Malaysian Journal of Microbiology, Vol 13(4) 2017, pp. 273-279

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Whole genome analysis of *Klebsiella*: Unique genes associated with isolates from Indonesian tempeh

Mahaldika Cesrany[1], Adi Yulandi[2], Iman Rusmana[1], Antonius Suwanto[1,2]*

[1]Department of Biology, Faculty of Mathematics and Natural Science, Bogor Agricultural University, Dramaga Campus, Bogor 16680, Indonesia.
[2]Faculty of Biotechnology, Atma Jaya Indonesia Catholic University, Jakarta 12930, Indonesia.
Email: asuwanto@indo.net.id

Received 28 December 2016; Received in revised form 21 March 2017; Accepted 23 March 2017

ABSTRACT

Aims: Our previous study demonstrated that *Klebsiella* IIEMP-3 associated with tempeh was genetically different from those of medical isolates. In addition to the whole genome sequence of *Klebsiella* IIEMP-3, the draft genome sequence of another isolate, i.e. IWJB-6 was employed for comparison. In this study, the details of the virulence genes and unique gene in both *Klebsiella* isolates were compared employing *in silico* and *in vitro* analysis.
Methodology and results: Whole genome of *Klebsiella* IIEMP-3 and IWJB-6 were annotated to investigate the virulence factor. *Klebsiella* IIEMP-3 and IWJB-6 were obtained from tempeh producers in Bogor, West Java - Indonesia. Genome sequences were analyzed employing BLAST Ring Image Generator (BRIG) software. The results showed that all of the samples, including isolates IIEMP-3 and IWJB-6 did not harbor *rmpA*, i.e. DNA sequence for *K. pneumoniae* virulence factor.
Conclusion, significance and impact of study: *Klebsiella* could be found in almost all tempeh samples from Indonesia and could be harmless for human due to the absence of *rmpA* and other virulence-associated genes. The significance of this study showed that IIEMP-3 and IWJB-6 isolates were more closely related to *K. variicola*. However, *K. variicola* At-22 harbored *sdsA* gene which is lacking in those two tempeh isolates. Combined with PCR analysis for specific gene/s; our study suggested that isolates from Indonesian tempeh were closely related to *K. variicola*, and proposed to be designated as *K. variicola* subsp. *tempehensis*.

Keywords: Genome annotation, *Klebsiella variicola* subsp. *tempehensis*, *sdsA*, virulence factor

INTRODUCTION

Tempeh is an Indonesian indigenous soy fermented food which is also one of the most important protein source for most Indonesian. During the process of fermentation, in addition to *Rhizopus* spp., bacteria also play important roles in the formation of flavor and nutrient content. In tempeh, one of the bacteria that contributes to tempeh nutrient content is *Klebsiella pneumoniae*. Isolates of *K. pneumoniae* in tempeh are well known as vitamin B12 producers (Keuth and Bisping, 1994).

Klebsiella spp., especially *K. pneumoniae*, is found in the diverse environment, associated with plant and human, and is known as human pathogens that show the mucoid phenotype. *Klebsiella pneumoniae* of medical isolates are usually associated with human and cause infectious disease in human (Holt *et al.*, 2015). Mucoviscosity associated gene A (*magA*) and regulator of mucoid phenotype (*rmpA*) are the genes that act in the production of mucoid phenotype in *K. pneumoniae* of medical isolates (Nassif *et al.*, 1989; Fang *et al.*, 2004). The gene encoding *rmpA* is only 536 bp in length (Nadasy *et al.*, 2007).

However, *Klebsiella* spp. have also been found in tempeh and these isolates usually did not indicate the presence of mucoid phenotype. Keuth and Bisping (1994) reported that *Klebsiella* from tempeh is safe for consumption because it did not contain enterotoxin genes. Genetically, based on 16S rRNA followed by Enterobacterial Repetitive Intergenic Consensus - Polymerase Chain Reaction (ERIC-PCR), *K. pneumoniae* from tempeh were shown to be different from those of medical isolates (Ayu *et al.*, 2014).

Whole genome data of *Klebsiella* IIEMP-3 and IWJB-6 was used to scan for the presence of virulence genes and other genes that can distinguish the diversity of *Klebsiella* from tempeh. Genome annotation indicated that isolates from Indonesian tempeh were lack of virulence gene/s associated with pathogenic *Klebsiella*. Although the tempeh isolates were closely related to *K. variicola* At-22, they did not possess the unique genes available in *K. variicola* At-22, such as *sdsA*.

***Corresponding author**

Our previous study demonstrated that *Klebsiella* IIEMP-3 associated with tempeh was genetically different from those of medical isolate. In addition to the whole genome sequence of *Klebsiella* IIEMP-3, the draft genome sequence of another isolate, i.e. IWJB-6 was employed for comparison. In this study, the details of the virulence genes in both *Klebsiella* isolates were compared employing *in silico* and *in vitro* analysis.

MATERIALS AND METHODS

Tempeh samples

Tempeh samples were obtained from Jakarta (KA, KOP, KJA, RA, and CA); West Java (MWR and CIO); Central Java (BTG and CPU); Jogjakarta (SLM); East Java (SU, MA, PA, GW, and PCTN); Sulawesi (MKS and TRJ); and Kalimantan (BP). The tempeh samples were sent in fresh condition at 0 h. When it arrived at laboratory, tempeh was incubated until it reaches the fermentation time 48 h. After 48 h, 1 g tempeh samples was taken for total DNA extraction and the rest of tempeh samples put in freezer 4 °C.

Isolates collection

IIEMP-3, IWJB-6, and *K. pneumoniae* FK were in our collection and as described previously (Ayu *et al.*, 2014).

Genome annotation

Klebsiella IIEMP-3 genome assembly was performed employing Velvet version 1.2.07 and evaluated by REAPR software (Yulandi *et al.*, 2016a). *Klebsiella* IIEMP-3 genome annotation was conducted employing Rapid Annotation of Transfer Tool (RATT) software (Otto *et al.*, 2011). *Klebsiella pneumoniae* subsp. *pneumoniae* NTUH-K2044 and *K. variicola* At-22 were used as genome reference. Meanwhile, *Klebsiella* IWJB-6 genome assembly and genome annotation process were done employing integrative bacterial genome analysis for Ion Torrent sequence data (IonGAP) (Baez-Ortega *et al.*, 2015).

BLAST Ring Image Generator (BRIG)

The genome assembly of *Klebsiella* IIEMP-3 and IWJB-6 were visualized by BLAST Ring Image Generator (BRIG) software as described previously (Alikhan *et al.*, 2011) employing *K. pneumoniae* subsp. *pneumoniae* NTUH-K2044 and *K. variicola* At-22 as reference strains. BRIG result was used as a reference to choose the genes that could be used for PCR primer design. The primers were used to group the diversity of *Klebsiella* isolates from tempeh.

Polymerase Chain Reaction (PCR) primer design

The gene/s or specific sequences that could be used to distinguish the diversity of *Klebsiella* from tempeh were further analyzed to construct specific pairs of primers by primer3 (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Rozen and Skaletsky, 2000). Primer pairs were verified by NetPrimer (http://www.premierbiosoft.com/netprimer/) and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Specific pairs of new primers used to amplify the *sdsA* gene were sdsA-F 5'- AGCATCTCGTCGAGCTTAGC -3' and sdsA-R 5'- TCGCTGGAGAAACAGTGGTC-3'. The other primers also used in this study, i.e. 16S rRNA, *rmpA* and *cbiG* (Table 1). The *cbiG* primer was used as a specific marker for *Klebsiella* (Alvin, 2014). The *rmpA* primer was used to confirm the presence of virulence gene responsible for the mucoid phenotype in *Klebsiella* (Nassif *et al.*, 1989), while 16S rRNA was used as a positive control for bacterial DNA amplification (Marchesi *et al.*, 1998).

Table 1: List PCR primers.

Genes	Primer	Oligonucleotide primers	Size of amplicons (bp)	Reference
16S rRNA	63f	5′-CAGGCCTAACACATGCAAGTC-3′	1300	(Marchesi *et al.*, 1998)
	1387r	5′-GGGCGGWGTGTACAAGGC-3′		
cbiG	*cbiG*-F	5'-TGCTGCCGCTCACCTGCTAC-3'	755	(Alvin, 2014)
	cbiG-R	5'-GCAACCCCGGCTCGTTTGC-3'		
rmpA	*rmpA* F	5'-ACTGGGCTACCTCTGCTTCA-3'	536	(Nassif *et al.*, 1989)
	rmpA R	5'-CTTGCATGAGCCATCTTTCA-3'		

DNA preparation

Klebsiella pneumoniae FK, *Klebsiella* IIEMP-3, and IWJB-6 were refreshed in Luria Broth (LB) at 37 °C overnight (Ayu *et al.*, 2014). Total DNA from the isolates were extracted employing Presto™ Mini gDNA Bacteria Kit protocols (Geneaid Biotech Ltd, Taiwan). *Klebsiella pneumoniae* FK was used as a positive control for virulence gene. Total DNA from tempeh samples were extracted employing Power Food® Microbial DNA Isolation Kit protocols (MOBIO, Canada).

PCR

Total PCR volume was 10 µL containing 5 µL KAPA2G Robust HotStart (KAPABiosystems, MA, USA), 10 pmol of forward and reverse primers, 100 ng of a DNA template, and nuclease-free water (Promega, WI, USA). The amplifications were performed using PCR (Applied Biosystems 2720 Thermal Cycler; Life Technologies, CA,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

USA; GeneAmp PCR System 2400; PerkinElmer, MA, USA) with a predetermined PCR condition (Table 2). After polymerization process was completed, the samples were verified by electrophoresis (Mini-Sub Cell GT Cell; Bio-Rad Laboratories, CA, USA) in 1% (w/v) agarose in 1× TAE buffer for 35 min, 80 V. Agarose gel was visualized under UV light (Biometra T1; Biometra, Göttingen, DE).

Table 2: Predetermined PCR condition.

Gene	PCR	Cycle	Reference
16S rRNA	Pre-denaturation (94 °C, 5 min); denaturation (92 °C, 30 sec); annealing (55 °C, 30 sec); extension (72 °C, 1 min); post-extension (72 °C, 5 min)	35 ×	(Marchesi *et al.*, 1998)
cbiG	Pre-denaturation (94 °C, 5 min); denaturation (94 °C, 30 sec); annealing (60 °C, 30 sec); extension (72 °C, 1 min); post-extension (72 °C, 20 min)	35 ×	(Alvin, 2014)
rmpA	Pre-denaturation (95 °C, 10 min); denaturation (94 °C, 30 sec); annealing (55 °C, 1 min); extension (72 °C, 1 min); post-extension (72 °C, 5 min)	35 ×	(Nassif *et al.*, 1989)
sdsA	Pre-denaturation (94 °C, 5 min); denaturation (94 °C, 30 sec); annealing (53 °C, 30 sec); extension (72 °C, 1 min); post-extension (72 °C, 10 min)	35 ×	-

RESULTS

Identification of virulence gene

The whole genome of IIEMP-3 and IWJB-6 isolates was analyzed employing RATT and IonGAP software to identify the virulence gene/s (Table 3). Comparative genomic sequence analysis indicated that the virulence genes were absence in either IIEMP-3 or IWJB-6. Unlike the virulence genes, multidrug resistance protein was presence in both IIEMP-3 and IWJB-6 isolates. Multidrug resistance proteins might be one form of defense to a number of antibacterial secreted by *Rhizopus* spp. in tempeh.

Table 3: Genome annotation data employing RATT and IonGAP program.

Microbes	Gene or protein[a]			
	virB1-virB11	*magA*	*rmpA*	Multidrug resistance protein
K. pneumoniae subsp. *pneumoniae* NTUH-K2044	P	P	P	P
K. variicola At-22	A	A	A	P
Klebsiella IIEMP-3	A	A	A	P
Klebsiella IWJB-6	A	A	A	P

[a] P denotes presence of the gene/s or protein.
A denotes absence of the gene/s or protein.

The BRIG profile showed that there was no *magA*, *rmpA*, *irp1*, *irp2* genes on *Klebsiella* IIEMP-3, IWJB-6, and *K. variicola* At-22 (Figure 1). PCR results showed that all of DNA samples from tempeh Jakarta, Bogor, Central Java, Jogjakarta, East Java, Sulawesi, Kalimantan, IIEMP-3, and IWJB-6 did not contain *rmpA* gene when compared to *K. pneumoniae* FK used as a positive control for virulent isolate (Table 4). The BRIG profile based on Yulandi *et al.* (2016b) showed that IIEMP-3 isolates did not show *virB1-virB11*, which encodes type IV system secretion (T4SS). This study showed that T4SS was absence in *K. variicola* At-22 and IWJB-6 isolates (Figure 1). General features from *K. pneumoniae* subsp. *pneumoniae* NTUH-K-2044, *K. variicola* At-22, IIEMP-3 and IWJB-6 isolates showed that all of the parameters were similar (Table 5).

Klebsiella isolates from tempeh possessed unique genes

Bioinformatics analysis indicated that *cbiG* gene was found in all of *Klebsiella* species in this study; i.e. *K. pneumoniae* subsp. *pneumoniae* NTUH-K2044, *K. variicola* At-22, *Klebsiella* IIEMP-3 and IWJB-6. PCR with our specific primers confirmed that all of the DNA samples derived from tempeh, contained sequences of *cbiG* gene. The DNA bands formation of *cbiG* gene in agarose gel was thick and the size of product from PCR analysis was 755 bp. MWR tempeh was the only tempeh that was failed to show *cbiG* (Table 4). The absence of *cbiG* gene in MWR might indicate the absence of *Klebsiella* or vitamin B12 in this tempeh (Alvin, 2014).

The BRIG analysis employing *K. variicola* At-22 as a reference showed that *sdsA* gene was found in *K.*

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

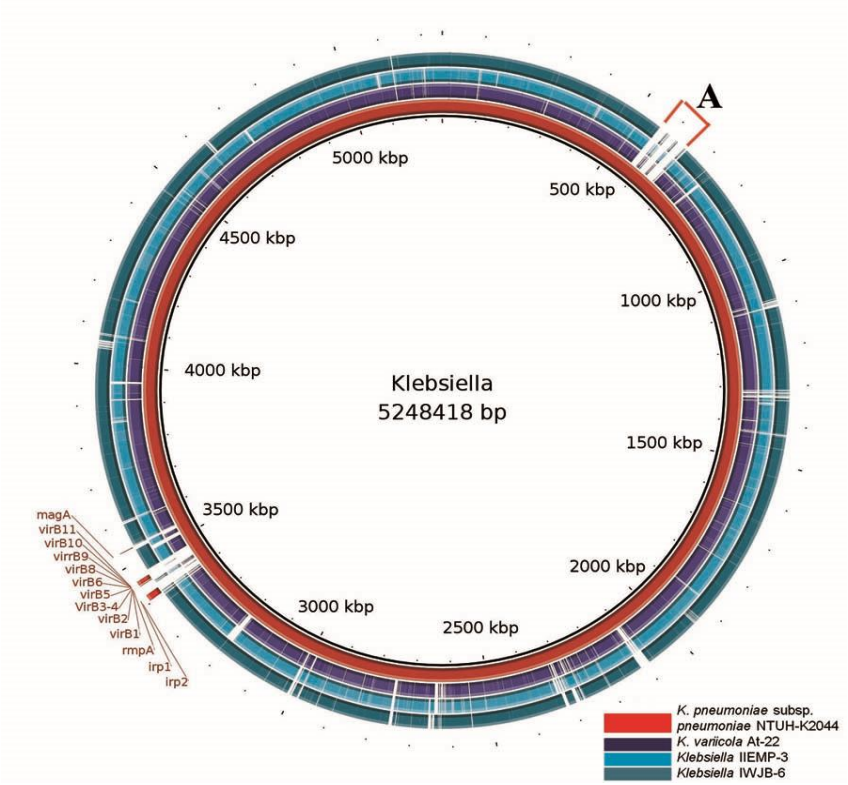

Figure 1: Whole genome comparison of *K. variicola* At-22, *Klebsiella* IIEMP-3, IWJB-6, and NTUH-K2044 based on BRIG analysis (Alikhan *et al.*, 2011). Please note the absence of virulence gene/s in IIEMP-3, IWJB-6, and At-22. Sequence in area A is only presence in NTUH-K2044, which contained hypothetical protein and conserved hypothetical protein compared to At-22, IIEMP-3, and IWJB-6.

Table 4: Presence of 16S rRNA, *cbiG*, *rmpA*, and *sdsA* genes in some *Klebsiella* isolates and tempeh samples.

Bacterial isolates/ tempeh samples	Source of isolates/ tempeh samples	Gene[a]			
		16S rRNA	*cbiG*	*rmpA*	*sdsA*
Klebsiella IIEMP-3	(Ayu *et al.*, 2014)	+	+	-	-
Klebsiella IWJB-6	(Ayu *et al.*, 2014)	+	+	-	-
K. pneumoniae FK	(Ayu *et al.*, 2014)	+	+	+	-
Tempeh KA	Karet – Central Jakarta	+	+	-	+*
Tempeh KOP	Kopro – West Jakarta	+	+	-	+
Tempeh KJA	Koja – North Jakarta	+	+	-	+*
Tempeh RA	Ranco – South Jakarta	+	+	-	-
Tempeh CA	Cakung – East Jakarta	+	+	-	+
Tempeh CIO	Ciomas – Bogor	+	+	-	-
Tempeh MWR	Mawar – Bogor	+	-	-	-
Tempeh BTG	Batang – Central Java	+	+	-	-
Tempeh CPU	Cepu – Central Java	+	+	-	+*
Tempeh SLM	Sleman – Jogjakarta	+	+	-	-
Tempeh SU	Sukorejo – East Java	+	+	-	+*
Tempeh MA	Malang – East Java	+	+	-	-
Tempeh PA	Pasuruan – East Java	+	+	-	-
Tempeh GW	Gedong Wetan – East Java	+	+	-	-
Tempeh PCTN	Pacitan – East Java	+	+	-	+
Tempeh MKS	Makassar – South Sulawesi	+	+	-	-
Tempeh TRJ	Toraja – South Sulawesi	+	+	-	+
Tempeh BP	Balikpapan – East Kalimantan	+	+	-	+*

[a] + : presence of PCR products. +* : Presence of PCR products, but the DNA bands from PCR products were very thin.
- : Absence of PCR products.

Table 5: General features of *K. pneumoniae* subsp. *pneumoniae* NTUH-K2044, *variicola* At-22, *Klebsiella* IIEMP-3 and IWJB-6 genome.

Microbes	Genome size (bp)	Coding DNA sequence	G+C content (%)	Reference
K. pneumoniae subsp. *pneumoniae* NTUH-K2044	5248520	5006	57.7	(Wu *et al.*, 2009)
K. variicola At-22	5458505	4979	57.6	(Pinto-Tomás *et al.*, 2009)
Klebsiella IIEMP-3	5362779	5096	57.8	(Yulandi *et al.*, 2016a)
Klebsiella IWJB-6	5159329	4911	57.5	-

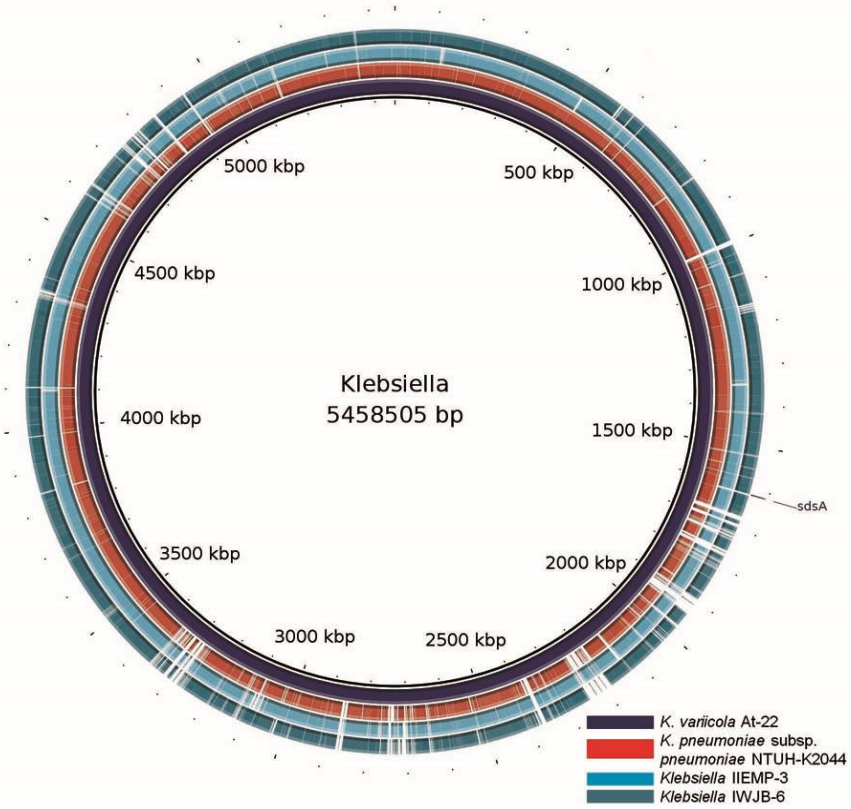

Figure 2: *sdsA* gene was absence in either IIEMP-3 or IWJB-6 but presence in At-22. *sdsA* in NTUH-K2044 was only found as a truncated gene.

variicola At-22, but absence in *Klebsiella* IIEMP-3 and IWJB-6. A specific primer was designed to amplify this region in *K. variicola* At-22 chromosome and used them to explore similar gene/s in IIEMP-3 and IWJB-6, as well as a number of tempeh samples (Figure 2). The result of *sdsA* gene amplification was tabulated in Table 4.

sdsA gene was found in some samples of tempeh (Table 4). However, the DNA bands derived from PCR products of KA, KJA, SU, CPU, or BP samples were very thin, which might indicate only a small number *K. variicola* presence in tempeh. Although IIEMP-3 and IWJB-6 isolates were similar to *K. variicola*, these tempeh isolates and nine tempeh samples did not have *sdsA* gene (Table 4), which indicated that *Klebsiella* commonly associated with tempeh was a different subspecies.

DISCUSSION

Vitamin B12 is not only complex structurally but also unique, because this vitamin only formed by bacteria or archaea, and tempeh is one of a few vegan diet known to contain vitamin B12 (Liem *et al.*, 1977; Keuth and Bisping, 1994). There are genes that could indicate the presence of *Klebsiella* in tempeh, such as *cbiG* gene (Alvin, 2014). *cbiG* gene has an important role in tempeh fermentation to create vitamin B12 through an anaerobic pathway (Rodionov *et al.*, 2003; Moore *et al.*, 2013). Alvin (2014) reported that the thickness of *cbiG* gene PCR bands

could be used to estimate semiquantitavely *Klebsiella* population in tempeh.

This study indicated that *Klebsiella* from tempeh is different from those of medical isolates. *magA*, *rmpA*, *irp1*, *irp2* genes are known as virulence genes in pathogenic *K. pneumoniae* (Nassif and Sansonetti, 1986; Nassif *et al.*, 1989; Carniel, 2001; Fang *et al.*, 2004). *rmpA*, a *Klebsiella* virulence gene, was not found in all *Klebsiella* associated with tempeh and tempeh samples. The absence of virulence gene in *Klebsiella* from tempeh supported the previous study, which is showed genetically by ERIC-PCR analysis and the absence of mucoid phenotype on solid media in both IIEMP-3 and IWJB-6 isolates (Ayu *et al.*, 2014). Yeh *et al.* (2007) reported that *rmpA* negative isolate is less resistant and less virulent than the *rmpA* positive isolate. T4SS may be important to cause diseases (Fodah *et al.*, 2014). The absence of T4SS in IIEMP-3 and IWJB-6 isolates also suggested that the isolates might not be pathogenic to human.

Smillie *et al.* (2010) reported that T4SS, type IV coupling protein (T4CP), the origin of transfer locus (*ori*T), and relaxase were needed in a conjugative plasmid. T4SS was found in plasmid of *K. variicola* Bz19, one of clinical isolates strain from *K. variicola*; pKPC-NY79 from 258 *K. pneumoniae* strain that was isolated from a patient hospitalized in New York, United States; and plasmid from *K. pneumoniae* KpQ3 (Ho *et al.*, 2013; Tobes *et al.*, 2013; Andrade *et al.*, 2014). However, *K. variicola* At-22 did not have both a plasmid and T4SS (Pinto-Tomás *et al.*, 2009). Either IIEMP-3 or IWJB-6 might not harbor this plasmid because of the absence of T4SS in their genome.

The gene that could differentiate *K. variicola* At-22 from either IIEMP-3 or IWJB-6 isolates was *sdsA*. *sdsA* gene was first described as a member of the group III sulfatase which was distinguished by the existence of metallo-β-lactams domain and have responsibility in alkyl sulfatase activity of *sdsA* protein, SdsA, involved in sodium dodesyl sulfate (SDS) degradation (Hagelueken *et al.*, 2006; Navais *et al.*, 2014). Based on these data, we proposed that IIEMP-3 and IWJB-6, and some *Klebsiella* isolates from tempeh, were renamed as *Klebsiella variicola* subsp. *tempehensis*. Further research is needed on the role of these isolates in tempeh, especially on the microbial community during tempeh fermentation and the impacts on tempeh as a functional food.

ACKNOWLEDGMENTS

We thank Biotechnology R&D, PT. Wilmar Benih Indonesia, Cikarang, for bioinformatics analysis.

REFERENCES

Alikhan, N. F., Petty, N. K., Zakour, N. L. B. and Beatson, S. A. (2011). BLAST ring image generator: Simple prokaryote genome comparisons. *BMC Genomics* **12, 1-10.**

Alvin, C. (2014). Korelasi antara konsentrasi vitamin B12 pada tempe dengan jumlah kopi gen *cbiG* dari *Klebsiella pneumoniae*. M.Sc. Thesis. Atma Jaya Catholic University, Indonesia. **pp. 1-8.**

Andrade, B. G. N., de Veiga Ramos, N., Marin, M. F. A., Fonseca, E. L. and Vicente, A. C. P. (2014). The genome of a clinical *Klebsiella variicola* strain reveals virulence-associated traits and pl9-like plasmid. *FEMS Microbiology Letter* **360, 13-16.**

Ayu, E., Suwanto, A. and Barus, T. (2014). *Klebsiella pneumoniae* from Indonesian tempeh were genetically different from that of pathogenic isolates. *Microbiology Indonesia* **8, 9-15.**

Baez-Ortega, A., Lorenzo-Diaz, F., Hernandez, M., Gonzalez-Vila, C. I., Roda-Garcia, J. L., Colebrook, M. and Flores, C. (2015). IonGAP: Integrative bacterial genome analysis for ion torrent sequence data. *Bioinformatics* **31, 2870-2873.**

Carniel, E. (2001). The *Yersinia* high-pathogenicity island: An iron-uptake island. *Microbes and Infection* **3, 561-569.**

Fang, C. T., Chuang, Y. P., Shun, C. T., Chang, S. C. and Wang, J. T. (2004). A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. *The Journal of Experimental Medicine* **199, 697-705.**

Fodah, R. A., Scott, J. B., Tam, H. H., Yan, P., Pfeffer, T. L., Bundschuh, R. and Warawa, J. M. (2014). Correlation of *Klebsiella pneumoniae* comparative genetic analyses with virulence profiles in a murine respiratory disease model. *PLoS ONE* **9, e107394.**

Hagelueken, G., Adams, T. M., Wiehlmann, L., Widow, U., Kolmar, H., Tümmler, B., Heinz, D. W. and Schubert, W. D. (2006). The crystal structure of SdsA1, an alkylsulfatase from *Pseudomonas aeruginosa*, defines a third class of sulfatases. *Proceeding of the National Academy of Science* **103, 7631-7636.**

Ho, P. L., Cheung, Y. Y., Lo, W. U., Li, Z., Chow, K. H., Lin, C. H., Chan, J. F. W. and Cheng, V. C. C. (2013). Molecular characterization of an atypical IncX3 plasmid pKPC-NY79 carrying bla_{KPC-2} in *Klebsiella pneumoniae*. *Current Microbiology* **67, 493-498.**

Holt, K. E., Wertheim, H., Zadoks, R. N., Baker, S., Whitehouse, C. A., Dance, D., Jenney, A., Connor, T. R., Hsu, L. Y., Severin, J., Brisse, S., Cao, H., Wilksch, J., Gorrie, C., Schultz, M. B., Edwards, D. J., Nguyen, K. V., Nguyen, T. V., Dao, T. T., Mensink, M., Minh, V. L., Nhu, N. T. K., Schultsz, C., Kuntaman, K., Newton, P. N., Moore, C. E., Strugnell, R. A. and Thomson, N. R. (2015). Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proceeding of the National Academy of Science* **112, E3574-E3581.**

Keuth, S. and Bisping, B. (1994). Vitamin B12 production by *Citrobacter freundii* and *Klebsiella pneumoniae* during tempeh fermentation and proof of enterotoxin absence by PCR. *Applied and Environmental Microbiology* **60, 1495-1499.**

Liem, I. T. H., Steinkraus, K. H. and Cronk, T. C. (1977). Production of vitamin B-12 in tempeh, a fermented soybean food. *Applied and Environmental Microbiology* **34, 773-776.**

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J. and Wade, W. G. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology* **64, 795-799.**

Moore, S. J., Lawrence, A. D., Biedendieck, R., Deery, E., Frank, S., Howard, M. J., Rigby, S. E. J. and Warren, M. J. (2013). Elucidation of the anaerobic pathway for the corrin component of cobalamin (vitamin B12). *Proceeding of the National Academy of Science* **110, 14906-14911.**

Nadasy, K. A., Saad, R. D. and Tribble, M. A. (2007). Invasive *Klebsiella pneumoniae* syndrome in North America. *Clinical Infectious Diseases* **45, e25-e28.**

Nassif, X. and Sansonetti, P. J. (1986). Correlation of the virulence of *Klebsiella pneumoniae* K1 and K2 with presence of a plasmid encoding aerobactin. *Infection and Immunity* **54, 603-608.**

Nassif, X., Fournier, J. M., Arondel, J. and Sansonetti, J. (1989). Mucoid phenotype of *Klebsiella pneumoniae* is a plasmid-encoded virulence factor. *Infection and Immunity* **57, 546-552.**

Navais, R., Mendez, J., Cascales, D., Reimundo, P. and Guijarro, J. A. (2014). The heat sensitive factor (HSF) of *Yersinia ruckeri* is produced by an alkyl sulphatase involved in sodium dodecyl sulphate (SDS) degradation but not in virulence. *BMC Microbiology* **14, 1-13.**

Otto, T. D., Dillon, G. P., Degrave, W. S. and Berriman, M. (2011). RATT: Rapid annotation transfer tool. *Nucleic Acids Research* **39, e57.**

Pinto-Tomás, A. A., Anderson, M. A., Suen, G., Stevenson, D. M., Chu, F. S. T., Cleland, W. W., Weimer, P. J. and Currie, C. R. (2009). Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. *Science* **326, 1120-1123.**

Rodionov, D. A., Vitreschak, A. G., Mironov, A. A. and Gelfand, M. S. (2003). Comparative genomics of the vitamin B12 metabolism and regulation in prokaryotes. *Journal of Biological Chemistry* **278, 41148-41159.**

Rozen, S. and Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. *In:* Bioinformatics Methods and Protocols - Methods in Molecular Biology. Krawetz, S. and Misener, S. (eds.). Humana Press, Totowa, New Jersey. **pp. 365-386.**

Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P. and de la Cruz, F. (2010). Mobility of plasmids. *Microbiology and Molecular Biology Reviews* **74, 434-452.**

Tobes, R., Codoñer, F. M., López-Camacho, E., Salanueva, I. J., Manrique, M., Brozynska, M., Gómez-Gil, R., Martínez-Blanch, J. F., Alvarez-Tejado, M., Pareja, E. and Mingorance, J. (2013). Genome sequence of *Klebsiella pneumoniae* KpQ3, a DHA-1 β-lactamase-producing nosocomial isolate. *Genome Announcements* **1, e00167-12.**

Wu, K. M., Li, L. H., Yan, J. J., Tsao, N., Liao, T. L., Tsai, H. C., Fung, C. P., Chen, H. J., Liu, Y. M., Wang, J. T., Fang, C. T., Chang, S. C., Shu, H. Y., Liu, T. T., Chen, Y. T., Shiau, Y. R., Lauderdale, T. L., Su, I. J., Kirby, R. and Tsai, S. F. (2009). Genome sequencing and comparative analysis of *Klebsiella pneumoniae* NTUH-K2044, a strain causing liver abscess and meningitis. *Journal of Bacteriology* **191, 4492-4501.**

Yeh, K. M., Kurup, A., Siu, L. K., Koh, Y. L., Fung, C. P., Lin, J. C., Chen, T. L., Chang, F. Y. and Koh, T. H. (2007). Capsular serotype K1 or K2, rather than *magA* and *rmpA*, is a major virulence determination for *Klebsiella pneumoniae* liver abscess in Singapore and Taiwan. *Journal of Clinical Microbiology* **45, 466-471.**

Yulandi, A., Sugiokto, F. G., Febrilina and Suwanto, A. (2016a). Genomic sequence of *Klebsiella pneumoniae* IIEMP-3, a vitamin B12-producing strain from Indonesian tempeh. *Genome Announcements* **1, e01724-15.**

Yulandi, A., Cesrany, M. and Suwanto, A. (2016b). Comparative genomics of *K. pneumonia* IIEMP-3, isolated from Indonesian Tempeh with some pathogenic strains. *Genome Annotation* doi: 10.13140/RG.2.1.1141.8002.