGCGCCAAGGAACAGG
pAF	CATGAACATGACCGTTATATCG
pAR	GATGCGCCCCGCGATCTCGAC
pBF	CCTGGACCCGGAATTCGCG
pBR	CAGTTCATCGCCCCCGGTGT
nF	GACGGGGCGGACACCATGG
nR	CTGGGGCCATCGCCCATGCC
pA-5F	GCGGAATTCACCACCGAAATCCTGCTGCC
pA-5R	CCGCCCGGGAGGGCGGACGTGCAGATCAC
pA-3F	GCGCCCGGGGCGCTGGAACACCGTGCC
pA-3R	CCGTCTAGAGGAAGCGGTGATGTCCAGC
PgF	CCGGGATCGAGGACCACGTG
PgR	GCCTCTAGAACCCGGTCTGTCAGGGTTGC
PpAF	GCGCCCGGGTCCGGGACGGGAACCCGCATG
PpAR	GGCTCTAGACATGGCCTGCGCCAGTGCTG
S1-gF	GTTCGATGGCCGCAACGCAC
S1-gR	GTGTCCAGCCCCGCATTACG
S1-pAF	GACGGACCGCATATCCATGC
S1-pAR	CGGGAGTGTCTTCCGTCATG
S1-pBF	TGCTGCAGGACAATGCCCTC
S1-pBR	TCCAGCCGTTCCACCAGCAC
S1-nF	CAGCCTGACGGACTGGTGAC
S1-nR	TGCGATAGCGGCCGCACGTC
1F	GGATGGACGGGAGACAGATC
2F	GCTACATCGACCGCAACCGC
3F	CGATGATCGCCTGCGTGCGC
4F	GACGGACCGCATATCCATGC
5F	AACGGCTGGCCGCCTTCCTG
6F	TGGCTGTGGCCTGTGCATGG
1R, 4R	TCTCGGTTGTACAGCCGAGG
2R	GGCCGGTATAGGACGGGTTG
3R, 6R	CACGGGTGATGTTGCGGGCC
5R	CCCACAGGGATTCCGCGGCC
RACE-gltA-S1	ATGTTGCGCAGCAGGTGCTG
RACE-gltA-S2	CATGGCACAGCGGGTTCGAC
RACE-gltA-A1	GATAGGTGGCGCGGTACCAG
RACE-gltA-A2	GATTGGGCGAATGGCCCAGC
RACE-cdg1a-S1	GGAATGGAGCGCAGTTTTGG
RACE-cdg1a-S2	TTGGCGACCGGCAGGAATTC
RACE-cdg1a-A1	CGGCCGATGATTATGAGGC
RACE-cdg1a-A2	GAGGCTGCGTTAAAGACATC
RACE-pdeB-S1	CAACATCCGCTCCACCCATG
RACE-pdeB-S2	CGACCTGATCCGCCACAACC
RACE-pdeB-A1	CGCGGCACAAGCACGTTGAC
RACE-pdeB-A2	TTGCGCCCGATCACGTCTCTG

*The following sites are underlined: a *SacI* site in gBBF; an *EcoRI* site in pA-5F; *SmaI* sites in pA-5R, pA-3F and PpAF; *XbaI* sites in gBBR, pA-3R, PgR and PpAR.

cloning of the *GP0603* homologue named *nagA*, a 0.7 kb fragment containing a portion of the *GP0603* sequence in *G. polyoxogenes* NCI1028 was amplified by PCR with primers nF and nR (Table 1).

The amplified fragment was used as a probe for Southern hybridization. Standard DNA manipulation detected a 2.0 kb *PstI* fragment, which contained the *nagA* gene. The 2.0 kb *PstI* fragment was cloned into pUC19.

Disruption of *gltA*, *pdeA*, *pdeB* and *nagA*. To disrupt *gltA*, the 2.2 kb *PstI* fragment cloned into pUC19 was digested with *EcoRV*. A *SmaI* fragment carrying the neomycin/kanamycin resistance gene from Tn5 (Beck *et al.*, 1982) was then inserted in the *EcoRV* site within the *gltA*-coding sequence. This plasmid was introduced into *G. intermedius* NCI1051 by electroporation, and kanamycin-resistant colonies were selected as candidates for *gltA*-disrupted mutants. Correct *gltA*-disrupted mutants were selected by Southern hybridization with a 1.3 kb fragment that had been amplified by PCR with primers gF and gR (Table 1) as the ³²P-labelled probe.

To disrupt *pdeA*, a 0.6 kb fragment containing a 5' portion of *pdeA* was amplified by PCR using the primers pA-5F, containing an *EcoRI* site, and pA-5R, containing a *SmaI* site (Table 1); a 0.4 kb fragment containing the 3' portion of *pdeA* was amplified by PCR using the primers pA-3F, containing a *SmaI* site, and pA-3R, containing an *XbaI* site (Table 1). The two fragments were placed between the *EcoRI* and *XbaI* sites of pUC19 (plasmid pPA53). A *SmaI* fragment carrying the neomycin/kanamycin resistance gene was then inserted in the *SmaI* site of pPA53. This plasmid was introduced into *G. intermedius* NCI1051 by electroporation, and kanamycin-resistant colonies were selected as candidates for *pdeA*-disrupted mutants. Correct *pdeA*-disrupted mutants were selected by Southern hybridization with a 0.9 kb fragment that had been amplified by PCR with primers pAF and pAR (Table 1) as the ³²P-labelled probe.

To disrupt *pdeB*, the 3.0 kb *NaeI* fragment cloned into pUC19 was digested with *EcoRV*. A *SmaI* fragment carrying the neomycin/kanamycin resistance gene from Tn5 was then inserted in the *EcoRV* site within the *pdeB*-coding sequence. This plasmid was introduced into *G. intermedius* NCI1051 by electroporation, and kanamycin-resistant colonies were selected as candidates for *pdeB*-disrupted mutants. Correct *pdeB*-disrupted mutants were selected by Southern hybridization with a 1.0 kb fragment that had been amplified by PCR with primers pBF and pBR (Table 1) as the ³²P-labelled probe.

To disrupt *nagA*, the 2.0 kb *PstI* fragment cloned into pUC19 was digested with *NruI*. A *SmaI* fragment carrying the neomycin/kanamycin resistance gene from Tn5 was then inserted in the *NruI* site within the *nagA*-coding sequence. This plasmid was introduced into *G. intermedius* NCI1051 by electroporation, and kanamycin-resistant colonies were selected as candidates for *nagA*-disrupted mutants. Correct *nagA*-disrupted mutants were selected by Southern hybridization with a 0.7 kb fragment that had been amplified by PCR with primers nF and nR (Table 1) as the ³²P-labelled probe.

Plasmid construction. For construction of the plasmid for expression of *gltA*, the 2.2 kb *PstI* fragment cloned into pUC19 was digested by *EcoRI* and *ApaI*, generating a 1.9 kb *EcoRI*–*ApaI* fragment carrying a 5' portion of *gltA*. A fragment containing a 3' portion of *gltA* was amplified by PCR with primers PgF and PgR containing an *XbaI* site (Table 1) and treated with *ApaI* and *XbaI*, generating a 1.7 kb *ApaI*–*XbaI* fragment. The 1.9 kb *EcoRI*–*ApaI* fragment and the 1.7 kb *ApaI*–*XbaI* fragment were placed between the *EcoRI* and *XbaI* sites of pMV24, generating plasmid pGltA. Nucleotide sequencing was used to confirm that no errors were made during PCR. Note that *gltA* on pGltA was under the control of both its own promoter and the *E. coli lac* promoter of pMV24.

For construction of the plasmid for expression of *pdeA*, a 4 kb fragment containing *pdeA* was amplified by PCR with primers PpAF, containing a *SmaI* site, and PpAR, containing an *XbaI* site (Table 1), and placed between the *EcoRI* and *SmaI* sites of pMV24, generating

plasmid pPdeA. Nucleotide sequencing was carried out to confirm that no errors were produced during PCR. Note that *pdeA* on pPdeA was under the control of the *E. coli lac* promoter of pMV24.

For construction of the plasmid for expression of *nagA*, the 2.0 kb *PstI* fragment cloned into pUC19 was digested with *KpnI* and *PstI*, generating a 1.8 kb *KpnI*–*PstI* fragment containing *nagA*. The 1.8 kb *KpnI*–*PstI* fragment was placed between the *KpnI* and *PstI* sites of pMV24, and the resulting plasmid was named pNagA. Note that *nagA* on pNagA was under the control of both its own promoter and the *E. coli lac* promoter of pMV24.

S1 nuclease mapping. Total RNA was isolated by the hot phenol method (Okamoto-Kainuma *et al.*, 2002) from cells grown at 30 °C to exponential and stationary phases in YPG medium containing 2 % (v/v) ethanol and 1 % (v/v) cellulase in a shaking flask. S1 nuclease mapping was conducted as described previously (Kelemen *et al.*, 1998). The hybridization probes were prepared by PCR using a pair of ³²P-labelled and non-labelled primers. The primer pairs were as follows: S1-gF and S1-gR* for *gltA*, S1-pAF and S1-pAR* for *pdeA*, S1-pBF and S1-pBR* for *pdeB*, and S1-nF and S1-nR* for *nagA* (Table 1). The primers marked with asterisks were labelled with ³²P at their 5' ends using a T4 polynucleotide kinase before PCR.

RT-PCR. Total RNA was isolated by the hot phenol method (Okamoto-Kainuma *et al.*, 2002) from cells grown at 30 °C for 8 h in YPG medium containing 2 % (v/v) ethanol and 1 % (v/v) cellulase in a shaking flask. Reverse transcription (RT) was performed using the total RNA with SuperScript III Reverse Transcriptase (Invitrogen). The cDNA was then amplified by PCR using the RT reaction mixture as the template and specific primers (Table 1). The RNA samples were tested with and without RT to verify the complete removal of genomic DNA. PCR using the genomic DNA from *G. intermedium* NCI1051 as the template was performed to ensure the fidelity for each primer pair.

5'-Rapid amplification of cDNA ends (RACE). 5'-RACE was performed using the 5'-Full RACE Core set (Takara Biochemicals) according to the manufacturer's instructions. Total RNA was isolated with the RNeasy kit (Qiagen) from cells grown at 30 °C for 8 h in YPG medium containing 2 % (v/v) ethanol and 1 % (v/v) cellulase in a shaking flask. RT was performed using primer S1-gR for *gltA*, primer S1-pAR for *cdg1a*–*pdeA*, primer S1-pBR for *pdeB* and primer S1-nR for *nagA* (Table 1). The first PCR was performed using the S1

and A1 primers for the gene in question (Table 1), and the nested PCR was performed using the corresponding S2 and A2 primers (Table 1). PCR products were cloned into pT7Blue T-vector (Novagen) and sequenced.

RESULTS

Cloning of *gltA*, *pdeA*, *pdeB* and *nagA* from *G. intermedium* NCI1051

By transcriptome analysis (T. Kaga, personal communication) of mutants *ginA*::Km and *ginI*::Km using an oligonucleotide microarray for an industrial vinegar producer, *G. polyoxogenes* NCI1028, which is closely related to *G. intermedium* NCI1051, four putative *GinA*-inducible genes had been identified. In that analysis, RNA was isolated from cells grown at 30 °C to the exponential phase or stationary phase in YPG medium containing 1 % (v/v) cellulase and 2 % (v/v) ethanol, and transcriptomes of mutants *ginA*::Km and *ginI*::Km were compared with those of the parent strain. We performed the transcriptome analyses for the RNAs prepared from two different growth phases to see possible differences depending on the growth phase. Transcription of *GP0586*-, *GP0611*-, *DV214*- and *GP0603*-homologous genes during both exponential and stationary phases was found to be downregulated in both the *ginA*::Km and *ginI*::Km mutants of *G. intermedium* NCI1051 compared with the parent strain (Table 2). To confirm the transcriptional activation of these four genes by the quorum-sensing system, and to characterize their functions, we cloned these four genes from *G. intermedium* NCI1051. Briefly, we amplified *G. polyoxogenes* NCI1028 DNA fragments containing a part of the *GP0586*, *GP0611*, *DV214* and *GP0603* genes by PCR using appropriate primers, which were designed on the basis of the genome sequence of *G. polyoxogenes* NCI1028 (K. Kondo, personal communication). These DNA fragments were ³²P-labelled and used as probes for cloning their homologues from *G.*

Table 2. Results of transcriptional analysis of *gltA*, *pdeA*, *pdeB* and *nagA* using the *G. polyoxogenes* DNA microarray

Gene number	Description	Change in gene expression (fold)*			
		Exponential phase		Stationary phase	
		<i>ginI</i> ::Km vs w.t.	<i>ginA</i> ::Km vs w.t.	<i>ginI</i> ::Km vs w.t.	<i>ginA</i> ::Km vs w.t.
<i>GP0586</i>	Glycosyltransferase	−4.91	−5.74	−2.06	−2.06
<i>GP0611</i>	Cyclic-di-GMP phosphodiesterase A	−3.00	−3.54	−3.54	−2.90
<i>DV214-1</i> †	Phosphodiesterase (N-terminal)	−11.20	−15.06	−11.76	−11.58
<i>DV214-2</i> †	Phosphodiesterase (C-terminal)	−9.60	−13.42	−10.09	−8.69
<i>GP0603</i>	<i>N</i> -Acetylglucosamine-6-phosphate deacetylase	−2.12	−2.45	−1.42	−1.63

*Changes in gene expression (fold) in the *ginI*::Km or *ginA*::Km mutants harbouring pMV24 compared with the wild-type (w.t.) strain NCI1051 harbouring pMV24. RNA was isolated from cells grown at 30 °C to the exponential and stationary phases in YPG medium containing 1 % (v/v) cellulase and 2 % (v/v) ethanol.

†*DV214* is disrupted by an insertion sequence in *G. polyoxogenes*, but *pdeB*, a *DV214* homologue in *G. intermedium* NCI1051, is not disrupted.

intermedius NCI1051. Finally, the nucleotide sequences of the cloned fragments were determined.

The GP0586 homologue encoded a 983 aa protein having two glycosyltransferase motifs, glycosyltransferase family 2 (Pfam 00535) and glycosyltransferase group 1 (Pfam 00534). Therefore, we tentatively named it *gltA*. The primary sequence of GltA showed approximately 43 % identity in amino acid sequence with proteins annotated as putative glycosyltransferases, including GDI2292 (GenBank accession no. YP_001602536.1) of *Gluconacetobacter diazotrophicus* PAL5, GbCGDNIH1_1539 (YP_745360.1) of *Granulibacter bethesdensis* (Greenberg *et al.*, 2007), Acry_2183 (YP_001235300.1) of *Acidiphilium cryptum* JF-5 and GOX0595 (YP_191027.1) of *Gluconobacter oxydans* 621H (Prust *et al.*, 2005).

The GP0611 homologue encoded a 774 aa protein having GGDEF (Pfam 00990) and EAL (Pfam 00563) domains, which were assumed to be involved in the turnover of cyclic-dimeric GMP (cyclic-di-GMP). We tentatively named the GP0611 homologue *pdeA*. The primary sequence of PdeA showed 95 % identity in amino-acid sequence to PdeA1 (AAC61683.1), which is a cyclic-di-GMP phosphodiesterase A in *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) (Tal *et al.*, 1998). *G. xylinus* contains three *cdg* operons, *cdg1*, *cdg2* and *cdg3*, all of which control the cellular turnover of cyclic-di-GMP, a positive effector of a cellulose synthase. *pdeA1* is located in

the *cdg1* operon (Tal *et al.*, 1998). The *cdg1* operon consists of four genes orientated in the same direction: *cdg1a*, encoding a protein similar to members of the Crp–Fnr family transcriptional regulators, *pdeA1*, *dgc1*, encoding a diguanylate cyclase, and *cdg1d*, encoding a hypothetical protein. Nucleotide sequencing of a 5.5 kb *Sma*I fragment containing *pdeA* of *G. intermedius* NCI1051 revealed the presence of *cdg1a* and *dgc* homologues upstream and downstream, respectively, from *pdeA* in *G. intermedius* NCI1051 (Fig. 1a).

We cloned a 3.0 kb *Nae*I fragment containing the DV214 homologue, named *pdeB*, and a 2.0 kb *Pst*I fragment containing the GP0603 homologue, named *nagA*, from *G. intermedius* NCI1051. The nucleotide sequence of the cloned fragments indicated that the two fragments overlapped. The *nagA* gene was located just downstream from *pdeB* in the same direction. The primary sequence of PdeB showed approximately 40 % identity with proteins annotated as phosphodiesterase/diguanylate cyclase or cAMP phosphodiesterase, including Bpet1368 (GenBank accession no. YP_001629973.1) from *Bordetella petrii* DSM 12804, CKO_00437 (YP_001452030.1) from *Citrobacter koseri* ATCC BAA-895, GDI3812 (YP_001604034.1) from *G. diazotrophicus* PAL5, and PdeA from *G. intermedius* NCI1051 described above. In contrast, the primary sequence of NagA showed approximately 65 % identity with proteins annotated as putative *N*-acetylglucosamine-

Fig. 1. Transcriptional analysis of *gltA*, *cdg1a-pdeA*, *pdeB* and *nagA*. (a) Agarose gel electrophoresis of RT-PCR products. RT-PCR was performed using total RNA from the wild-type strain NCI1051. The RNA samples were analysed with (+ RT) and without (–RT) RT to verify the absence of genomic DNA. Lane P was a control lane in which the genomic DNA of *G. intermedius* NCI1051 was amplified with the respective pair of primers. For example, the primer combination 1F and 1R is presented as 1F1R. The positions of the *cdg1a*, *pdeA* and *dgc* genes and PCR products are also shown schematically. (b) S1 nuclease mapping of *gltA*, *cdg1a-pdeA*, *pdeB* and *nagA* in the wild-type (w.t.) strain NCI1051 harbouring pMV24, the *ginI*::Km mutant harbouring pMV24 and the *ginA*::Km mutant harbouring pMV24 in the exponential and stationary phases. rRNA was used to check the amount of RNA used.

6-phosphate deacetylases, including GOX1473 (GenBank accession no. YP_191872.1) from *Gluconobacter oxydans* 621H (Prust *et al.*, 2005) and GDI2579 (YP_001602823.1) from *G. diazotrophicus* PAL5, and 33 % identity with Acry_1419 (YP_001234548.1) from *Acidiphilium cryptum* JF-5.

Dependence of *gltA*, *pdeA*, *pdeB* and *nagA* transcription on *ginA*

As described above, *pdeA* is apparently encoded in an operon with its neighbouring genes in *G. intermedius* NCI1051 as well as *G. xylinus*. In fact, we failed to detect the *pdeA* transcript from the putative *pdeA* promoter in front of the *pdeA*-coding sequence by S1 nuclease mapping (data not shown). Therefore, we assumed that *pdeA* was co-transcribed with *cdg1a*, which is located just upstream from *pdeA*, and we confirmed this assumption by RT-PCR (Fig. 1a). RT-PCR analysis using cDNA synthesized by RT from RNA prepared from the wild-type strain gave a distinct amplified fragment for each gene: fragment 1F1R synthesized with primers 1F and 1R for *cdg1a*, fragment 2F2R for *pdeA* and fragment 3F3R for *dgc* (Fig. 1a). A region spanning *cdg1a*–*pdeA* was also amplified by RT-PCR using primers 5F and 5R, yielding fragment 5F5R, which indicated that *cdg1a* and *pdeA* were co-transcribed. In contrast, a region spanning *pdeA*–*dgc* was not amplified by RT-PCR using primers 6F and 6R. Furthermore, a DNA fragment (4F4R) containing a region upstream from *cdg1a* and its 5' portion was not amplified by RT-PCR, suggesting that *cdg1a* was transcribed from its own promoter. Transcription from the *cdg1a* promoter was confirmed by S1 nuclease mapping as described below.

We determined the transcription of *gltA*, *cdg1a*–*pdeA*, *pdeB* and *nagA* in the wild-type strain NCI1051, the *ginI*::Km mutant and the *ginA*::Km mutant, all of which harboured the vector plasmid pMV24, by low-resolution S1 nuclease mapping (Fig. 1b). RNA was isolated by the hot phenol method from cells grown at 30 °C to the exponential and stationary phases in YPG medium containing 1 % (v/v) cellulase and 2 % (v/v) ethanol. In strain NCI1051, *gltA*, *cdg1a*–*pdeA* and *pdeB* were actively transcribed in both the exponential and the stationary phase, but *nagA* was actively transcribed only in the exponential phase (Fig. 1b). In contrast, in both the *ginI*::Km and the *ginA*::Km mutant, all of these transcripts were hardly detectable in both the exponential and the stationary phase (Fig. 1b). From these results, we concluded that *gltA*, *pdeA* (*cdg1a*–*pdeA*), *pdeB* and *nagA* are positively regulated by the GinI/GinR quorum-sensing system via *GinA*.

By 5'-RACE, we determined the transcriptional start sites of *gltA*, *cdg1a*–*pdeA* and *pdeB*, all of which were actively transcribed in both the exponential and the stationary phase, as described below. *gltA* was transcribed from a C, which was located 28 nt upstream of the first nucleotide of the start codon. *cdg1a*–*pdeA* was transcribed from an A,

which was located 101 nt upstream from the first nucleotide of the start codon of *cdg1a*. *pdeB* was transcribed from a G, which was located 11 nt upstream from the first nucleotide of the start codon. These transcriptional start sites determined by 5'-RACE showed good correlation with those predicted by the low-resolution S1 nuclease mapping.

Growth of the *gltA*, *pdeA*, *pdeB* and *nagA* disruptants in the presence of ethanol

To examine the possible functions of the novel *GinA*-inducible genes, we disrupted the chromosomal *gltA*, *pdeA*, *pdeB* and *nagA* genes in *G. intermedius* NCI1051, generating mutants *gltA*::Km, *pdeA*::Km, *pdeB*::Km and *nagA*::Km, respectively. First, we analysed the growth of the mutants in YPG medium containing 2 % (v/v) ethanol.

The growth rate of mutant *nagA*::Km in the exponential phase was much lower than that of the parental strain in YPG medium containing 2 % (v/v) ethanol (Fig. 2e). However, the final cell mass of mutant *nagA*::Km was almost the same as that of the parental strain. The deficiency in the exponential growth of the *nagA*::Km mutant was due to the mutation in *nagA*, because introduction of *nagA* on pNagA into the mutant increased the growth rate to the same level as that of the parental strain (Fig. 2e). Because the growth rates of mutants *ginI*::Km and *ginA*::Km, in which *nagA* was scarcely transcribed, were greater than that of the parental strain in YPG medium containing 2 % (v/v) ethanol, the low growth rate of the *nagA*::Km mutant was surprising. This unexpected finding showed a new aspect of the GinI/GinR quorum-sensing system: it accelerates exponential growth by induction of *nagA*.

The growth rate and cell mass of mutants *gltA*::Km and *pdeA*::Km during the exponential growth phase were slightly higher than those of the parental strain (Fig. 2b, c). These growth profiles of mutants *gltA*::Km and *pdeA*::Km were almost the same as those of mutants *ginI*::Km (Fig. 2a), *ginR*::Km and *ginA*::Km (Iida *et al.*, 2008a). The higher growth rates of the mutants were due to the mutations in *gltA* and *pdeA*, because introduction of *gltA* on pGltA and *pdeA* on pPdeA into the corresponding mutants reduced the growth rates to the same level as that of the parental strain (Fig. 2b, c). In medium containing no ethanol, however, the *gltA*::Km and *pdeA*::Km mutants grew with the same time-course and cell mass as the parental strain (Fig. 2f). Therefore, the higher growth rate and cell mass of mutants *gltA*::Km and *pdeA*::Km were dependent on the presence of ethanol, indicating that *gltA* and *pdeA* are involved in acetic acid fermentation. In contrast, the *pdeB*::Km mutant grew with almost the same time-course and cell mass as the parental strain in medium containing 2 % (v/v) ethanol (Fig. 2d), suggesting that *pdeB* is not involved in acetic acid fermentation.

Fig. 2. Growth of *G. intermedium* strains in YPG medium containing 2% (v/v) ethanol (a–e) and YPG medium (f) as described in Methods. (a–f) Wild-type strain NCI1051 harbouring pMV24 (●). (a) The *ginI*::Km mutant harbouring pMV24 (△) and the *ginI*::Km mutant harbouring pGinIA (▲). (b) The *gltA*::Km mutant harbouring pMV24 (△) and the *gltA*::Km mutant harbouring pGltA (▲). (c) The *pdeA*::Km mutant harbouring pMV24 (△) and the *pdeA*::Km mutant harbouring pPdeA (▲). (d) The *pdeB*::Km mutant (△). (e) The *nagA*::Km mutant harbouring pMV24 (△) and the *nagA*::Km mutant harbouring pNagA (▲). (f) The *gltA*::Km mutant harbouring pMV24 (△) and the *pdeA*::Km mutant harbouring pMV24 (▲). The growth of the strains was measured by recording the OD₆₆₀ of the culture broths. The values given are means ($n=3$). SD values are not shown because they would complicate the figure.

Repression of oxidative fermentation by *gltA* and *pdeA*

Because the *gltA*::Km and *pdeA*::Km mutations affected cell growth in medium containing ethanol, we examined the involvement of the *gltA* and *pdeA* genes in oxidative fermentation. We measured acetic acid and gluconic acid production by the *gltA*::Km and *pdeA*::Km mutants. The strains were cultured at 30 °C in YPG medium containing ethanol in a 3 l mini-jar fermenter. The ethanol concentration was initially 3% (v/v) and then automatically maintained at 2% (v/v) by adding ethanol during cultivation.

Fig. 3(a) and Fig. 3(b) show the acetic acid and gluconic acid fermentation profiles, respectively, of the wild-type strain NCI1051, the *ginI*::Km mutant and the *gltA*::Km mutant, all of which harboured the vector pMV24. Like mutant *ginI*::Km, the *gltA*::Km mutant accumulated acetic acid and gluconic acid in higher yields than the parental strain NCI1051. The final yields of acetic acid (5.32 ± 0.23 % w/v) and gluconic acid (2.07 ± 0.04 %) produced by mutant *gltA*::Km were larger than those (acetic acid, 2.68 ± 0.08 %; gluconic acid, 1.73 ± 0.12 %) produced by the parental strain NCI1051. The observed increase in the rate of acetic acid production and in the total amount of acetic acid produced by the *gltA*::Km

mutant was in agreement with the increased growth rate of the mutant compared with the parental strain during the late exponential growth phase (Fig. 3c). The increased final yields of acetic acid and gluconic acid in the *gltA*::Km mutant were reduced by introducing *gltA* on pGltA into this mutant (data not shown). In contrast, the wild-type strain NCI1051 harbouring pGltA produced almost the same amounts of acetic acid and gluconic acid as the wild-type strain harbouring the empty vector pMV24 (Fig. 3a, b). Mutant *gltA*::Km continued to produce acetic acid in the stationary phase, like the *gmpA*::Km mutant (Iida *et al.*, 2008b), although production of acetic acid in the stationary phase by the *ginI*::Km (Fig. 3a) and *ginA*::Km (Iida *et al.*, 2008a) mutants ceased. Thus, the phenotypes of the *gltA*::Km mutant were not completely the same as those of the *ginI*::Km and *ginA*::Km mutants. In addition, mutant *gltA*::Km began to grow and produce both acetic acid and gluconic acid after a delay of about 2 h compared with the parental strain and mutant *ginI*::Km (Fig. 3a–c), like mutant *ginA*::Km (Iida *et al.*, 2008a). This result suggested that the time lag at the beginning of exponential growth and oxidative fermentation in mutant *ginA*::Km is caused by the repression of *gltA* in the mutant.

Fig. 3(d) and Fig. 3(e) show the acetic acid and gluconic acid fermentation profiles, respectively, of the wild-type

Fig. 3. Courses of acetic acid (a, d) and gluconic acid (b, e) fermentation by *G. intermedius* strains, together with their growth (c, f). The growth of the strains was measured by recording the OD₆₆₀ of the culture broths. (a–c) Wild-type strain NCI1051 harbouring pMV24 (●), the *ginI*::Km mutant harbouring pMV24 (▲), the *gltA*::Km mutant harbouring pMV24 (△), and the wild-type strain NCI1051 harbouring pGltA (□) were cultured as described in Methods. (d–f) Wild-type strain NCI1051 harbouring pMV24 (●), the *pdeA*::Km mutant harbouring pMV24 (△), and the wild-type strain NCI1051 harbouring pPdeA (□) were cultured as described in Methods. The medium contained 3% (v/v) ethanol initially, and the ethanol concentration of the medium was automatically maintained at 2% (v/v) by adding ethanol during cultivation. The values given are means $\pm$ SD ($n=3$).

strain NCI1051 and the *pdeA*::Km mutant, both of which harboured the vector pMV24. The *pdeA*::Km mutant accumulated acetic acid with a slightly higher yield than the parental strain NCI1051, although the increased final yield of acetic acid in the *pdeA*::Km mutant was smaller than those in the *ginI*::Km and *gltA*::Km mutants. The final yields of acetic acid ($2.96 \pm 0.36\%$ w/v) produced by mutant *pdeA*::Km were larger than that ($2.68 \pm 0.08\%$) produced by the parental strain NCI1051. The *pdeA*::Km mutant produced almost the same amount of gluconic acid as the wild-type strain. The increased final yields of acetic acid in the *pdeA*::Km mutant were restored to the wild-type level by introducing *pdeA* on pPdeA into this mutant (data not shown). Consistent with the observation that the

pdeA mutation led to production of acetic acid at high yields, introduction of *pdeA* under the control of the *E. coli* *lac* promoter on the pMV24-derived plasmid pPdeA into the wild-type strain NCI1051 reduced the final yields of acetic acid ($1.83 \pm 0.39\%$ w/v) and gluconic acid ($0.80 \pm 0.04\%$) to below those of the parental strain (Fig. 3d, e). The copy number of pMV24 is about 10 (Iida *et al.*, 2008a). The observed decrease in the rate of acetic acid production and in the total amount of acetic acid produced by the *pdeA*-overexpressing strain was in agreement with the decreased growth rate of the strain compared with that of the wild-type strain during the late exponential growth phase (Fig. 3f). These findings suggested that *gltA* and *pdeA*, novel targets of the GinI/GinR quorum-sensing

system in *G. intermedius* NCI1051, repressed oxidative fermentation, including acetic acid and gluconic acid fermentation.

gltA represses antifoam activity

We previously showed that the GinI/GinR quorum-sensing system represses antifoam activity via GinA (Iida *et al.*, 2008a). Therefore, antifoam activities of the *gltA*::Km, *pdeA*::Km, *pdeB*::Km and *nagA*::Km mutants were examined. The *gltA*::Km mutant formed foams of smaller volumes during growth in the exponential phase than the wild-type strain NCI1051 when the strains were grown in YPG medium containing ethanol in a shaking flask (Fig. 4a) or a 3 l mini-jar fermenter (Fig. 4b). The reduced foam production in the *gltA*::Km mutant was restored to the wild-type level by introducing *gltA* on pGltA into this mutant (Fig. 4a). In contrast, the *pdeA*::Km, *pdeB*::Km and *nagA*::Km mutants formed foams of almost the same volume as the wild-type strain NCI1051 (data not shown). Therefore, *gltA* also repressed antifoam activity in addition to oxidative fermentation, but *pdeA*, *pdeB* and *nagA* had no apparent effects on antifoam activity.

DISCUSSION

We identified four novel GinA-inducible genes, *gltA*, *pdeA*, *pdeB* and *nagA*, in the GinI/GinR quorum-sensing system of *G. intermedius*. Of these genes, *gltA* and *pdeA* repressed oxidative fermentation, including acetic acid and gluconic acid fermentation, and growth in medium containing ethanol. In addition, *gltA* also repressed antifoam activity. We had previously identified *gmpA*, encoding an outer-membrane protein, as a GinA-inducible gene that repressed oxidative fermentation (Iida *et al.*, 2008b). Therefore, the mechanism for the repression of oxidative fermentation by the GinI/GinR quorum-sensing system is apparently complex; at least three GinA-inducible genes, *gltA*, *pdeA* and *gmpA*, are involved in the repression of oxidative fermentation via unknown mechanisms (Fig. 5).

gltA was the first GinA-inducible gene that repressed both oxidative fermentation and antifoam activity. We observed that disruption of *gltA* led to an increase in the yield of acetic acid of about twofold (Fig. 3a). The final yield of acetic acid in the *gltA*::Km mutant was highest amongst several mutants related to the GinI/GinR quorum-sensing system of *G. intermedius*. In addition to the enhancement

Fig. 4. Foam production during growth in YPG medium containing ethanol. (a) Foam production during growth in YPG medium containing 2% (v/v) ethanol in a shaking flask for 10 h. 1, Wild-type strain NCI1051 harbouring pMV24; 2, mutant *ginI*::Km harbouring pMV24; 3, mutant *ginI*::Km harbouring pGinIA; 4, mutant *gltA*::Km harbouring pMV24; 5, mutant *gltA*::Km harbouring pGltA. (b) Foam production during growth in YPG medium containing ethanol in a mini-jar fermenter. Wild-type strain NCI1051 harbouring pMV24 and mutant *gltA*::Km harbouring pMV24 were cultured as described in Methods.

Fig. 5. A possible model for the quorum-sensing system in *G. intermedius* NCI1051. The GinI/GinR quorum-sensing system in *G. intermedius* activates transcription of *gltA*, *pdeA* and *gmpA* (Iida *et al.*, 2008b) via GinA, which in turn represses oxidative fermentation, including acetic acid and gluconic acid fermentation, via GltA, PdeA and GmpA, and antifoam activity via GltA. R, GinR; I, GinI; P, promoter; A, GinA.

of acetic acid fermentation, the *gltA* mutant formed smaller volumes of foams during growth in the exponential phase than the wild-type strain NCI1051 (Fig. 4). These properties of the *gltA* mutant are advantageous for industrial vinegar fermentation. GltA is composed of two domains: the N-terminal domain contains a glycosyltransferase 2 (GT2) motif (Pfam 00535) and the C-terminal domain contains a glycosyltransferase 1 (GT1) motif (Pfam 00534). The GT2-family glycosyltransferases transfer sugar from UDP-glucose, UDP-*N*-acetylgalactosamine, GDP-mannose or CDP-abequose to a range of substrates, including cellulose, dolichol phosphate and teichoic acids. The GT1-family glycosyltransferases transfer UDP-, ADP-, GDP- or CMP-linked sugars to a variety of substrates, including glycogen, fructose 6-phosphate and lipopolysaccharides. Tandem glycosyltransferase modules within a single peptide are commonly found in glycosyltransferases that are involved in the biosynthesis of alternating polysaccharides such as hyaluronan, chondroitin and heparin (Coutinho *et al.*, 2003). These alternating polysaccharides are formed by the two glycosyltransferase modules with different substrate specificities, which function alternately. GltA may be involved in the biosynthesis of alternating polysaccharides, although our previous study showed that *ginI* and *ginR* have insignificant effects on exopolysaccharide production, including production of bacterial cellulose and water-soluble polysaccharides (Iida *et al.*, 2008a). We speculate that GltA represses antifoam activity by synthesizing lipopolysaccharide or exopolysaccharide to change some character-

istics of the cell surface. This physical change of the cell surface may repress oxidative fermentation by an unknown mechanism. If some polysaccharide(s) is modified or not formed by the disruption of *gltA*, the substrate (ethanol or glucose) of oxidative fermentation may enter the periplasmic space more efficiently by diffusion, thereby accounting for the gradual increase in oxidative fermentation. Because an outer-membrane protein, GmpA, also represses oxidative fermentation, the properties of the outer membrane (or cell surface) appear to have a great influence on oxidative fermentation, although two major enzymes for oxidative fermentation, alcohol dehydrogenase and aldehyde dehydrogenase, are localized in the inner membrane. The involvement of the outer membrane, which is generally thought to function as a structural barrier against environmental factors, in oxidative fermentation by acetic acid bacteria requires further investigation.

pdeA, encoding a putative cyclic-di-GMP phosphodiesterase, was shown to repress oxidative fermentation. Although the disruption of *pdeA* had less effect on oxidative fermentation than the disruption of *ginI* and *gltA*, overexpression of *pdeA* clearly repressed oxidative fermentation (Fig. 3d, e). Because *G. polyoxogenes* contains several sets of phosphodiesterases and diguanylate cyclases (K. Kondo, personal communication) and *G. xylinus* had three sets (Tal *et al.*, 1998), *G. intermedius* NCI1051 may also contain multiple sets of these enzymes to control the concentration of cyclic-di-GMP. The redundancy of the phosphodiesterases and diguanylate cyclases might compensate for the

intermedius. Our studies have revealed that many genes involved in oxidative fermentation are under the control of the GinI/GinR quorum-sensing system. This finding emphasizes the importance of the quorum-sensing system in *G. intermedius*, which has presumably evolved to adapt to an environment in which oxidative fermentation is effective. Although molecular mechanisms for repression of oxidative fermentation and antifoam activity by GinA-inducible genes, as well as the molecular function of GinA, remain to be elucidated, our findings will be helpful for strain improvement in industrial vinegar fermentation by acetic acid bacteria.

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