Supplementary information

Title: The type VI secretion system protein AsaA in *Acinetobacter baumannii* is a periplasmic protein physically interacting with TssM and required for T6SS assembly

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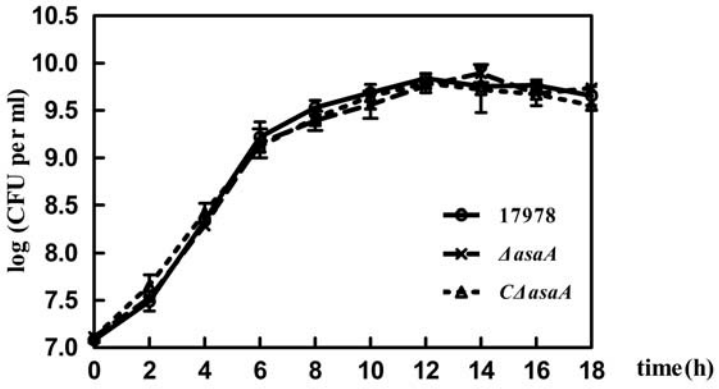
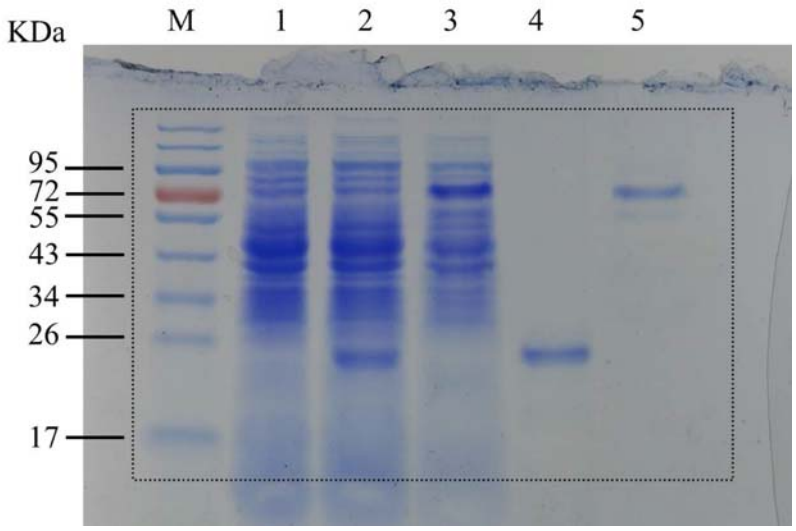
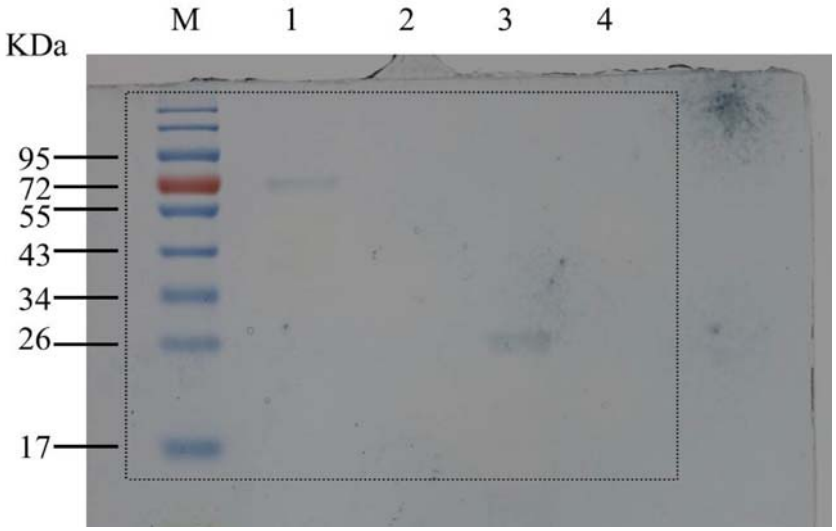


Supplementary Figure 1. A. baumannii strains growth curve in LB medium. The wild type 17978, AsaA mutant and complementation strains were used to inoculate LB medium at 10^7 CFU per ml. Samples were taken in triplicate at intervals of 2 h, diluted and plated on LB plates. Bacterial CFU were counted after incubation for 2 days. The experiments were repeated twice with similar results, and one representative result is presented.



Supplementary Figure 2. The full-length gel of His_6-tagged fusion proteins were over expressed and purified. Lanes: 1, crude BL21/pET30a extract; 2, crude BL21/pET30a-AsaA extract induced with IPTG; 3, crude BL21/pET30a-$TssM_{436\text{-}1041}$ extract induced with IPTG; 4, affinity-purified His_6-AsaA protein; 5, affinity-purified His_6-$TssM_{436\text{-}1041}$ protein; M, molecular mass marker. Inside the box is the figure 4B.



Supplementary Figure 3. The full-length gel of Pull-down assays. Lanes: 1, pull-down of His_6-TssM by immobilized His_6-AsaA; 2, His_6-TssM mixed with streptavidin sepharose beads(negative control); 3, pull-down of His_6-AsaA by immobilized His_6-TssM; 4, His_6-AsaA mixed with streptavidin sepharose beads(negative control) ; M, molecular mass marker. Inside the box is the figure 4C.

Table S1. Strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Reference or source
E. coli strains		
JM109	*RecA*1, *endA*1, *gyrA*96, *thi, supE*44, *relA*1 Δ (*lac-proAB*)/F′ [*traD36*, *lacI*^{q}, *lacZ* ΔM15]	[1]
JM109/pk18*mob*	JM109 harboring recombinant plasmid pk18*mob*, Kan^r	This work
BL21(DE3)	F$^-$ *ompT gal dcm lon hsdS*$_B$ (*r*^-_B*m*^-_B) λ(DE3)	Novagen,Germany
BL21/ pET30a	BL21(DE3) harboring plasmid pET30a, Kan^r	This work
BL21/pET30a-AsaA	BL21(DE3) harboring plasmid pET30a-AsaA, Kan^r	This work
BL21/pET30a-TssM$_{34\text{-}370}$	BL21(DE3) harboring plasmid pET30a-TssM$_{34\text{-}370}$, Kan^r	This work
XL1-Blue MRF′	Reporter strain, *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 hisB sup E44 thi1 recA1 gyrA96relA1 lac* [F′ lacI^q *HIS3 aadA* Kan^r]	Stratagene
X/pBA-pTB	XL1-Blue MRF′ harboring plasmid pBA and pTB, Kan^r Tc^r Cm^r	This work

X/pBA-pTL	XL1-Blue MRF′ harboring plasmid pBA and pTL, Kan^r Tc^r Cm^r	This work
X/pBA-pTM_{1303}	XL1-Blue MRF′ harboring plasmid pBA and pTM_{1303}, Kan^r Tc^r Cm^r	This work
X/pBA-$pTM_{33\text{-}415}$	XL1-Blue MRF′ harboring plasmid pBA and $pTM_{33\text{-}415}$, Kan^r Tc^r Cm^r	This work
X/pBA-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid pBA and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/pBA-pT	XL1-Blue MRF′ harboring plasmid pBA and pTRG, Kan^r Tc^r Cm^r	This work
X/pB-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid pBT and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/pBhpaM-pThrcJ	XL1-Blue MRF′ harboring plasmid pBhpaM and pThrcJ, Kan^r Tc^r Cm^r	[2]
X/$pBA_{25\text{-}70}$-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid $pBA_{25\text{-}70}$ and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/$pBA_{25\text{-}110}$-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid $pBA_{25\text{-}110}$ and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/$pBA_{25\text{-}150}$-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid $pBA_{25\text{-}150}$ and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/$pBA_{25\text{-}190}$-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid $pBA_{25\text{-}190}$ and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/$pBA_{25\text{-}230}$-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid $pBA_{25\text{-}230}$ and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/$pBA_{110\text{-}230}$-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid $pBA_{110\text{-}230}$ and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/$pBA_{150\text{-}230}$-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid $pBA_{150\text{-}230}$ and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/$pBA_{190\text{-}230}$-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid $pBA_{190\text{-}230}$ and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/$pBA_{70\text{-}150}$-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid $pBA_{70\text{-}150}$ and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/$pBA_{70\text{-}110}$-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid $pBA_{70\text{-}110}$ and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work
X/$pBA_{110\text{-}150}$-$pTM_{436\text{-}1041}$	XL1-Blue MRF′ harboring plasmid $pBA_{110\text{-}150}$ and $pTM_{436\text{-}1041}$, Kan^r Tc^r Cm^r	This work

A. Baumannii strains

17978	wild type strain	[3]
ΔasaA	As 17978, but *asaA* gene (*A1S_1292*) deleted.	This work
ΔtssM	As 17978, but *tssM* gene (*A1S_1302*) deleted.	This work
CΔasaA	ΔasaA harboring the recombinant plasmid pTrc99AasaA , Amp^r	This work
17978/pThcpH6	17978 harboring recombinant plasmid pTHcpH6, Amp^r	This work
ΔasaA /pThcpH6	*ΔasaA* harboring recombinant plasmid pTHcpH6, Amp^r	This work
ΔtssM /pThcpH6	*ΔtssM* harboring recombinant plasmid pTHcpH6, Amp^r	This work
ΔasaA / pTasaAH6	*ΔasaA* harboring the recombinant plasmid pTrc99AasaAH6 , Amp^r	This work
17978/pTpglCH6	17978 harboring recombinant plasmid pTpglCH6, Amp^r	This work
17978/pTompAH6	17978 harboring recombinant plasmid pTompA H6, Amp^r	This work
17978/pTdsbAH6	17978 harboring recombinant plasmid pTdsbAH6, Amp^r	This work

Plasmids

pET30a	Expression vector, allow the production of fusion proteins containing amino terminal 6×His -tagged sequences. Kan^r	Novagen
pET30a-AsaA	pET30a containing a 618-bp fragment of partial *asaA* gene sequence encoding the 25^{th}–230^{th} amino acids. Kan^r	This work
pET30a- $TssM_{436\text{-}1041}$	pET-30a containing a 1818-bp fragment of partial *tssM* gene sequence encoding the 436^{th}–1041^{th} amino acids. Kan^r	This work
pTrc99A	P*trc*, pBR322ori, *rrnB* T1, *rrnB* T2, lac^{Iq}, *bla*, template for P*trc* Promoter, Amp^r	[4]
pThcpH6	pTrc99A containing the encoding sequence of Hcp with 6×His tag in its C-terminus, Amp^r	This work

pTasaAH6	pTrc99A containing the encoding sequence of AsaA with 6×His tag in its C-terminus, Amp^r	This work
pTpglCH6	pTrc99A containing the encoding sequence of PglC with 6×His tag in its C-terminus, Amp^r	This work
pTompAH6	pTrc99A containing the encoding sequence of OmpA with 6×His tag in its C-terminus, Amp^r	This work
pTdsbAH6	pTrc99A containing the encoding sequence of DsbA with 6×His tag in its C-terminus, Amp^r	This work
pBT	Two-hybrid system bait plasmid containing the *cm* gene, p15A origin of replication and λcI ORF, Cm^r	Stratagene
pBA	pBT derivative carrying a 621-bp fragment encoding the 25^{th}–230^{th} amino acids of AsaA, Cm^r	This work
$pBA_{25\text{-}70}$	pBT derivative carrying a 138-bp fragment encoding the 25^{th}–70^{th} amino acids of AsaA, Cm^r	This work
$pBA_{25\text{-}110}$	pBT derivative carrying a 258-bp fragment encoding the 25^{th}–110^{th} amino acids of AsaA, Cm^r	This work
$pBA_{25\text{-}150}$	pBT derivative carrying a 378-bp fragment encoding the 25^{th}–150^{th} amino acids of AsaA, Cm^r	This work
$pBA_{25\text{-}190}$	pBT derivative carrying a 498-bp fragment encoding the 25^{th}–190^{th} amino acids of AsaA, Cm^r	This work
$pBA_{70\text{-}230}$	pBT derivative carrying a 483-bp fragment encoding the 70^{th}–230^{th} amino acids of AsaA, Cm^r	This work
$pBA_{110\text{-}230}$	pBT derivative carrying a 363-bp fragment encoding the 110^{th}–230^{th} amino acids of AsaA, Cm^r	This work
$pBA_{150\text{-}230}$	pBT derivative carrying a 243-bp fragment encoding the 150^{th}–230^{th} amino acids of AsaA, Cm^r	This work
$pBA_{190\text{-}230}$	pBT derivative carrying a 123-bp fragment encoding the 190^{th}–230^{th} amino acids of AsaA, Cm^r	This work
$pBA_{70\text{-}150}$	pBT derivative carrying a 243-bp fragment encoding the 70^{th}–150^{th} amino acids of AsaA, Cm^r	This work
$pBA_{70\text{-}110}$	pBT derivative carrying a 123-bp fragment encoding the 70^{th}–110^{th} amino acids of AsaA, Cm^r	This work
$pBA_{110\text{-}150}$	pBT derivative carrying a 123-bp fragment encoding the 110^{th}–150^{th} amino acids of AsaA, Cm^r	This work
pTRG	Two-hybrid system target plasmid containing the *tet* gene, ColE1 origin of replication, and RNA polymerase α subunit ORF, Tc^r	Stratagene
pTB	pTRG derivative carrying the 150-bp of *tssB* gene, Tc^r	This work
pTL	pTRG derivative carrying the 648-bp of *tssL* gene, Tc^r	This work
pTM_{1303}	pTRG derivative carrying the 567-bp of *A1S_1303* gene, Tc^r	This work
$pTM_{33\text{-}415}$	pTRG derivative carrying a 1149-bp fragment encoding the 33^{th}–415^{th} amino acids of *tssM* gene, Tc^r	This work
$pTM_{436\text{-}1041}$	pTRG derivative carrying a 1818-bp fragment encoding the 436^{th}–1041^{th} amino acids of *tssM* gene, Tc^r	This work

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Table S2. Primers used in this study

Primer	Nucleotide sequence (5′→3′)	The amplified fragment or the utilization
asaA-F	TATTCTAAAAATATTAACTTTTCGGTAAGGCCTTGAATAAATAAGAAAA CAGAGGAGTGTAGGCTGGAGCTGCTTC	DNA fragment of 1581-bp of the kanamycin resistance ORF sequence and coding sequence fusing with *asaA* upstream and downstream encoded sequence. Used for construction of *asaA* deletion mutant.
asaA-R	TTCGCTGAAGCTTCTTTTGTACGCTTTCTCTCTTAGCCATAAGATTCTA TTCCGGACATATGAATATCCTCCTTAG	
tssM-F	GGCAGTACATCACGAATCCTAAAGCAATTATCGCCTTGTCATTGTTCGT GGCATTGGTGTAGGCTGGAGCTGCTTC	DNA fragment of 1581-bp of the kanamycin resistance ORF sequence and coding sequence fusing with *tssM* upstream and downstream encoded sequence. Used for construction of *tssM* deletion mutant.
tssM-R	TGCCATTCCATTTGTAGGTGCAACATAGCGTTGGAAGTTCATAACAAA TTGTGGGTCATATGAATATCCTCCTTAG	
asaA-CF	AAGAATTCAAATATTAACTTTTCGGTAAGG	DNA fragment of 759-bp DNA fragment spans 48 upstream to the stop codon of the *asaA* ORF sequence, which fusing with 6×His tag encoding sequences. Used for construction of *ΔasaA* complement and Western blot analysis.
asaA-CR	AAGGATCCTTAGTGGTGGTGGTGGTGGTGTTTAATTAAAGGGTCAAA AGG	
hcp-qF	ATATATACGTTGAGTTTCG	381-bp internal fragment of *hcp*, used for qRT-PCR.
hcp-qR	AACCGAATGCTTCTGTAG	
16s-F	GTGCCAGCAGCCGCGGT	846-bp internal fragment of the 16S rDNA sequence, used for qRT-PCR.
16s-R	GACGGGCGGTGTGTACA	
hcp-HF	ATGGATCCGACCACATTTCCAGCTGG	DNA fragment of 627-bp *hcp* coding sequence fusing with 6×His tag encoding sequences. Used for Western blot analysis.
hcp-HR	AGTCTAGATTAGTGGTGGTGGTGGTGGTGCGCTGCGTAAGAAGC	
pglC-HF	CCGAATTCGTGAATGAAAATATGAA	DNA fragment of 633-bp *pglC* coding sequence fusing with 6×His tag encoding sequences. Used for Western blot analysis.
pglC -HR	AAGGATCCTTAGTGGTGGTGGTGGTGGTGCTTAACAGAAGAAT	
dsbA-HF	CCGAATTC ATGAAAAAATTGGTTTTGG	DNA fragment of 618-bp *dsbA* coding sequence fusing with 6×His tag encoding sequences. Used for Western blot analysis.
dsbA-HR	AAGGATCCTTAGTGGTGGTGGTGGTGGTGTTTTGCCTTACGTT	
ompA-HF	CCGAATTCATGAAATTGAGTCGTATT	DNA fragment of 1071-bp *ompA* coding sequence fusing with 6×His tag encoding sequences. Used for Western blot analysis.
ompA -HR	AAGGATCCTTAGTGGTGGTGGTGGTGGTGTTGAGCTGCTGCAG	
$tssM_{(33\text{-}415)}$-F	CCGGATCCCAGCGTAAAAAGCATGCT	1149-bp DNA fragment spans nucleotides 97 to 1245 bp of the *tssM* coding sequence, encoding the 33^{th} - 415^{th} amino acids. Used for bacterial two-hybrid assay.
$tssM_{(33\text{-}415)}$-R	AAGGATCCACGCTGACGCTTTTTGCT	
$tssM_{(436\text{-}1041)}$-F	CCGGATCCTCATATCGTAACAATCAA	1818-bp DNA fragment spans nucleotides 1306 to 3123 bp of the *tssM* coding sequence, encoding the 436^{th} - 1041^{th} amino acids. Used for bacterial two-hybrid assay.
$tssM_{(436\text{-}1041)}$-R	AAGAATTCAACTAATCCCCAAATCTT	

tssB-F	CCGGATCCGTGTTCAGACTTACATAT	150-bp DNA fragment of the *tssB* ORF sequence. Used for bacterial two-hybrid assay.
tssB-R	AAGAATTCCTAAATCGACATTAATGA	
tssL-F	CCGGATCCATGTCACAATCTACAGGT	648-bp DNA fragment spans nucleotides 1 to 648 bp of the *tssM* coding sequence, encoding the 1^{th} - 216^{th} amino acids. Used for bacterial two-hybrid assay.
tssL-R	AAGAATTCAGGTAGCTCACGATGGAT	
Tss1303-F	CCGGATCCATGGCAACAGGTGAGTTA	567-bp DNA fragment of the *A1S_1303* ORF sequence. Used for bacterial two-hybrid assay.
Tss1303-R	AAGAATTCTCATGGCTTAACTCCCGC	
asaA-OF	AAGGATCCCAAGCAGCAGAACTAGAG	621-bp DNA fragment spans nucleotides 73 to 693 bp of the *asaA* coding sequence, encoding the 25^{th} - 230^{th} amino acids. Used for pull-down assay.
asaA-OR	CGAAGCTTTTATTTAATTAAAGGGTC	
tssM-OF	AAGGATCCTCATATCGTAACAATCAA	1821-bp DNA fragment spans nucleotides 1306 to 3126 bp of the *tssM* coding sequence, encoding the 436^{th} - 1041^{th} amino acids. Used for pull-down assay.
tssM-OR	CCGTCGACTTAAACTAATCCCCAAATCTT	
asaA-1F	CCGAATTCACAAGCAGCAGAACTAGAG	621-bp DNA fragment spans nucleotides 73 to 693 bp of the *asaA* coding sequence, encoding the 25^{th} - 230^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-1R	AAGGATCCTTATTTAATTAAAGGGTC	
asaA-2F	CCGAATTCACAAGCAGCAGAACTAGAG	138-bp DNA fragment spans nucleotides 73 to 210 bp of the *asaA* coding sequence, encoding the 25^{th} - 70^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-2R	AAGGATCCATTTTTTTGAGTTGTATT	
asaA-3F	CCGAATTCACAAGCAGCAGAACTAGAG	258-bp DNA fragment spans nucleotides 73 to 330 bp of the *asaA* ORF sequence, encoding the 25^{th} - 110^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-3R	AAGGATCCTAAGACATCTGTGATTAA	
asaA-4F	CCGAATTCACAAGCAGCAGAACTAGAG	378-bp DNA fragment spans nucleotides 73 to 450 bp of the *asaA* ORF sequence, encoding the 25^{th} - 150^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-4R	AAGGATCCATGAATATTTTCAACTAA	
asaA-5F	CCGAATTCACAAGCAGCAGAACTAGAG	498-bp DNA fragment spans nucleotides 73 to 570 bp of the *asaA* ORF sequence, encoding the 25^{th} - 190^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-5R	AAGGATCCAGCACTCACTTTATTACG	
asaA-6F	CCGAATTCATCTAGAACATCAAATAAA	123-bp DNA fragment spans nucleotides 571 to 693 bp of the *asaA* ORF sequence, encoding the 190^{th} - 230^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-6R	AAGGATCCTTATTTAATTAAAGGGTC	
asaA-7F	CCGAATTCAACTGCAATGCCGGCTCCT	243-bp DNA fragment spans nucleotides 451 to 693 bp of the *asaA* ORF sequence, encoding the 150^{th} - 230^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-7R	AAGGATCCTTATTTAATTAAAGGGTC	
asaA-8F	CCGAATTCATTTTTATCTGGTGTGAGC	363-bp DNA fragment spans nucleotides 331 to 693 bp of the *asaA* ORF sequence, encoding the 110^{th} - 230^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-8R	AAGGATCCTTATTTAATTAAAGGGTC	
asaA-9F	CCGAATTCAGATTTATTAGCTAAAGCT	483-bp DNA fragment spans nucleotides 211 to 693 bp of the *asaA* ORF sequence, encoding the 70^{th} - 230^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-9R	AAGGATCCTTATTTAATTAAAGGGTC	
asaA-10F	CCGAATTCAGATTTATTAGCTAAAGCT	240-bp DNA fragment spans nucleotides 211 to 450 bp of the *asaA* ORF sequence, encoding the 70^{th} - 150^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-10R	AAGGATCCATGAATATTTTCAACTAA	
asaA-11F	CCGAATTCAGATTTATTAGCTAAAGCT	120-bp DNA fragment spans nucleotides 211 to 330 bp of the *asaA* ORF sequence, encoding the 70^{th} - 110^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-11R	AAGGATCCTAAGACATCTGTGATTAA	

asaA-12F	CCGAATTCATTTTTATCTGGTGTGAGC	120-bp DNA fragment spans nucleotides 331 to 450 bp of the *asaA* ORF sequence, encoding the 110^{th} -150^{th} amino acids. Used for bacterial two-hybrid assay.
asaA-12R	AAGGATCCATGAATATTTTCAACTAA	

The underlined sequences indicate the restriction sites for *Eco*RI, *Hin*dIII, *Sal*I , *Xba*I and *Bam*HI, respectively.

Table S3. AsaA homologues in the family *Moraxellaceae*

Species	Gene	Signal peptide	Length (aa)	Identity(%) /similarity(%)	Function predicted	Sequence Types (ST)	Reference
Acinetobacter baumannii AC29	*BL01_13280*	1^{th}-24^{th} aa	230	100/100	signal peptide protein	195	[1]
Acinetobacter baumannii BJAB0868	*BJAB0868_01408*	1^{th}-24^{th} aa	230	100/100	hypothetical protein	218	[2]
Acinetobacter baumannii 1656-2	*ABK1_1741*	1^{th}-24^{th} aa	230	100/100	putative exported protein	423	[3]
Acinetobacter baumannii AB307-0294	*ABBFA_002240*	1^{th}-24^{th} aa	230	99.6/99.6	hypothetical protein	231	[4]
Acinetobacter baumannii AB0057	*AB57_1478*	1^{th}-24^{th} aa	230	99.1/99.6	hypothetical protein	207	[4]
Acinetobacter baumannii BJAB0715	*BJAB0715_01479*	1^{th}-24^{th} aa	230	99.1/99.6	hypothetical protein	642	[5]
Acinetobacter baumannii AbH12O-A2	*LX00_06400*	1^{th}-24^{th} aa	230	99.1/99.1	signal peptide protein	924	[6]
Acinetobacter pittii	*BDGL_000638*	1^{th}-26^{th} aa	229	90.9/92.6	hypothetical protein	1527	[7]
Acinetobacter calcoaceticus	*BUM88_06735*	1^{th}-26^{th} aa	229	87.8/92.2	hypothetical protein	1043	accession CP020000.1
Acinetobacter nosocomialis	*RR32_11560*	1^{th}-24^{th} aa	229	80.9/87.8	signal peptide protein	1162	accession CP010368.1
Acinetobacter baylyi	*ACIAD2693*	1^{th}-22^{th} aa	218	43.5/57.8	hypothetical protein		[8]

Acinetobacter indicus	*CTZ23_07445*	1th-22 th aa	222	33.3/46.8	hypothetical protein	[9]

Searching the AsaA homologues were carried out by blast the genome sequences in NCBI database with the AsaA sequence. No, no significant similarity protein (similarity>20%) was found within the genome sequence. Signal peptide predictions was carried with the SignalP program. Identity(%)/similarity(%) analysis was performed using the Align X program in Vector NTI software.

Reference

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