Effect of *gacS* and *gacA* Mutations on Colony Architecture, Surface Motility, Biofilm Formation and Chemical Toxicity in *Pseudomonas* sp. KL28

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GacS and GacA proteins form a two component signal transduction system in bacteria. Here, Tn5 transposon *gacS* and *gacA* (Gac) mutants of *Pseudomonas* sp. KL28, an alkylphenol degrader, were isolated by selecting for smooth colonies of strain KL28. The mutants exhibited reduced ability to migrate on a solid surface. This surface motility does not require the action of flagella unlike the well-studied swarming motility of other *Pseudomonas* sp. The Gac mutants also showed reduced levels of biofilm and pellicle formation in liquid culture. In addition, compared to the wild type KL28 strain, these mutants were more resistant to high concentrations of *m*-cresol but were more sensitive to H_2O_2, which are characteristics that they share with an *rpoS* mutant. These results indicate that the Gac regulatory cascade in strain KL28 positively controls wrinkling morphology, biofilm formation, surface translocation and H_2O_2 resistance, which are important traits for its capacity to survive in particular niches.

Keywords: biofilm, colony phenotype, GacA, GacS, *Pseudomonas*, RpoS

A bacterial two component signal transduction system, which consists of a membrane-bound histidine sensor kinase GacS and a cytoplasmic transcriptional response regulator GacA, responds to signal(s) that accumulate during growth and positively controls the expression of small regulatory RNA(s), a messenger for the downstream control of the expression of certain genes (Heeb and Haas, 2001; Kay *et al.*, 2005). This Gac two-component signaling system (GacTSS), which is present in a broad range of Gram-negative bacteria, has been shown to be involved in controlling the expression of genes related to virulence factors, biofilm, motility, quorum-sensing signal molecules, survival, and secondary metabolites (Heeb and Haas, 2001). Extensive studies on GacTSS have been carried out in animal and plant *Pseudomonas* pathogens, such as *P. aeruginosa* (Parkins *et al.*, 2001; Goodman *et al.*, 2004; Park *et al.*, 2005; Davies *et al.*, 2007), *P. syringae* (Kinscherf and Willis, 1999; Chatterjee *et al.*, 2003) and the root colonizing biocontrol strains, such as *P. fluroescens* (Whistler *et al.*, 1998; Heeb *et al.*, 2005), *P. chlororaphis* (Kang *et al.*, 2004; Poritsanos *et al.*, 2006), and *P. putida* (Dubern and Bloemberg, 2006). However, the role played by GacTSS in the degradation of organic pollutants by *Pseudomonas*, which is frequently found in polluted environments (Lee *et al.*, 2006), has not been investigated; especially with regard to how GacTSS contributes to the catabolism of, and resistance to chemical pollutants, as well as to how it affects the environmental fitness of these bacteria.

Pseudomonas sp. KL28 has been shown to grow on a range of alkylphenols, such as 3- and 4-*n*-alkylphenol (C_1-C_5) using a catabolic pathway encoded by the *lap* catabolic gene cluster (Jeong *et al.*, 2003), which is distantly related to that used for the catabolism of phenol and methylphenols (Shingler *et al.*, 1992). In our previous study, an *rpoS* mutant of strain KL28 (S23) was shown to possess increased tolerance to *m*-cresol, a wrinkled morphology, and increased motility on the surface of LB agar, as well as enhanced biofilm formation in polystyrene micro-dishes (Yun *et al.*, 2007). The *rpoS* gene is known to encode a sigma factor of RNA polymerase that controls the expression of certain genes at stationary phase and under various stresses (Suh *et al.*, 1999). In this study, transposon mutants of strain KL28 showing a smooth phenotype on the surface of LB agar were selected with the hope of identifying the regulatory genes that control phenotypes opposite to those of the *rpoS* mutant. We found that mutants of GacTSS had this characteristic. The role of GacTSS in determining the resistance of strain KL28 to *m*-cresol and H_2O_2 is further investigated. The results show that GacSTT and RpoS regulate some pathways that counteract each other and result in distinct phenotypes for strain KL28.

Materials and Methods

Bacterial strains and culture conditions

Pseudomonas sp. KL28 was cultured in Luria-Bertani (LB) medium (Bertani and Bertani, 1970) or in minimal salts basal medium (MSB) (Stanier *et al.*, 1966) with an appropriate carbon and energy source at 30°C. Strains S23, C16,

and C19 are *Pseudomonas* sp. KL28 derivatives with Tn5 mutations in *rpoS*, *gacA*, and *gacS*, respectively, which were generated by the method described in the following section. Southern hybridization with the *aph* gene encoding resistance to kanamycin as a probe was performed as described previously (Park et al., 2001) and showed that the mutants had single mutations. *Escherichia coli* cells were grown in LB medium at 37°C. The detailed culture conditions for the strains and the amounts of antibiotics added to the culture media for plasmid maintenance have been previously described (Yun et al., 2007). The P_{lapB}-GFP reporter vector, pJJR1 (Jeong et al., 2003), was introduced into KL28 and its derivative strains by conjugation as described (Yun et al., 2007).

Transposon mutagenesis and identification of the mutated gene from mutants

For transposon mutagenesis of strain KL28, biparental mating was carried out between strains KL28 and *E. coli* BW20767, which bears the transposon delivery vector pRL27 (Larsen et al., 2002), as previously described (Yun et al., 2007). Transconjugants were selected on LB agar containing ampicillin, chloramphenicol and kanamycin. The transposon junction plasmids were obtained by self-ligation of *Bam*HI or *Bgl*II-digested chromosomal DNA obtained from the transconjugants. Identification and sequencing of the mutated genes were carried out as previously described (Yun et al., 2007). The deduced amino acid sequences were compared with the protein sequence database (GenBank) using the BLASTX Algorithm (http://www.ncbi.nlm.nih.gov).

Complementation of the gacA gene

The plasmid pGacA, which expresses the *gacA* gene, was constructed as follows. The *gacA* gene was amplified by PCR (94°C for 5 min, 35 cycles consisting of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, followed by a final incubation at 72°C for 10 min) with primers C16F; AAGGTGGC GGGATCCAATTGC and C16R; CCGCTGGAGCTCGCC AGGAAA from strain KL28. The amplified PCR product was purified and ligated into pGEM®-T Easy (Promega Co.) vector, yielding pT-gacA. The presence of the intact *gacA* gene in pT-gacA was confirmed by DNA sequencing. The amplified *Bam*HI-*Sac*I fragment from pT-gacA was ligated into the same sites of the broad-host range pBBR1MCS-5 (Kovach et al., 1995). The resulting plasmid (pGacA) was transformed into the mutant C16 by triparental mating as previously described (Yun et al., 2007) to yield strain C16 (pGacA).

Transmission electron microscopy (TEM)

Pellicles obtained from standing culture which had been incubated at 25°C for 72 h were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide for 2 h each at 4°C. The fixed samples were washed with phosphate buffer (0.1 M, pH 7.2) and dehydrated in graded ethanols. After further dehydration in propylene oxide, the samples were treated with a 1:1 mixture of propylene oxide-Epon 812 for 1 h and then embedded in Epon 812. The samples were polymerized for 12 h at 37°C and sectioned (60-90 nm). Sections were stained with uranyl acetate and lead citrate. Samples were

examined with a transmission electron microscope (JEM-1200EXII, JEOL, Japan).

m-Cresol and H₂O₂ sensitivity testing

For the measurement of sensitivity to *m*-cresol in liquid cultures, 250 ml-flasks containing 50 ml of LB medium with gentamycin (Gm) and *m*-cresol (0.075%) were inoculated with overnight-grown cultures of plasmid containing *Pseudomonas* cells to an initial $O.D._{660}$ of 0.01. Flasks were incubated at 30°C on an orbital shaker (160 rpm) and cell growth was monitored at $O.D._{660}$. The sensitivity of strain KL28(pBBR1MCS-5) and its mutants to *m*-cresol and H_2O_2 in LB agar was determined by measuring survival as follows. Cells cultured in LB liquid medium for 24 h were harvested by centrifugation and were diluted with saline to an $O.D._{660}$ of 0.4. Then, the samples were serially diluted and 5 μl from each dilution was spotted on overnight-dried LB agar plates containing Gm with *m*-cresol (0.075%) or H_2O_2 (2 mM) and the plates were incubated at 30°C for 24 h or 48 h. For H_2O_2 sensitivity testing in suspension, the plasmid-containing cells harvested after 24 h cultivation were washed and suspended in saline to an $O.D._{660}$ of 0.1. H_2O_2 was added to a final concentration of 50 mM. The suspensions were subjected to shaking incubation (100 rpm) at room temperature (20-24°C) for 10 min. The treated cells were quickly diluted with saline and spread on LB agar plates containing Gm for viability measurements.

Quantification of biofilm formation and GFP expression

Biofilms were elaborated in 96-well polystyrene dishes (U-plate, Medi Land Co., Korea) as described previously (O'Toole and Kolter, 1998). In brief, plasmid containing cells were grown in LB medium with Gm at 30°C for 24 h and spun down and washed with sterile saline. Then, the samples were diluted to an $O.D._{660}$ of 0.1 and used as seed. Then 15 μl of seed and 150 μl fresh sterile LB with Gm were incubated at 25°C for 24 h. To prevent drying, 96-well polystyrene dishes were loosely sealed with Parafilm. The amount of biofilm formed was measured by crystal violet staining as described previously (Yun et al., 2007). All strains were tested five times to obtain the SDs. For GFP measurements, samples were adjusted to an $O.D._{660}$ of 0.4 in saline and the intensity of fluorescence were determined using a spectrofluorometer (model RF-5301PC, Shimadzu Co., Japan) at an excitation wavelength of 393 nm and an emission wavelength of 509 nm, with each 3.0 nm of wavelength split. All strains were tested three times to obtain the SDs.

Nucleotide sequence accession numbers

The 5,017 bp and 2,500 bp regions around *gacS* and *gacA*, respectively, have been sequenced (Fig. 1) and deposited in the GenBank database, under accession numbers EF583623 and EF583622, respectively.

Results and Discussion

Identification of gacS and gacA as genes that control the architecture of Pseudomonas sp. KL28 colonies

Pseudomonas sp. KL28 forms slightly wrinkled colonies when growing on LB medium with 1.5% agar. In order to
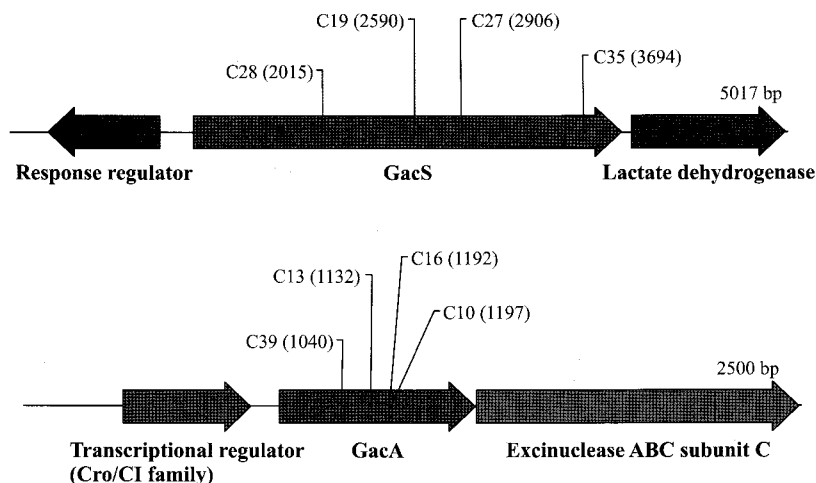
Fig. 1. Physical map of the transposon-inserted chromosomal segments in the *gacS* and *gacA* mutants. Mutant names are placed on the gene with the insertion positions obtained from the sequenced fragments in parenthesis.
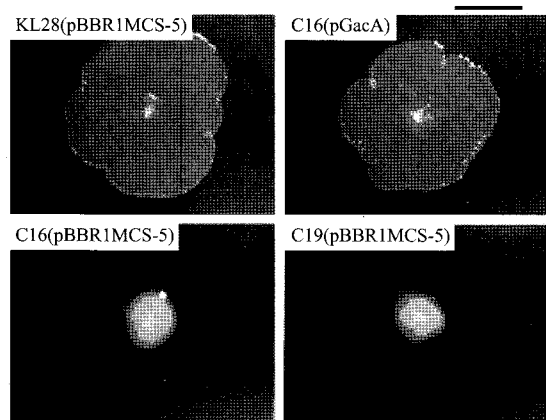
Fig. 2. Surface translocation by strain KL28(pBBR1MCS-5) and its derivatives. The pictures were taken by an SMZ1500 stereomicroscope (Nikon Co., Japan) with an Infinity 1 camera (Lumenera Scientific, Canada). Bar: 0.5 cm.

identify the genes controlling these colony phenotypes, pRL27 transposon mutants of strain KL28 showing smooth surface colonies were selected and the mutated genes were identified as described in Materials and Methods. Among them, C19, C27, C28, and C35 mutants were shown to have mutations in the *gacS* gene and C10, C13, C16, C39 mutants were shown to have mutations in *gacA* as shown in Fig. 1. Because the genes comprising GacTSS have not been previously shown to be involved in morphological phenotypes, we further studied the role of GacTSS in colony morphology. The deduced 917 amino acids of *gacS* had highest identity (83%) to that of *P. entomophila* L48 and 72-82% identities to those of *P. putida* KT2440 and F1, *P. chlororaphis, P. fluorescens* Pf-5 and *P. syringae*. The deduced 207 amino acids of *gacA* had highest identity (96%) to that of *P. entomophila* L48 and 92% identities to those of *P. flurorescens, P. syringae,* and *P. chlororaphis,* which are well studied strains for *gac* signal transduction. Further database analysis showed that the sequences flanking the *gac* genes in strain KL28 are also

conserved in other *Pseudomonas* strains, such as *P. entomophila* L48, *P. putida* F1 and KT2440 and *P. fluorescens* Pf-5 as shown in Fig. 1.

Mutation of gacS/gacA affects the surface motility of Pseudomonas sp. KL28

In order to further confirm the role of the *gac* genes in colony morphology and surface motility, *gacS* (C19) and *gacA* (C16) mutants (*gac* mutants) carrying the control vector pBBR1MCS-5, the *gacA* complemented C16 mutant [C16(pGacA)] and the KL28(pBBR1MCS-5) strain were stab inoculated on LB plates with 1.2% agar containing Gm followed by incubation at 25°C for 24 h. Strain KL28 (pBBR1MCS-5) showed enhanced surface motility with slight wrinkling of the edges of the colonies whereas C16 (pBBR1MCS-5) and C19(pBBR1MCS-5) formed small, smooth and shiny colonies (Fig. 2). Strain C16(pGacA) showed a colony morphology similar to that of strain KL28 (pBBR1MCS-5), suggesting that GacTSS positively controls the surface motility of strain KL28, in addition to its rough colony architecture on LB media containing higher levels of agar. Because *Pseudomonas* sp. KL28 with mutations in genes encoding flagellin or in genes involved in swimming still showed surface motility (data not shown), this type of motility would appear to be different from the swarming defined in *P. aeruginosa* and other bacteria, which requires flagella (Kohler *et al.,* 2000; Overhage *et al.,* 2007). There have been reports showing the positive effect of GacTSS on swarming motility in *P. syringae* B728a (Kinscherf and Willis, 1999), *P. syringae* pv. tomato DC3000 (Chatterjee *et al.,* 2003), *P. fluorescens* CHA0 (Kay *et al.,* 2005) and *P. chlororaphis* PA23 (Poritsanos *et al.,* 2006). In contrast, the same motility has been shown to be negatively controlled by GacA in *P. aeruginosa* (Parkins *et al.,* 2001; Kay *et al.,* 2006) and GacS in *P. aeruginosa* PA14 (Davies *et al.,* 2007). The detailed mechanism by which the GacTSS exerts its effect on swarming motility and surface translocation observed by strain KL28 remains unclear. Because strain KL28 did not show consistent swimming motility in LB medium
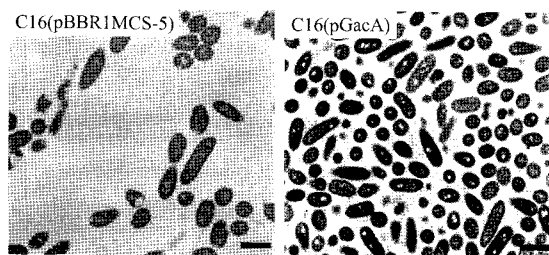
Fig. 3. TEM photographs of pellicles formed by KL28 derivatives. Photographs were taken of a horizontal cross section of the pellicle formed after 72 h incubation. Bar: 1 μm.

with 0.3% agar, the effect of *gac* mutations on swimming could not be confirmed.

GacTSS positively regulates the formation of biofilm in Pseudomonas sp. KL28

Next, the biofilm-forming ability of the mutants was tested to find out if it bore any relationship to their surface translocation and colony morphology. In the biofilm assays as described in Materials and Methods, the optical density at 590 nm of crystal violet formed by strains KL28(pBBR1MCS-5), C16(pBBR1MCS-5), C19(pBBR1MCS-5), and C16(pGacA) were 4.5±0.7 (Mean±SD), 1.2±0.2, 1.4±0.2, 5.1±0.6, respectively, showing that the *gac* mutants formed three-fold less biofilm compared to strain KL28. In addition, both *gac* mutants also had a delayed capacity to form cell aggregates (pellicles) at the air-liquid interface in standing cultures in test tubes; strain KL28(pBBR1MCS-5) and C16(pGacA) formed dense pellicles after 24 h incubation but the *gac* mutants started to form pellicles only after 48 h incubation at 25°C. Besides, the amount of pellicle formed by the *gac* mutants was very small compared to that formed by strain KL28 as shown by TEM photographs (Fig. 3) and as was also observed by the naked eyes (data not shown). There were also fewer inclusion bodies inside the pellicles of strain C16(pBBR1MCS-5) than inside those of C16(pGacA). Thus, our data show that GacTSS in strain KL28 positively regulates the formation of biofilms, including pellicles. The positive involvement of GacTSS in the formation of biofilm has already been documented for other *Pseudomonas* strains (Parkins *et al.*, 2001; Poritsanos *et al.*, 2006; Davies *et al.*, 2007). In *P. aeruginosa* the GacTSS is known to be an upstream regulator of *pel* and *psl* gene clusters, which encode genes for synthesis of an exopolymer that is required for biofilm formation (Goodman *et al.*, 2004). It is interesting to note that, in *P. aeruginosa* PA14, biofilm formation and movement on the surface, namely, swarming motility, are inversely regulated via a regulatory protein, SadB (Caiazza *et al.*, 2007). In strain KL28, variants having enhanced biofilm ability show enhanced surface movement, and vice versa. These complex interactions between biofilm formation and bacterial movement may be rooted in fundamental differences in the nature of the movement and/or exopolymer(s) generated by *Pseudomonas* sp. We are currently studying the characteristics and the genes involved in surface translocation in strain KL28.
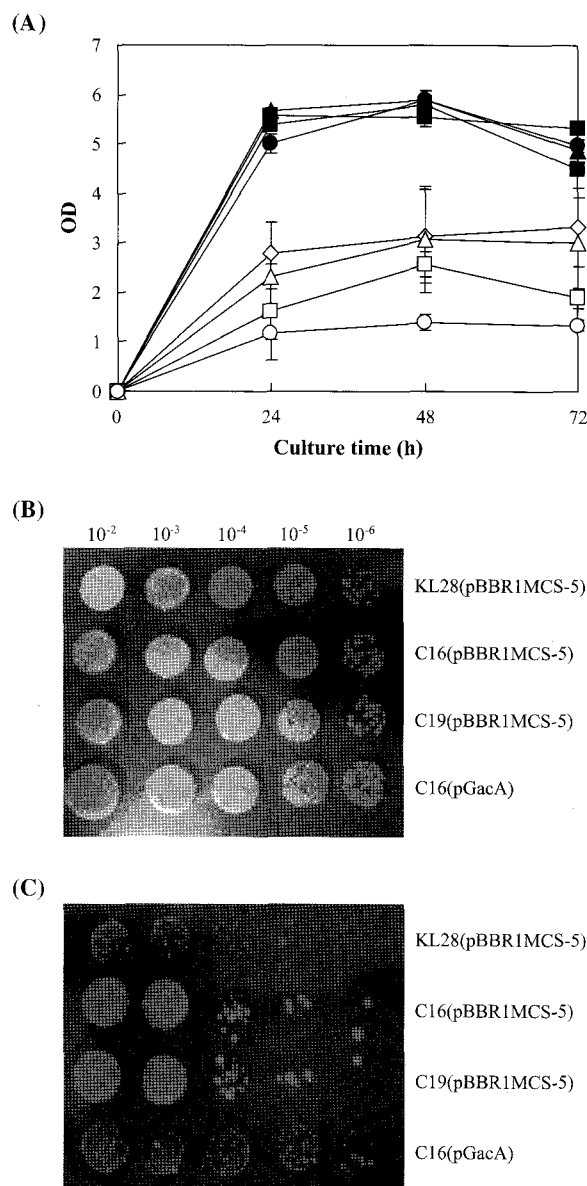

Fig. 4. Effect of *m*-cresol on growth rates of KL28(pBBR1MCS-5) and its derivatives. (A) KL28(pBBR1MCS-5), (□, ■); C16(pBBR1MCS-5), (◇, ◆); C19(pBBR1MCS-5), (△, ▲) and C16(pGacA) (○, ●) in LB medium and Gm with (open symbols) or without (filled symbols) 0.075% *m*-cresol. (B) Growth of KL28(pBBR1MCS-5) and its derivatives on an LB plate with Gm after 24 h incubation. (C) Growth of KL28(pBBR1MCS-5) and its derivatives on an LB plate with 0.075% *m*-cresol and Gm after 48 h incubation.

Mutation of the gac genes contribute to m-cresol resistance and H2O2 susceptibility

The level of resistance of strain KL28(pBBR1MCS-5) and its *gac* mutants to *m*-cresol and H_2O_2 toxicity were determined to examine the role of GacTSS in the detoxification of those chemicals. The effect of *gac* mutations on *m*-cresol tolerance was measured in liquid LB medium containing Gm and 0.075% *m*-cresol. The specific growth rates under these culture conditions decreased in the following order; C16(pBBR1MCS-5), C19(pBBR1MCS-5), KL28(pBBR1MCS-5)

and C16(pGacA) as shown in Fig. 4A. This was not due to a growth defect conferred by the mutations because the wild type and the mutants showed very similar growth patterns in the absence of m-cresol (Fig. 4A). In addition, when the serially diluted 24 h cultured cells were spotted on LB plates supplemented with m-cresol as described in Materials and Methods, only C16(pBBR1MCS-5) and C19 (pBBR1MCS-5) formed colonies at 10^{-3} dilutions after 24 h incubation at 30°C (data not shown). After 48 h of incubation of the spotted plate, the gac mutants showed bigger, visible colonies even at a 10^{-4} dilution whereas KL28 (pBBR1MCS-5) and C16(pGacA) formed tiny colonies at the same dilutions (Fig. 4C), although they showed normal growth on LB agar without m-cresol (Fig. 4B). Three independent experiments produced consistent results. On the other hand, the growth rates of strains KL28(pJJR1) and C16(pJJR1) and the levels of transcriptional expression of the lap catabolic promoter, as monitored by the expression of GFP from pJJR1 (Jeong et al., 2003), from the same strains were almost identical each other during growth in the presence of a relatively lower concentration of 0.02% m-cresol in MSB (data not shown), indicating that the expression of alkylphenol catabolic genes is insensitive to mutation of gac genes. Taken together, our results demonstrate that the GacTSS negatively influences the m-cresol resistance trait of strain KL28 and has no effect on the expression of alkylphenol catabolic genes.

When C16(pBBR1MCS-5), C19(pBBR1MCS-5), and S23 (pBBR1MCS-5) mutants at the stationary phase were exposed to 50 mM H_2O_2 for 10 min as described in Materials and Methods, viabilities dropped to less than 0.1%. In contrast, C16(pGacA) and an rpoS-complemented rpoS mutant [S23(pRpoS)], showed resistance to H_2O_2 at levels that were similar to that of strain KL28(pBBR1MCS-5) (Table 1). In addition, when the serially diluted 48-h cultured cells were spotted on LB plates with 2 mM H_2O_2, C16(pBBR1MCS-5), C19(pBBR1MCS-5) and S23(pBBR1MCS-5) cells showed growth only at a 10^{-2} dilution whereas KL28(pBBR1MCS-5) and C16(pGacA) and S23(pRpoS) strains showed growth even at a 10^{-4} dilution following a 24 h incubation (data not shown). These results showed that, like RpoS, GacTSS also positively controls the resistance to H_2O_2 toxicity probably via the increased formation of catalase. The planktonic and biofilm cells of P. aeruginosa PA14 gacS mutants show increased susceptibility to H_2O_2 (Davies et al., 2007). The authors proposed that this was due to the reduced production of an autoinducer, N-acyl-L-homoserine lactone, which is known as a positive regulator of catalase production (Hassett et al., 1999). In other Pseudomonas, the susceptibility to H_2O_2 conferred by gac mutations was proposed to be due to a deregulation of a Gac-rpoS cascade (Whistler et al., 1998; Kang et al., 2004; Heeb et al., 2005; Poritsanos et al., 2006) where RpoS positively controls the expression of catalase. We are at present investigating the effect of GacTSS on the expression and post-transcriptional control of the rpoS gene to elucidate the detailed mechanism beneath this result.

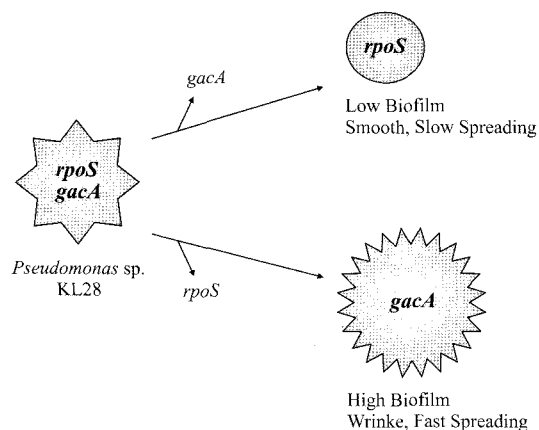**Table 1.** Survival of various KL28 derivatives upon exposure to 50 mM H_2O_2 for 10 min*

Strain	Number of viable cells	
	0 min	10 min (%)[#]
KL28(pBBR1MCS5)	3.64×10^5	1.94×10^5 (46.8)
C19(pBBR1MCS5)	2.59×10^5	446 (99.9)
C16(pBBR1MCS5)	3.44×10^5	0 (100)
C16(pGacA)	3.43×10^5	1.45×10^5 (58.0)
S23(pBBR1MCS5)	3.89×10^5	181 (99.9)
S23(pRpoS)[§]	3.83×10^5	1.6×10^5 (58.3)

* Experimental details are described in Materials and Methods. Results are averages of duplicate experiments.
[#] % reduction
[§] pRpoS is a RpoS expression vector (Yun et al., 2007).

Conclusions

Recently, we have shown that an rpoS mutant of KL28, S23, displays m-cresol resistance and a highly wrinkled colony phenotype with increased spreading. S23 also formed higher levels of biofilms and pellicles in liquid cultures (Yun et al., 2007). Here, we have shown that the gac mutants of strain KL28 have a small, smooth colony phenotype and reduced levels of biofilms and pellicles. These findings suggest that GacTSS plays a different role to that of RpoS in controlling colony morphology, surface motility and biofilm synthesis in strain KL28 as summarized in Fig. 5. This contrast between the colony morphologies of rpoS and gacS/gacA cells has not been reported in other bacteria. On the other hand, for some traits, both of these regulators exert the same regulatory effects, as shown by the increased resistance to m-cresol and increased susceptibility to H_2O_2 of the two mutants. The regulatory consequences of mutations in rpoS and gacS/gacA may reflect the existence of diverse intermediate steps in the RpoS and GacTSS signal transduction networks as was observed in P. fluorescens CHA0 (Heeb et al., 2005). Overall, our findings demonstrate that GacTSS and RpoS play an important role in strain KL28 in moderating colony architecture, surface movement, biofilm formation and chemical susceptibility, which are traits that are essential for bacterial fitness in continuously changing

Fig. 5. Colony variations of the gacA and rpoS mutants of Pseudomonas sp. KL28. The details on the phenotypic changes by the mutants are described in the 'Conclusions' section.

environments. In practice, these traits should be considered when designing biofilters for the treatment of contaminated air or when developing *Pseudomonas* biocontrol inoculants (Mark *et al.*, 2006).

Acknowledgements

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