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Identification and characterization of KvgAS, a two-component system in *Klebsiella pneumoniae* CG43

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Abstract

A two-component system encoding gene cluster *kvgAS* that is present only in virulent *Klebsiella pneumoniae* CG43 was isolated and its sequence determined. RT-PCR and Southern analysis demonstrated that *kvgAS* is organized as an operon. No apparent effect of a *kvgS* deletion on bacterial virulence was observed in a mouse peritonitis model. In the presence of paraquat or 2,2-dipyridyl, the activity of *kvgAS* promoter in the *kvgS* mutant was found to be reduced to half of the level in the wild-type strain. The data suggest that the KvgAS system is autoregulated and plays a role in countering free radical stresses and sensing iron-limiting conditions.

Keywords: Two-component system; Response regulator; Histidine kinase; *Klebsiella* gene

1. Introduction

Klebsiella pneumoniae, an important nosocomial pathogen, causes suppurative infections, urinary tract infection and septicemia in humans. As an opportunistic pathogen, it primarily attacks immunocompromised individuals who are hospitalized and/or suffering from severe underlying diseases, such as diabetes mellitus, chronic alcoholism or pulmonary obstruction [1]. Furthermore, many clinical strains of *K. pneumoniae* are highly resistant to antibiotics indicating the relative ineffectiveness of current therapy.

During infection, bacterial pathogens must adapt to various changes in order to persist and proliferate in appropriate locations and to circumvent host defenses [2,3]. Many bacteria possess specific molecules that sense environmental signals and turn on or off a subset of genes in response to the stress. The most common signal transduction systems belong to the two-component family that consist of a sensory histidine kinase and a response regulator. Upon sensing external signals, a transmembrane kinase activates the response regulator by phosphorylation which, in turn, modulates the transcription of structural genes [4,5].

In an earlier study, we have employed a DNA subtractive hybridization technique to identify genes specifically present in the virulent strain *K. pneumoniae* CG43. Among the subtractive DNA fragments isolated, we found three independent clones that carried a *bvgAS*-like sequence [6]. Since the BvgAS two-component system has been demonstrated to play a critical role in regulation of virulence gene expression in *Bordetella* spp. [4], it would be of interest to know whether the *bvgAS*-like genes play a similar role in *K. pneumoniae*.

2. Materials and methods

2.1. Plasmids, bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains, and *K. pneumoniae* CG43 [7] and its derivatives were propagated at 37°C in Luria–Bertani (LB) broth unless otherwise indicated. Growth of the bacteria was determined by measuring the absorbance at OD_{600} with a Shimatzu UV-1201 spectrophotometer.

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2.2. RT-PCR

Total RNA was isolated from mid-exponential phase *K. pneumoniae* CG43 cells (OD_{600} = 0.6–0.8) by extraction with the TRI reagent (Molecular Research Center, Cincinnati, OH, USA). Contaminating DNA was eliminated from RNA samples with RQ1 RNase-Free DNase (Promega, Madison, WI, USA). Reverse transcription coupled with PCR reaction in one step was performed with Ready-To-Go™ RT-PCR Beads (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) from 1–2 μg of DNA-free RNA. RT-PCR amplification (30 cycles) was carried out in a reaction mixture containing the first-strand primer, PCR primers, 1.5 mM $MgCl_2$, 50 mM Tris–HCl, M-MuLV reverse transcriptase and *Taq* DNA polymerase. The primers used in amplification of the *kvgAS* transcript were A01 and S06, corresponding to the *kvgA* coding sequence and the complement to the *kvgS*, respectively (Table 1). The other set of primers used to investigate *kvgS* and *orfX* were S01 and Q02, corresponding to the *kvgS* coding sequence and the complement to the *orfX* coding sequence, respectively (Table 1).

2.3. Recombinant DNA manipulation, library screening and sequence analysis

All restriction endonucleases and DNA modifying enzymes were obtained from either Promega or Amersham-Pharmacia and used under the conditions recommended by the manufacturers. All recombinant DNA techniques were performed essentially as described previously [8]. The chromosomal DNA of *K. pneumoniae* CG43 was purified by proteinase K digestion, phenol/chloroform extraction, followed by ethanol precipitation [8]. The *K. pneumoniae* CG43 genomic library was constructed by ligating the *Sau*3AI partially digested chromosomal DNA fragments into the *Bam*HI site of the λDASH vector (Stratagene, La Jolla, CA, USA). The nucleotide sequence was determined using the *Taq* DyeDeoxy terminator cycle kit with an Applied Biosystems Model 377 automated sequencer. The nucleotide sequence was analyzed using the Vector NTI Suite 6 program (Informax, Bethesda, MD, USA).

2.4. Construction of a kvgS deletion mutant in K. pneumoniae CG43

The plasmid used to specifically disrupt *kvgS* was constructed by deletion of an internal 1.0-kb *Bam*HI fragment from pYC020 (Fig. 1), which contains a 2.9-kb fragment encoding *kvgS* in pGEM-T easy vector (Promega, Madison, WI, USA). The *kvgS* deleted insert was then subcloned into the suicide vector pKAS46 (a generous gift of Dr. Skorupski, University of New Hampshire) [9], resulting in plasmid pYC030. A *rpsL* gene contained in the plasmid confers on the recipient cell sensitivity to streptomycin and serves as a positive marker in the selection for the loss of the plasmid following homologous recombination. Subsequently, pYC030 was transformed into *E. coli* S17-1 λ *pir* and then mobilized to the streptomycin-resistant strain *K. pneumoniae* CG43-S3 via conjugation. A kanamycin-resistant transconjugant was initially picked and propagated in 2 ml LB overnight, and a small aliquot of the culture was plated on LB agar containing 500 μg ml^{-1} streptomycin. A total of 50 streptomycin-resistant

Table 1
Strains, plasmids, and primers used in this study

Name	Description	Source
K. pneumoniae		
CG43	A blood isolate	[7]
CG43-S3	CG43 with mutation on *rpsL* gene; Sm^r	This study
CG43-S3021	CG43-S3 Δ(*kvgS*)	This study
E. coli		
S17-1 λ *pir*	*recA*, *thi*, *pro*, *hsdR*$^-$ *M*$^+$ [RP4-2-Tc::Mu:Km^R Tn*7*] (λ *pir*); Tp^r Sm^r	[9]
Plasmids		
pKAS46	positive selection suicide vector, pGP704 with *rpsL* gene; Ap^r Km^r	[9]
pYC020	2.9-kb fragment of *K. pneumoniae* CG43 *kvgS* gene generated by PCR, and cloned into pGEM, Ap^r	This study
pYC030	1.9-kb fragment from pYC020, containing an internal 1.0-kb *Bam*HI deletion, cloned into pKAS46, Ap^r Km^r	This study
pUCD1752.1	derivative of pUCD1752, containing *V. fischeri luxAB* as a reporter and a kanamycin resistance gene, Ap^r Km^r	This study
pSL050	330-bp *Hin*dIII/*Eco*RI fragment containing *kvgAS* promoter, cloned into pUCD1752.1	This study
pSL043	0.6-kb fragment containing portion of *kvgS* sequence, cloned in pUC18	[6]
Primers		
A01	5′-GAACAGCGGTACCCACAATA-3′	
A07	5′-TGATTTGGTGCGCCACTAAC-3′	
S01	5′-ACGGGCACAACAATGAGCATAT-3′	
S04	5′-TATTCAAGGCACTAAGCGCC-3′	
S05	5′-CGAATGATTATTGTGCTGGC-3′	
S06	5′-TGTTGACTCACTGCCACATT-3′	
Q02	5′-GCGTTGTGTCGGTGACTCCATG-3′	

clones showing susceptibility to kanamycin were isolated and the deletion of *kvgS* was verified by PCR with primers flanking the *kvgS* gene and by Southern hybridization with fluorescein-labeled *kvgS* probes.

2.5. Luciferase activity assay

Bacteria to be tested were grown in LB overnight, inoculated at 1/20 dilution into fresh medium, and incubated at 25°C under the indicated conditions for 1 h. One hundred microliters of the bacterial culture was mixed with 100 μl of 0.1% (v/v) *n*-decyl aldehyde (Sigma-Aldrich, Milwaukee, WI, USA) for 10 s. The mixture was then read in full integral, autoranging model of a 10 s integral time with a luminometer (Bacterial system™, BG1™) and the result was normalized and expressed as relative light unit (RLU)/OD_{600}.

3. Results and discussion

3.1. Cloning, sequencing and organization of the bvgAS-like genes in K. pneumoniae CG43

The insert fragment of pSL043 [6] containing the *bvgS*-like sequence was used as a probe to screen a genomic library of *K. pneumoniae* CG43 constructed in the λDASH vector. One of the positive phage clones was obtained and subclones in pUC18 were generated to facilitate sequencing analysis. We determined an 8.4-kb sequence and identified four ORFs, designated *kvgA*, *kvgS*, *orfX*, and *kvhR*. The gene organization and the direction of transcription are depicted in Fig. 1.

In *Bordetella* spp., BvgS is responsible for sensing external signals, and transducing them to its cytoplasmic domain, which possesses kinase activity and switches on the transcriptional activator BvgA [4]. The deduced amino acid sequence of KvgS was 42% identical to that of BvgS. The identity between KvgS and EvgS [10], the BvgS homologue of *E. coli*, was even higher, reaching 51%. The amino acid motif –LAT(R)MSHEIRTP– that has been found to be essential for the histidine kinase activity in BvgS and EvgS was also conserved in KvgS [11], except that the threonine was replaced by an arginine in KvgS. BvgA receives the phosphate group from BvgS thereby activating the transcription of a specific set of genes responding to the signals transmitted from BvgS. Sequence analysis has shown that the gene product of *kvgA* resembles BvgA (42.6% similarity). More interestingly, *kvhR* is also a homologue of the response regulator related to BvgA (47.1% similarity) and KvgA (53.8% similarity). The aspartate residue in BvgA that accepts the phosphate group from BvgS is also conserved in both KvgA and KvhR. The deduced amino acid sequence of the *orfX* product did not exhibit similarity with any characterized protein and was not investigated in this study. These sequences are not present in the *K. pneumoniae* MGH78578 genome database generated by the Genome Sequencing Center, Washington University, St. Louis, MO, USA, and thus have been deposited in GenBank database under accession number AJ250891.

To investigate whether *kvgA*, *kvgS* and *orfX* are in the same transcript, RT-PCR was performed. Purified total RNA from *K. pneumoniae* CG43 was used as a template and a pair of oligonucleotide primers specific to either *kvgA* and *kvgS*, or *kvgS* and *orfX*, were employed. As demonstrated in Fig. 2, *kvgA* and *kvgS* are apparently transcribed from the same promoter since an RNA transcript containing both *kvgA* and *kvgS* can be readily detected. The amplified RT-PCR products were also validated by Southern hybridization with the DNA probe of *kvgA* or *kvgS* (Fig. 2C,D). In contrast, no RT-PCR product can be observed with the primer pair specific to *kvgS* and *orfX* (data not shown). The result suggests that *orfX* is transcribed from a promoter distinct from that of *kvgAS*.

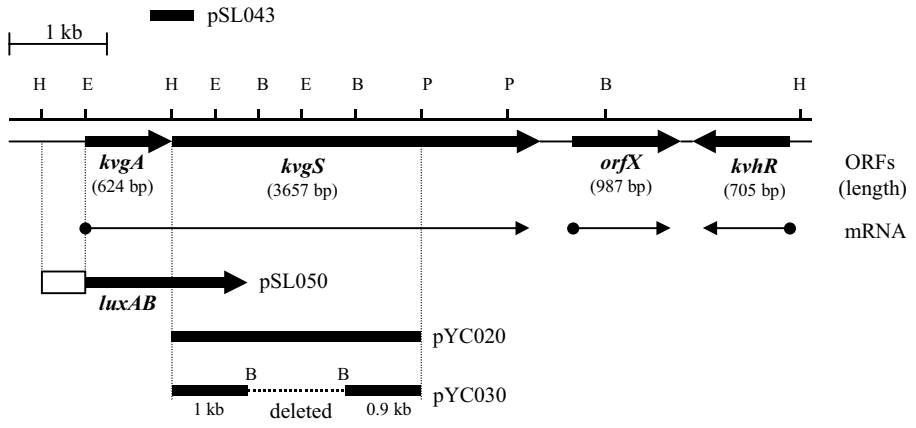

Fig. 1. Organization of the *kvgAS* gene cluster. The transcriptional direction of the four genes and the relative position of the insert in pSL043 are shown. Transcripts found after RT-PCR analysis are indicated with thin arrows. The DNA fragments used to construct the P_{kvgAS}-*luxAB* transcriptional fusion and the plasmids used for allelic exchange of the *kvgS* region are also indicated beneath the map. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I.

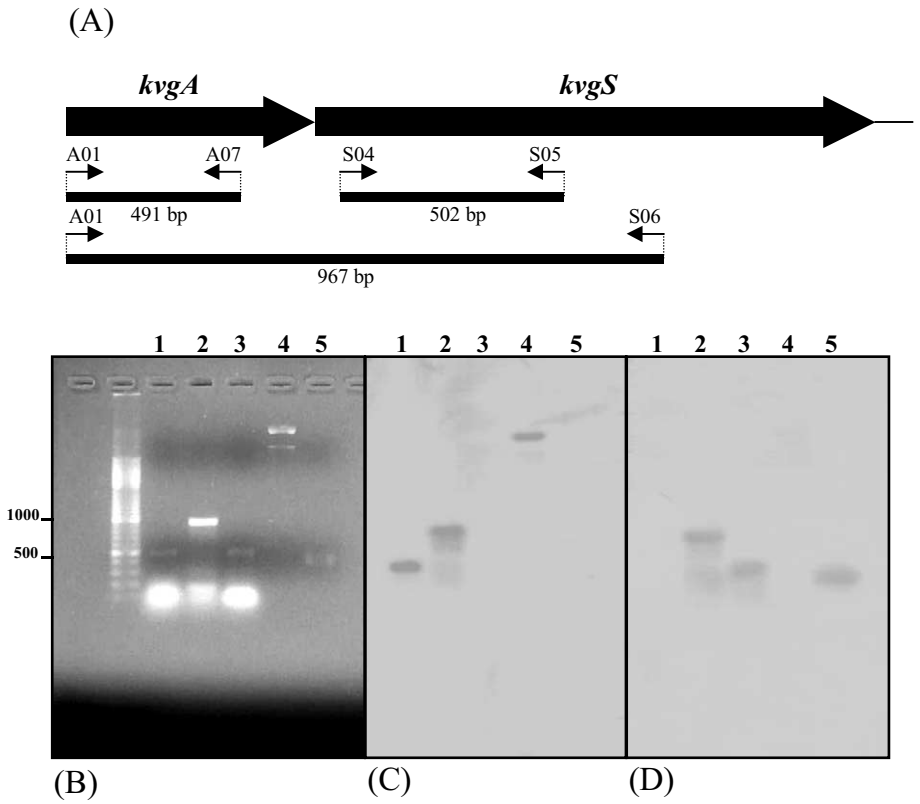

Fig. 2. Transcript analysis of the *kvgAS* region. Total RNA was extracted from mid-exponential phase *K. pneumoniae* CG43 and subjected to RT-PCR. A: Primers used in amplification of the *kvgAS* region (Table 1) and the expected sizes of product are listed. B: Gel electrophoresis of RT-PCR products amplified from different primer sets. Lanes 1, S04/S05; 2, A01/S06; 3, A01/A07. As the positive control, DNA fragments containing the coding region of *kvgS* (lane 4) and *kvgA* (lane 5) were also amplified. The DNA fragments were transferred to a nylon membrane and hybridized with a DNA probe specific to *kvgS* (C) or *kvgA* (D).

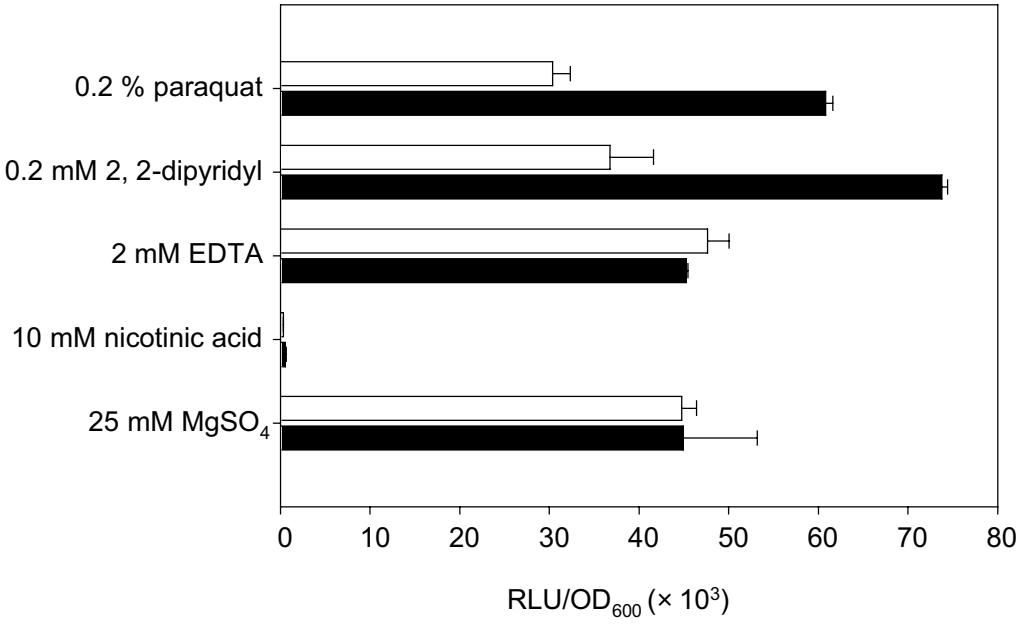

Fig. 3. Regulation of the *kvgAS* promoter. Activity of the *kvgAS* promoter was examined in wild-type CG43-S3 (closed bar) or in the *kvgS* mutant CG43-S3021 (open bar). Bacteria were inoculated in LB medium and incubated with different reagents at 25°C for 1 h. Luciferase activity is expressed in RLU/OD_{600} as described in Section 2.

3.2. Construction and characterization of a K. pneumoniae kvgS deletion mutant

In order to understand the physiological roles of the KvgAS two-component system, we constructed a *kvgS* deletion on *K. pneumoniae* CG43-S3, a streptomycin-resistant derivative of *K. pneumoniae* CG43. The *kvgS* deletion mutant obtained through the allelic exchange procedure was designated *K. pneumoniae* CG43-S3021 and was used for further study. *K. pneumoniae* CG43-S3021 displayed a heavily mucoid colony morphology on LB agar that was indistinguishable from its parental strain. The growth rate of CG43-S3021 was also similar to that of CG43-S3. The LD_{50} of CG43-S3021 to laboratory mice in a peritonitis model was found to be approximately 4000 CFU, which is comparable with that of its parental strain. Nevertheless, it may not be appropriate to conclude that KvgAS is not important for the bacterium to infect humans since the intraperitoneal route does not represent a common route for *Klebsiella* infection and the mouse is not a natural host.

In *Bordetella* spp., *bvgAS* is responsible for regulating the expression of many virulence genes including those encoding filamentous hemagglutinin [12], pertussis toxin [13,14], and adenylate cyclase toxin/hemolysin [12]. However, searching the *K. pneumoniae* genome database failed to identify homologues of such virulence-associated genes in *K. pneumoniae*. Therefore, despite its relatedness with BvgAS, the KvgAS two-component system is likely to play a very different role in *K. pneumoniae* from BvgAS in *Bordetella* spp.

3.3. Regulation of kvgAS expression

In *Bordetella* spp., it has been demonstrated that the expression of *bvgAS* genes is regulated by the addition of $MgSO_4$, nicotinic acid, or elevated growth temperatures [15]. To investigate whether these factors also exert similar effects on the expression of *kvgAS*, a 330-bp *Hin*dIII/*Eco*RI fragment (Fig. 1) that comprises the putative control region of *kvgAS* was cloned upstream of the *luxAB* reporter carried on pUCD1752.1. The resulting plasmid pSL050 was then transformed into the wild-type CG43-S3 and the *kvgS* mutant CG43-S3021. The luciferase activities of these strains grown in LB medium supplemented with either 25 mM $MgSO_4$, 10 mM nicotinic acid, 2 mM EDTA, 0.2 mM 2,2-dipyridyl, or 0.2% paraquat were determined and compared at 25°C. As shown in Fig. 3, upon exposure to the oxidative stress-generating agent paraquat or the iron-chelating agent 2,2-dipyridyl, the luciferase activity of *K. pneumoniae* CG43-S3021 [pSL050] was reduced to approximately half that of *K. pneumoniae* CG43-S3 [pSL050]. This phenomenon was not observed in the same strains harboring pUCD1752.1, in which the *luxAB* reporter was under the control of the *lac* promoter. The results suggest a positive regulatory role of KvgS on its own expression and a possible involvement of KvgS in sensing iron limitation and countering free radical stresses. OxyR, a regulator of the *E. coli* response to oxidative stress, has been reported to be able to activate the expression of Fur, the global repressor of ferric ion uptake [16]. Since *K. pneumoniae* possesses both the OxyR and Fur homologues, the linkage of iron response and free radical stress mediated by KvgAS implies the presence of a regulatory circuit between these systems. We also found that the susceptibility of the *kvgS* mutant CG43-S3021 to paraquat increased slightly by using the disk diffusion assay, consistent with the notion that the KvgAS two-component system may participate in the response to free radical stresses. It has been reported that approximately 200 genes in the *E. coli* genome are paraquat-regulated [17]. The gene expression pattern mediated by the KvgAS two-component system requires investigation.

In conclusion, we have identified a novel two-component system encoding operon *kvgAS* in *K. pneumoniae* CG43. Despite the availability of the *kvgS* deletion mutant, the role of the KvgAS two-component system in *K. pneumoniae* physiology remains elusive. Since only 15% of bacteremic *K. pneumoniae* strains contain *kvgAS* [6], the gene cluster is unlikely to carry out a housekeeping task as demonstrated by the lack of apparent effect on the growth rates of the *kvgS* mutant. Comparative analysis of the wild-type and *kvgS* mutant strains with technology such as DNA microarray or two-dimensional gel electrophoresis of proteins will be useful in providing important information towards understanding the function of this novel two-component system.

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