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Emergence of *Staphylococcus aureus* Carrying Multiple Drug Resistance Genes on a Plasmid Encoding Exfoliative Toxin B

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We report the complete nucleotide sequence and analysis of $pETB_{TY825}$, a *Staphylococcus aureus* TY825 plasmid encoding exfoliative toxin B (ETB). *S. aureus* TY825 is a clinical isolate obtained from an impetigo patient in 2002. The size of $pETB_{TY825}$, 60.6 kbp, was unexpectedly larger than that of the archetype $pETB_{TY4}$ (~30 kbp). Genomic comparison of the plasmids shows that $pETB_{TY825}$ has the archetype $pETB_{TY4}$ as the backbone and has a single large extra DNA region of 22.4 kbp. The extra DNA region contains genes for resistance to aminoglycoside [*aac*(6′)/*aph*(2″)], macrolide (*msrA*), and penicillin (*blaZ*). A plasmid deletion experiment indicated that these three resistance elements were functionally active. We retrospectively examined the resistance profile of the clinical ETB-producing *S. aureus* strains isolated in 1977 to 2007 using a MIC determination with gentamicin (GM), arbekacin (ABK), and erythromycin (EM) and by PCR analyses for *aac*(6′)/*aph*(2″) and *msrA* using purified plasmid preparations. The ETB-producing *S. aureus* strains began to display high resistance to GM, which was parallel with the detection of *aac*(6′)/*aph*(2″) and *mecA*, after 1990. Conversely, there was no significant change in the ABK MIC during the testing period, although it had a tendency to slightly increase. After 2001, isolates resistant to EM significantly increased; however, *msrA* was hardly detected in ETB-producing *S. aureus* strains, and only five isolates were positive for both *aac*(6′)/*aph*(2″) and *msrA*. In this study, we report the emergence of a fusion plasmid carrying the toxin gene *etb* and drug resistance genes. Prevalence of the $pETB_{TY825}$ carrier may further increase the clinical threat, since ETB-producing *S. aureus* is closely related to more severe impetigo or staphylococcal scalded-skin syndrome (SSSS), which requires a general antimicrobial treatment.

Exfoliative toxin (ET) is an exotoxin produced by staphylococcal species, causing blisters on human and animal skin (1). ET-producing *Staphylococcus aureus* is involved in staphylococcal scalded-skin syndrome (SSSS) or Ritter disease and in bullous impetigo in neonates (1–3). Serologically, ETs causing diseases in human have been divided into three major serotypes: ETA, ETB, and ETD (4–6). All types cause intraepidermal cleavage in the granular layer, without epidermal necrolysis or inflammatory response in the skin (4, 5, 7). ETs are serine proteases that selectively cleave desmoglein 1, a desmosomal protein connecting epidermal cells present in the epidermis (8).

Virulence factors of staphylococci such as ET are accessory proteins, which are not essential for cell growth or division. Genetic determinants for these factors are often associated with mobile genetic elements, such as phages, plasmids, and pathogenicity islands (9–11). The *eta* gene is located on the genome of a temperate phage (ϕ ETA) (12), the *etb* gene is on a large plasmid (4, 13), and the *etd* gene is chromosomally located in a pathogenicity island (6).

We previously reported the complete nucleotide sequence of the ETB plasmid of strain *S. aureus* TY4, isolated from skin lesions of patients diagnosed with staphylococcal scalded-skin syndrome (SSSS) (13). The ETB plasmid (pETB) contains three copies of IS*257*, which divides the pETB genome into three regions: (i) a cadmium resistance operon-containing region, (ii) a lantibiotic gene-containing region, and (iii) the region where genes for plasmid replication and/or maintenance are dispersed. These genes include two virulence-related genes, the *etb* gene, and the ADP-ribosyltransferase *ednC* gene, which belongs to the C3 exoenzyme family. Further, we reported significant size variation of the ETB plasmid from various clinical strains.

During our genome project, we determined the nucleotide sequence of a new ETB plasmid from *S. aureus* strain TY825 from an impetigo patient. Comparative analysis of $pETB_{TY4}$ and $pETB_{TY825}$ showed that $pETB_{TY825}$ carries three antibiotic resistance genes. Here we report a novel ETB plasmid contributing to the multidrug resistance of *S. aureus*. Additionally, we investigated the relevance of the $pETB_{TY825}$ type and antimicrobial susceptibilities of ETB-producing *S. aureus* strains isolated between 1977 and 2007 in Japan.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* TY825 was isolated from the skin lesions of patients diagnosed with impetigo. Other *S. aureus* strains used in this study were from our laboratory collection of clinical isolates producing ETB.

Manipulation of DNA. Routine DNA manipulations were performed using standard procedures (14). pETB was extracted from *S. aureus* TY825 and purified using a Qiagen midikit. The plasmid DNA was further purified by CsCl equilibration centrifugation, followed by isopropanol precipitation. Southern blotting of the DNA and hybridization were performed as described previously (15).

Shotgun sequencing, assembly, and annotation of $pETB_{TY825}$. The genome sequence of pETB DNA was determined using the random shot-

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TABLE 1 Oligonucleotides used for PCR amplification

Purpose and gene or region	Primer	Oligonucleotide sequence (5′-3′)	Product size (bp)	Primer design reference or source
PCR				
etb	ET-3	ATACACACATTACGGATAAT	629	13
	ET-4	CAAAGTGTCTCCAAAAGT		
aac(6′)/*aph*(2′)	aac/aph-F	TACAGAGCCTTGGGAAGATG	406	32
	aac/aph-R	CATTTGTGGCATTATCATCATATC		
msrA	msr-F	TGCAAATGGCATACTATCGTC	160	32
	msr-R	CAAGAACGCTCAAGTGCTTC		
PCR scanning				
Region 1	region_1-F	CCTAAAATTGTTTGAATAGTATC	3,949	This study
	region_1-R	GGATTGAACTTCTGATAATCATT		
Region 2	region_2-F	CTTGTGTCTTTTTATGTGGATTG	4,054	This study
	region_2-R	GACAATCTATTCATGATATAACT		
Region 3	region_3-F	TTTATCAAGATAATCCCTTATCG	3,164	This study
	region_3-R	CACTTTTAAAATATGAACTAGGA		
Region 4	region_4-F	TGTAAAGTATCTCTATTTTTAGC	3,150	This study
	region_4-R	CATTTAGGGGTATCTTATATATT		
Region 5	region_5-F	CTTAGACCTTATTTAAAATATCC	2,019	This study
	region_5-R	CATAATTTTTGATAAAGTCCGTA		
Region 6	region_6-F	AAATTTCTTTTCTACCATTTTCG	4,922	This study
	region_6-R	GTTAAAGATTTATTCCAACTACA		
Region 7	region_7-F	ATTTAGATAGAAAAGAAAGAGCG	5,012	This study
	region_7-R	GATAAGCTTAAAGTAACTTCTTT		

gun sequencing method as described previously (12). Collected sequences were assembled using SEQUENCHER DNA sequencing software (v3.0; Gene Codes). Gaps were closed by direct sequencing of the PCR products amplified with oligonucleotide primers designed to anneal to each end of the neighboring contigs. Initially, potential protein-encoding regions (open reading frames [ORFs]) that were ≥150 bp long were identified using MetaGeneAnnotator (16) and the InSilico molecular cloning software package, genomics edition (InSilico Biology Inc., Yokohama, Japan), and each ORF was reviewed manually for the presence of a ribosomal binding sequence. Functional annotation was assigned based on homology searches against the GenBank nonredundant protein sequence database using the program BLASTP (17). Protein and nucleotide sequences were compared with those in the sequence databases using the BLAST and FASTA programs implemented at the DDBJ (DNA Data Bank of Japan; http://www.ddbj.nig.ac.jp/).

Antimicrobial susceptibility testing. The MIC determination was performed using the microdilution broth method (14) with the MicroScan-WalkAway-96 system. The antibiotics tested were benzylpenicillin (PCG), ampicillin (ABPC), cefazolin (CEZ), cefotiam (CTM), cefozopran (CZOP), cefpirome (CPR), cefdinir (CFDN), cefditoren (CDTR), flomoxef (FMOX), imipenem (IPM), meropenem (MEPM), gentamicin (GM), arbekacin (ABK), erythromycin (EM), clindamycin (CLDM), minocycline (MINO), levofloxacin (LVFX), vancomycin (VCM), teicoplanin (TEIC), sulfamethoxazole-trimethoprim (ST), fosfomycin (FOM), and linezolid (LZD). Separately, the microdilution method was used to assess endpoints for the ABK, GM, and EM MICs according to the CLSI guidelines (18).

PCR scanning analysis. Plasmid DNAs were isolated from ETB-producing *S. aureus* clinical strains in our laboratory stock and were used as templates for PCR scanning analysis (36). All primers were designed according to the nucleotide sequence of pETB (Table 1).

Nucleotide sequence accession number. The nucleotide sequence described here has been deposited in GenBank under accession number AP012467.

RESULTS

General overview and comparative analysis of the ETB plasmid. *S. aureus* TY825 was clinically isolated in 2002 from a lesion of an impetigo patient and is positive for the plasmid carrying *etb* (pETB). As a part of the genome project of clinically isolated *S. aureus* strains in Japan, the complete nucleotide sequence of $pETB_{TY825}$ was determined using a shotgun approach. The fully assembled circular DNA sequence of $pETB_{TY825}$ was 60,563 bp (Fig. 1A). The average GC content of $pETB_{TY825}$ was 28.2%. We identified 63 potential protein-coding regions (Fig. 1A; Table 2). $pETB_{TY825}$, which is 38,211 bp, is significantly larger than the archetype pETB ($pETB_{TY4}$), which is ~35 kb (13) (Fig. 1C). Comparison of $pETB_{TY825}$ and $pETB_{TY4}$ shows that $pETB_{TY825}$ is a composite of $pETB_{TY4}$ and a single large extra DNA region (22,352 bp) (Fig. 1; Table 2). Sequence alignment of both plasmids shows the extra DNA region was inserted between *orf25* and *orf37* in $pETB_{TY4}$ (Fig. 1B). Examining the boundary nucleotide sequences of the extra DNA region, direct repeats of 25-bp sequences (5′-CTCTACTAACCAGTGTTATAATTTA-3′) were found (Fig. 1C). The genome organization of the backbone sequence of $pETB_{TY825}$ corresponding to the $pETB_{TY4}$ sequence was conserved (Fig. 1B). The genes *etb* and *ednC*, genetic elements for lantibiotic production, are present in the backbone sequence. Annotation of the extra DNA region identified a cadmium resistance element and three antibiotic resistance elements that confer resistance to aminoglycosides, macrolides, and β-lactams (Table 2; Fig. 1C) The aminoglycoside resistance gene, *aac*(6′)/*aph*(2″), encoding a bifunctional enzyme, is located between two IS*256* elements, forming the 4.5-kb Tn*4001*, which is most frequently observed as the mobile element of *aac*(6′)/*aph*(2″) in Gram-positive bacteria (19, 20). AAC(6′)/APH(2″) primarily confers resistance to gentamicin, kanamycin, and tobramycin (21). The macrolide resistance element is composed of *stpA*, *smpA*, and *msrA*, whose products act as an ATP-dependent efflux pump conferring the so-called MS phenotype, i.e., inducible resistance to 14- and 15-membered ring macrolides and resistance to streptogramin type B (22, 23). The

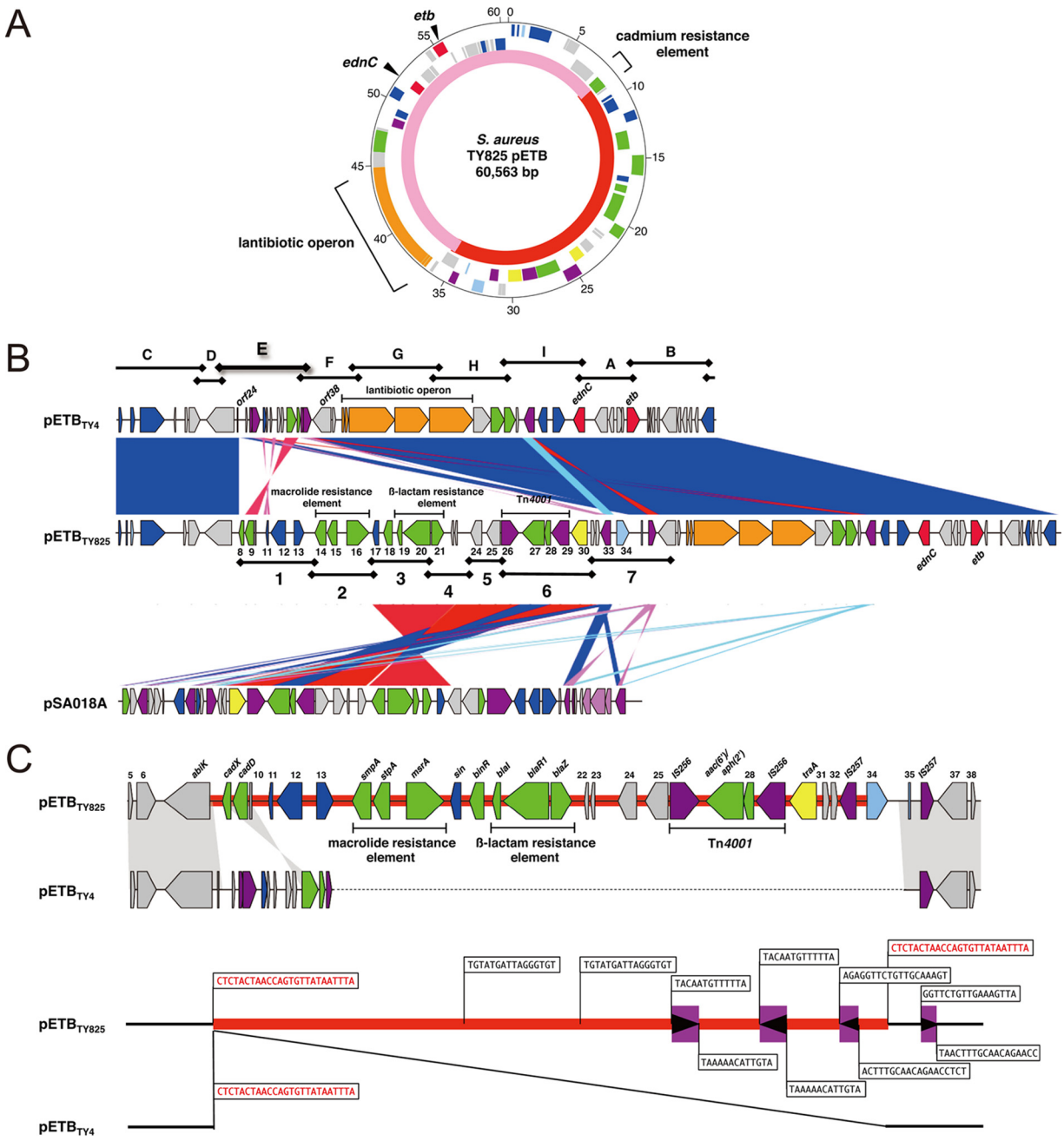

FIG 1 (A) Circular genetic map of pETB$_{TY825}$ from *S. aureus* TY825. From the outside in, the first circle shows the nucleotide sequence positions (in kb), the second and third circles show coding sequences transcribed clockwise and counterclockwise, respectively (red, pathogenic factor; green, antibiotic resistance gene; blue, DNA replication, recombination, and repair; light blue, transcription regulator; purple, transposase; yellow, conjugal transfer [*tra*]; orange, lantibiotic operon; and gray, conserved ORFs), and the fourth circle shows the backbone of pETB$_{TY4}$ (pink) (GenBank accession no. AP003088) and the acquired region (red). (B) Structural comparison of pETB$_{TY825}$ to pETB$_{TY4}$ and the *Staphylococcus* plasmid pSA018A. Color shading indicates homologous regions. The approximately 16-kb extra DNA region of pETB$_{TY825}$ was similarity matched with the *Staphylococcus* plasmid pSA018A (GenBank accession no. GQ900383). (C) IS elements are represented as purple boxes, and the directions of the transposase genes are indicated by arrowheads in the boxes. Sequences of the terminal inverted repeats of each IS elements are shown. Sequences of the terminal directed repeats of the acquired region (red) of pETB$_{TY825}$ are shown.

β-lactamase-dependent resistance element *blaZ*, two closely linked genes (*blaI* and *blaR*), and IS*257* form Tn*552*. This transposon is frequently observed on a large plasmid as well as in the chromosome of staphylococci (24). However, the β-lactam resistance element of pETB$_{TY825}$ and pSA018A lacks IS*257* downstream of *blaZ* (Fig. 1A and B; Table 2). Identification of the *sin* recombinase gene immediately downstream of the element and the partial 12-bp *resH* sequence (5′-TGTATGATTAGG-3′) (25) on both sides of the element, a direct repeat, strongly suggests that the element was acquired as a block through Sin-dependent re-

TABLE 2 Features of $pETB_{TY825}$ ORFs

ORF	Position (bp)		Strand	Gene	Length (aa)[a]	Translation signal[b]	Source	Description	Identity (%)	Overlap[c] (aa)	Accession no.
	Start	Stop									
1	231	455	+	*repA*	74	GAGGTTTTTATT**ATG**	*S. aureus*(pETB)	pETB_p18 (replication initiator protein A)	100	74/74	BAB78416
2	642	785	+	*rep*	47	GAGAATAATGAT**ATG**	*S. aureus* TCH130	Hypothetical protein (truncated replication protein)	60.6	33/47	ZP_04868980
3	1002	1217	+		71	AGGGCTATGTAAAGAA**TTG**	*S. aureus*(pETB)	pETB_p19 (transcriptional regulator protein)	100	71/71	NP_478362
4	1590	3164	+	*repR*	524	AGGAGGTGCAGACA**ATG**	*S. aureus*(pETB)	pETB_p20 (plasmid replication protein RepR)	100	524/524	NP_478363
5	4397	4564	+		55		*S. aureus*(pETB)	pETB_p22 (lipase)	100	55/55	NP_478365
6	4647	5378	+		243	GAGGTATTCTTAATAAA**ATG**	*S. aureus*(pETB)	pETB_p23 (cell wall-associated biofilm protein)	92.4	243/243	NP_478366
7	5738	7510	−	*abiK*	590	AGGAGAAAGGCT**ATG**	*S. aureus*(pETB)	Abortive infection protein K	100	590/590	NP_478367
8	7914	8261	−	*cadX*	115	AGGGTGCGATTTTAT**ATG**	*S. lugdunensis*(pLUG)	CadX	100	115/115	NP_054018
9	8280	8897	−	*cadD*	205	GAGGTGTAATT**ATG**	*S. aureus*(pETB)	Cadmium-binding protein	99.5	205/205	NP_478377
10	8966	9109	−		48		*S. epidernidis* BCM-HMP0060	Hypothetical protein	97.9	48/48	ZP_04824204
11	9670	9858	−		62	AGGATTATATCGAAAACGT**ATG**	*S. epidernidis* BCM-HMP0060	Replication protein Rep	93.4	62/62	ZP_04824202
12	9989	10960	−		323	AGAGGTTTTTGT**ATG**	*S. saprophyticus* ATCC 15305	Replication initiator protein	99.4	323/323	YP_302585
13	11475	12167	+		230	GGAGGCCATTAT**ATG**	*S. epidernidis* BCM-HMP0060	Partitioning protein	80.9	230/230	ZP_04824200
14	12788	13558	−	*smpA*	256	AGGAGGATCAATCGTAAA**ATG**	*S. epidernidis* 968	ABC transporter membrane protein	100	256/256	CAA83062
15	13560	14255	−	*stpA*	231	AGGAGATAATTGT**ATG**	*S. epidernidis* W23144	ABC transporter ATP-binding protein	100	231/231	ZP_04796098
16	14792	16258	+	*msrA*	488	AGGAGTGTATAAAT**ATG**	*S. epidernidis* W23144	ABC transpoter permease protein (erythromycin resistance protein, MsrA)	100	488/488	ZP_04796097
17	16482	16910	−	*Sin*	142	GGAGATCGATTCGTT**GTG**	*S. aureus* USA300_TCH959	Recombinase Sin	97.9	142/142	YP_001569089
18	17217	17795	−	*binR*	192	AGGAGGTTTGTATT**TTG**	*S. aureus* CF-Marseille	Tn*552* DNA invertase BinR	98.9	192/192	ZP_04839235
19	18059	18439	−	*blaI*	126		*S. epidernidis* ATCC 12228	Beta-lactamase repressor BlaI	100	126/126	NP_863211
20	18429	20228	−	*blaR1*	599		*S. aureus* JKD6008	Beta-lactamase regulatory protein BlaR1	100	585/599	ZP_03563212
21	20293	21138	+	*blaZ*	281	GGAGGGTTTATT**TTG**	*S. aureus* MRSA252	Beta-lactamase	99.6	281/281	NP_878023
22	21500	21685	−		61	AGGTTATGAAAGTAAATGT**ATG**	*S. epidernidis* RP62A	Conserved hypothetical protein	95.1	61/61	YP_189789
23	21757	21960	−		67	AGGGGGAGTATCT**TTG**	*S. epidernidis* RP62A	Conserved hypothetical protein	95.7	47/67	YP_189789
24	22751	23482	−		243	GGAGGTAAGTT**TTG**	*S. epidernidis* RP62A	Conserved hypothetical protein	97.1	243/243	YP_189787
25	23788	24666	−		292	AGGACTGTTAT**ATG**	*S. epidernidis* ATCC 12228	Hypothetical protein	93.2	281/292	NP_863227
26	24729	25901	+	IS*256*	390	AGGAGGACTTTTAC**ATG**	*E. faecalis* V583	IS*256* transposase	100	390/390	NP_813928
27	26041	27480	−	*aac(6′)-aph(2′)*	479	AGGTGATAAATAA**ATG**	*S. aureus* Mu50	Bifunctional AAC(6′)/APH(2″): 6′-aminoglycoside *N*-acetyltransferase and 2″-aminoglycoside phosphotransferase	100	479/479	NP_115315
28	27481	27885	−		134	AGGAGTCTGGAC**TTG**	*S. aureus*(pLW043)	Acetyltransferase GNAT family protein	100	134/134	NP_878007
29	27930	29102	−	IS*256*	390	AGGAGGACTTTTAC**ATG**	*E. faecalis* V583	IS*256* transposase	100	390/390	NP_813928
30	29203	30294	−	*traA*	363	AGAGGAGGTAAAATC**ATG**	*S. epidernidis* W23144	Nickase TraA	100	363/363	ZP_03986061
31	30498	30767	+		89	GGAGTTTTTTA**ATG**	*S. epidernidis* W23144	Conserved hypothetical protein	100	89/89	ZP_03986060
32	30784	31023	+		79		*S. epidernidis* W23144	Conserved hypothetical protein	100	79/79	ZP_03986059
33	31131	31805	−	IS*257*	224	AGGAGTCTTCTGT**ATG**	*S. aureus*(pV030-8)	IS*257* transposase	98.7	224/224	YP_001653101
34	32145	32966	+		273	AGGAGACCTAGTTA**ATG**	*S. aureus* MRSA252	LysR family regulatory protein	96.3	273/273	YP_040145
35	33683	33823	−		46		*S. aureus*(pEDINA)	pEDINA_p50 (transcriptional regulator)	97.8	45/46	YP_001573922

36	34209	34736	+	IS*257*	175		*S. aureus*(pETB)	pETB_p37 (IS*257* transposase)	100	175/175	NP_478380
37	34771	35913	−		380	AGGAGAAACT**ATG**	*S. aureus*(pETB)	pETB_p38 (putative ATP/GTP-binding protein)	99.7	380/380	NP_478381
38	36042	36272	+		76	TAAGCTGCTGCTGTATATT**ATG**	*S. aureus*(pETB)	pETB_p39 (conserved hypothetical protein)	100	76/76	NP_478382
39	36635	36823	+	*sacaA*	62	TAAAGCGTGGTGATTCTT**ATG**	*S. aureus*(pETB)	pETB_p40 (lantibiotic structural protein Sac-alpha-A)	100	62/62	NP_478383
40	36847	37050	+	*sacbA*	67	TAAGGTGGTATTTTT**ATG**	*S. aureus*(pETB)	pETB_p41 (lantibiotic structural protein Sac-beta-A)	100	67/67	NP_478384
41	37069	39966	+	*sacM1*	965	GGAGATAGTTCATA**ATG**	*S. aureus*(pETB)	pETB_p42 (lantibiotic mersacidin modifying enzyme SacM1)	100	965/965	NP_478385
42	39968	42130	+	*sacT*	720	GAGGTGTAAT**ATG**	*S. aureus*(pETB)	pETB_p43 (lantibiotic mersacidin ABC transporter system SacT)	100	720/720	NP_478386
43	42127	44880	+	*sacM2*	917	AAGGAGTGTGGAGT**TTG**	*S. aureus*(pETB)	pETB_p44 (lantibiotic mersacidin modifying enzyme SacM2)	99.9	917/917	NP_478387
44	44896	45996	+		366	AGGAGCGTAAATAT**TTG**	*S. aureus*(pETB)	pETB_p45 (conserved hypothetical protein)	100	365/366	NP_478388
45	46011	46880	+		289	AGGAGAATTCTG**ATG**	*S. aureus*(pETB)	pETB_p46 (multidrug efflux ABC transporter ATP-binding protein)	100	289/289	NP_478389
46	46864	47589	+		241	GGAGGTTCTAAAA**TTG**	*S. aureus*(pETB)	pETB_p47 (putative membrane protein)	100	241/241	NP_478390
47	47618	47791	+		57	GGAGGAATTTTA**ATG**	*S. aureus*(pETB)	pETB_p48 (conserved hypothetical protein)	100	57/57	NP_478391
48	48118	48792	−	IS*257*	224	GAGGTGCAGAGG**ATG**	*S. aureus*(pETB)	pETB_p49 (IS*257* transposase)	100	224/224	NP_478392
49	49007	49573	−	*res*	188	GAGGTTATATTTGA**ATG**	*S. aureus*(pETB)	pETB_p50 (recombinase Res)	100	188/188	NP_478393
50	50164	50955	+		263	AGGTACCAATTT**ATG**	*S. aureus*(pETB)	pETB_p01 (replication-associated protein)	100	263/263	NP_478344
51	51488	52231	−	*ednC*	247	AAGGAGTCTTTT**ATG**	*S. aureus*(pETB)	epidermal cell differentiation inhibitor EDINC	100	247/247	NP_478345
52	52801	53631	−		276	AAGGAGAATGAGGCA**TTG**	*S. aureus*(pETB)	pETB_p03 (conserved hypothetical protein)	99.6	276/276	NP_478346
53	53708	54022	−		104	AAGGAGAGAAAATA**ATG**	*S. aureus*(pETB)	pETB_p04 (conserved hypothetical protein)	100	104/104	NP_478347
54	54175	54591	+		138	GAGGTGTATTAAA**ATG**	*S. aureus*(pETB)	pETB_p05 (conserved hypothetical protein)	100	138/138	NP_478348
55	54833	55666	+	*etb*	277	AAGGAGGTTTTATAT**ATG**	*S. aureus*(pETB)	exfoliative toxin B	100	277/277	NP_478350
56	55760	55921	−		53		*S. aureus* MN8	conserved hypothetical protein	56.9	51/53	ZP_03987549
57	56732	56881	−		49	AGGAGGCATTTATT**ATG**	*S. aureus*(pETB)	pETB_p11 (conserved hypothetical protein)	100	49/49	NP_478354
58	57008	57958	−		316	AAGGAGTAGTTAAG**ATG**	*S. aureus*(pETB)	pETB_p12 (extracellular protein)	100	316/316	NP_478355
59	58022	58234	−		70	GGAGGTAACCTAAAT**ATG**	*S. aureus*(pETB)	pETB_p13 (conserved hypothetical protein)	100	70/70	NP_478356
60	58309	58653	−	*mutS*	114	GGAACAA**TTG**	*S. aureus*(pWBG749)	putative DNA mismatch repair protein MutS	83.1	98/118	NP_478357
61	58713	58925	−		70	GAGGGTTTTACAA**ATG**	*S. aureus*(pETB)	pETB_p15 (conserved hypothetical protein)	100	70/70	NP_478358
62	59090	59332	−		80	AGGAGAGATACT**ATG**	*S. aureus*(pETB)	pETB_p16 (conserved hypothetical protein)	100	80/80	NP_478359
63	59501	60307	−	*parA*	268	GGAGGTGGAAGCA**ATG**	*S. aureus*(pETB)	pETB_p17 (plasmid partition protein ParA)	100	268/268	NP_478360

[a] aa, amino acids.

[b] Underlining indicates a putative ribosome binding site complementary to the 3′ end of the 16S rRNA; boldface indicates the start codon.

[c] Overlap indicates the number of overlapping amino acids/total number of amino acids.

TABLE 3 Antimicrobial susceptibilities of *S. aureus* TY825 in the presence and absence of pETB[a]

Antibiotic[b]	pETB$^+$		pETB$^-$	
	MIC (μg/ml)[c]	Susceptibility	MIC (μg/ml)[c]	Susceptibility
PCG	>8	R	2	R
ABPC	>8	R	2	R
CEZ	≤2	S	≤2	S
CTM	≤2	S	≤2	S
CZOP	≤2	S	≤2	S
CPR	≤2	S	≤2	S
CFDN	≤0.5	S	≤0.5	S
CDTR	≤0.5	S	≤0.5	S
FMOX	≤4	S	≤4	S
IPM	≤1	S	≤1	S
MEPM	≤1	S	≤1	S
A/S	≤8	S	≤8	S
A/C	≤2	S	≤2	S
GM	>8	R	≤1	S
ABK	4	S	≤1	S
EM	>4	R	≤0.25	S
CLDM	≤0.5	S	≤0.5	S
MINO	≤1	S	≤1	S
LVFX	≤0.5	S	≤0.5	S
VCM	1	S	1	S
TEIC	≤2	S	≤2	S
ST	≤0.5	S	≤0.5	S
FOM	>16	R	>16	R
LZD	2	S	2	S

[a] Shading indicates antimicrobial agents whose susceptibility was altered by the loss of pETB.
[b] A/S, ampicillin-sulbactam; A/C, amoxicillin-clavulanic acid.
[c] MICs were determined by using the Microscan system panel of antibiotics (Siemens Healthcare Diagnostics, Tokyo, Japan). S, susceptible; R, resistant.

combination. A cadmium resistance element is also present in $pETB_{TY4}$ and was found at the extreme 5′ end of the extra DNA region with an inversion (Fig. 1B and C).

A homology search of the extra DNA region shows a ca. 16-kb extra DNA region in $pETB_{TY825}$ containing the aminoglycoside resistance element (Tn*4001*) and β-lactam resistant element showed nearly a perfect match with the sequence of pSA018A from a clinical coagulase-negative *Staphylococcus* sp. strain CDC 25 isolated from a human (Fig. 1B).

Antimicrobial susceptibilities of *S. aureus* TY825 in the presence or absence of pETB. To examine the functional activities of these resistance elements in $pETB_{TY825}$, we constructed a pETB-defective strain of TY825 (26), and compared its antimicrobial susceptibility profile to that of the wild type. We determined the MICs of several clinically relevant antibiotics using the broth microdilution method (Table 3). As expected, the wild type was resistant to benzylpenicillin (MIC ≥ 8 μg/ml), ampicillin (MIC ≥ 8 μg/ml), gentamicin (MIC ≥ 8 μg/ml), and erythromycin (MIC ≥ 4 μg/ml). Conversely, the pETB-defective strain TY825 showed significantly decreased MICs of gentamicin (MIC ≤ 1 μg/ml), arbekacin (MIC ≤ 1 μg/ml), erythromycin (MIC ≤ 0.25 μg/ml), benzylpenicillin (MIC ≤ 2 μg/ml), and ampicillin (MIC ≤ 2 μg/ml). TY825 was also resistant to fosfomycin (MIC ≥ 16 μg/ml); however, the deletion of $pETB_{TY825}$ did not alter the MIC of fosfomycin. These results clearly demonstrated that the resistance elements of $pETB_{TY825}$ were functionally active and conferred resistance to these antibiotics.

Antimicrobial susceptibility to EM and GM in clinically isolated ETB-producing *S. aureus* strains. For the treatment of impetigo/SSSS, GM is often used as an ointment, and a macrolide is one of the choices for empirical therapy. Additionally, ABK has frequently been used for the treatment of methicillin-resistant *S. aureus* (MRSA) in Japan since 1990, and *aac*(6′)/*aph*(2″) has been identified as one of the risk factors for ABK resistance in recent years (27, 28). Since the proportion of ETB-producing *S. aureus* causing impetigo/SSSS is significantly higher in Japan than in Western countries (29), we retrospectively examined the MICs of GM, ABK, and EM and genes for resistance to aminoglycosides [*aac*(6′)/*aph*(2′)] and macrolides (*msrA*) detected in $pETB_{TY825}$ by PCR (Table 1), using the purified plasmid fractions of 86 randomly selected ETB-producing clinical isolates (1977 to 2007) stored in our laboratory (Table 4). Of note, an increase in MRSA strains causing impetigo/SSSS has been reported in recent years (30). Therefore, *mecA* was also examined in the MRSA strains by using PCR.

ETB-producing *S. aureus* strains isolated in the 1970s and 1980s were largely susceptible to ABK, GM, and EM (Table 4). However, MICs of GM sharply changed after 1992, and ETB-producing *S. aureus* strains began to display high resistance to GM. This high resistance almost perfectly matched the detection of *aac*(6′)/*aph*(2″). Further, the detection of *aac*(6′)/*aph*(2″) paralleled the detection of *mecA*. Conversely, there was no significant change in the ABK MICs during the test period, with only a slight increase from 1 to 2 to 8 μg/ml after 1989. There was no correlation between ABK MIC and the presence or absence of *aac*(6′)/*aph*(2″). Resistance to EM was sporadically found in strains from the 1970s and 1980s. After 2001, strains resistant to EM significantly increased. Notably, however, *msrA* was rarely detected in ETB-producing *S. aureus* strains, and only five strains were positive for both *aac*(6′)/*aph*(2″) and *msrA* by PCR.

PCR scanning of ETB-producing *S. aureus* strains positive for *aac*(6′)/*aph*(2″) and *msrA*. Detection of both *aac*(6′)/*aph*(2″) and *msrA* suggests that these five strains (TY632, TY825, TY1020, TY1603, and TF3056) possess a TY825-type pETB. We therefore examined the genome organization of the 22-kb extra DNA region of the plasmids isolated from the four strains using the PCR scanning method. We generated seven pairs of primers whose PCR products cover all of the 22-kb extra DNA region. All pairs of primers yielded PCR products with the expected sizes in only one strain, TF3056, besides TY825 (Fig. 2). The other three strains were found to possess a DNA region containing macrolide and β-lactam resistance elements but lack the DNA region corresponding to the aminoglycoside resistance element.

DISCUSSION

In this study, we sequenced the pETB plasmid of the clinical isolate TY825, obtained in 2002 from a lesion of an impetigo patient. $pETB_{TY825}$ is significantly larger than the archetype $pETB_{TY4}$ and has a single extra DNA region (22,352 bp). Comparative analysis suggested that $pETB_{TY825}$ was generated from $pETB_{TY4}$ by acquiring a single 22-kb block of extra DNA. In a previous study, we reported that region D of $pETB_{TY4}$ is highly heterogeneous in size, based on PCR scanning analysis of plasmids from clinical isolates (13). However, the extra DNA region of $pETB_{TY825}$ was found to be inserted into the region corresponding to region E of $pETB_{TY4}$. A nearly perfect match of ca. 16 kb in the extra DNA region of $pETB_{TY825}$ with the partial sequence of a plasmid from a coagu-

TABLE 4 Antimicrobial susceptibility testing and PCR analysis of clinically isolated ETB-producing *S. aureus* strains

Strain	Yr	Diagnosis	MIC (μg/ml)			PCR result		
			ABK	GM	EM	*mecA*	*aac(6′)/aph(2″)*	*msrA*
TY468	1977	SSSS	1	1	0.125	−	−	−
TY469	1977	SSSS	1	1	0.125	−	−	−
TY470	1977	SSSS	2	1	0.125	−	−	−
TY471	1981	SSSS	1	1	0.125	−	−	−
TY472	1981	Impetigo	1	1	0.125	−	−	−
TY473	1982	SSSS	1	1	64	−	−	−
TY474	1982	SSSS	1	1	0.125	−	−	−
TY477	1978	Impetigo	0.5	1	2	−	−	−
TY478	1979	SSSS	1	1	32	−	−	−
TY479	1980	SSSS	1	1	1	−	−	−
TY480	1980	SSSS	1	0.5	>128	−	−	−
TY481	1980	SSSS	2	1	>128	−	−	−
TY482	1980	SSSS	2	1	1	−	−	−
TY484	1980	SSSS	1	2	0.125	−	−	−
TY485	1981	SSSS	1	2	0.125	−	−	−
TY487	1982	Impetigo	0.5	0.5	0.125	−	−	−
TY488	1982	Impetigo	>0.5	1	0.125	−	−	−
TY489	1982	SSSS	1	1	128	−	−	−
TY490	1982	SSSS	1	1	0.128	−	−	−
TY491	1983	SSSS	1	2	128	−	−	−
TY502	1983	Impetigo	1	2	2	−	−	−
TY507	1983	SSSS	2	4	0.25	−	−	−
TY519	1984	Impetigo	2	1	1	−	−	−
TY520	1984	Impetigo	2	4	0.125	−	−	−
TY522	1984	Impetigo	2	4	0.125	−	−	−
TY561	1987	SSSS	4	>128	0.125	−	+	−
TY564	1988	Impetigo	2	1	>128	−	−	−
TY565	1988	SSSS	1	1	>128	−	−	−
TY573	1989	Impetigo	4	4	0.125	−	−	−
TY576	1989	SSSS	4	16	0.125	−	−	−
TY4	1990	SSSS	2	32	>128	+	+	
TY580	1992	SSSS	4	>128	0.125	+	+	−
TY36	1999	Impetigo	8	>128	>128	+	+	−
TY49	1999	Impetigo	2	>128	2	+	+	−
TY54	1999	Impetigo	2	>128	0.125	+	+	−
TY56	1999	Impetigo	4	>128	0.125	+	+	−
TY64	1999	Impetigo	1	1	0.125	−	−	−
TY69	1999	Impetigo	4	>128	>128	+	+	−
TY93	1999	Impetigo	4	>128	>128	−	+	−
TY97	1999	Impetigo	8	>128	0.125	−	+	−
TY110	1999	Impetigo	4	>128	>128	+	+	−
TY119	2000	ND	32	>128	0.5	+	−	−
TY145	2000	ND	1	8	0.5	−	+	−
TY146	2000	ND	1	8	0.5	−	+	−
TY162	2000	Atopy	32	>128	0.25	−	+	−
TY174	2000	Atopy	8	>128	0.5	−	+	−
TY189	2001	SSSS	>128	>128	>128	+	+	−
TY213	2001	SSSS	>128	>128	>128	+	+	−
TY219	2001	SSSS	16	>128	0.25	+	+	−
TY226	2001	ND	8	16	>128	−	+	−
TY228	2001	Abscess	1	8	0.25	−	−	−
TY229	2001	SSSS	1	4	>128	−	−	−
TY632	2002	Impetigo	32	>128	2	−	+	+
TY825	2002	Impetigo	4	>128	16	−	+	+
TY1020	2002	Impetigo	4	>128	16	−	+	+
TY1603	2002	Impetigo	4	>128	32	−	+	+
TF2753	2005	Impetigo	16	>128	0.125	+	+	−
TF2754	2005	Impetigo	8	>128	>128	+	+	−
TF2778	2005	Impetigo	16	>128	1	−	+	−
TF2780	2005	Impetigo	4	>128	>128	−	+	−

(Continued on following page)

TABLE 4 (Continued)

Strain	Yr	Diagnosis	MIC (μg/ml)			PCR result		
			ABK	GM	EM	*mecA*	*aac(6′)/aph(2″)*	*msrA*
TF2791	2005	Impetigo	8	>128	>128	+	+	−
TF2799	2005	Impetigo	2	>128	0.125	+	+	−
TF2800	2005	Impetigo	8	>128	>128	+	+	−
TF2802	2005	Impetigo	16	>128	>128	+	+	−
TF2809	2005	Impetigo	8	>128	0.125	+	+	−
TF2815	2005	Impetigo	4	>128	>128	−	+	−
TF2816	2005	Impetigo	4	>128	2	−	+	−
TF2817	2005	Impetigo	4	>128	2	−	+	−
TF2818	2005	Impetigo	2	>128	>128	−	+	−
TF2825	2005	ND	2	>128	>128	+	+	−
TF2829	2005	Impetigo	4	>128	0.125	+	+	−
TF2846	2005	Impetigo	2	>128	>128	+	+	−
TF2848	2005	Impetigo	8	>128	0.125	−	+	−
TF2920	2005	Impetigo	64	>128	>128	−	+	−
TF2932	2005	Impetigo	>16	>128	0.125	−	+	−
TF2939	2005	Impetigo	>16	>128	>128	+	+	−
TF3056	2005	Impetigo	2	>128	8	−	+	+
TF3371	2006	SSSS	4	>128	128	+	+	−
TF3516	2007	ND	2	64	1	−	+	−
TF3520	2007	ND	2	32	128	−	+	−
TF3526	2007	ND	4	>128	128	+	+	−
TF3543	2007	ND	4	>128	128	+	+	−
TF3546	2007	ND	2	128	128	+	+	−
TF3563	2007	ND	2	>128	>128	+	+	−
TF3564	2007	ND	4	>128	>128	+	+	−
TF3571	2007	ND	1	>128	>128	+	+	−
TF3578	2007	ND	2	>128	>128	+	+	−
TF3583	2007	ND	1	>128	>128	+	+	−
TF3585	2007	ND	2	>128	>128	+	+	−
TF3586	2007	ND	1	2	0.125	−	−	−
TF3591	2007	ND	2	2	0.25	−	−	−
TF3598	2007	ND	8	>128	>128	+	+	−
TF3600	2007	ND	2	128	0.25	−	+	−
TF3602	2007	ND	8	>128	2	+	+	−
TF3612	2007	ND	8	>128	>128	+	+	−

[a] Boldface indicates strains that were selected for PCR scanning analysis. ND, no diagnosis data.

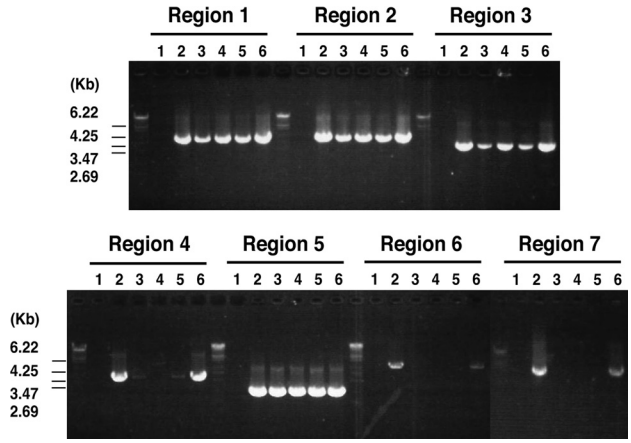

FIG 2 PCR scanning analysis of pETB plasmids. The gene organization of the acquired region in the $pETB_{TY825}$ plasmid was examined using PCR scanning analysis. Various combinations of the 14 primers that target the selected seven genes were used. A schematic view is shown in Fig. 1B. The results of the PCR analysis of regions 1 to 7 are shown. By comparing the length of each amplified fragment with that from pETB, the regional heterogeneity was determined. Results with pETB from the following strains are shown in the indicated lanes: 1, TY4; 2, TY825; 3, TY632; 4, TY1020; 5, TY1603; and 6, TF3056.

lase-negative staphylococcus (CNS) may imply that *S. aureus* acquired this region by horizontal transfer from resident CNS on the skin.

According to the PCR analysis for *aac(6′)/aph(2″)* and *msrA* and subsequent PCR scanning analysis of the pETB plasmid from the clinical isolates, the $pETB_{TY825}$ type was rare and found in only two strains, TY825 and TF3056. It should be noted that the frequency of strains positive for both *mecA* and *aac(6′)/aph(2″)* markedly increased after 1990. In recent studies, community-associated MRSA with type IVc SCC*mec* was shown to possess Tn*4001* in the J3 region (30–32). Tn*4001* is composed of two IS*256* elements flanking *aac(6′)/aph(2″)* and *orf28*. We therefore screened for SCC*mec* type IVc in the ETB-producing MRSA strains isolated after 1990. Only two strains (TF3371 and TF3571) among the all *mecA*-positive strains were typed as SCC*mec* type IVc, suggesting that SCC*mec* type IVc was rare among ETB-producing MRSA strains. Therefore, *aac(6′)/aph(2″)* in ETB-producing strains isolated after 1990 may be attributable to a plasmid other than pETB or a chromosome site other than SCC*mec*.

Antimicrobial susceptibility testing of TY825 and the pETB-defective strain indicated that *aac(6′)/aph(2″)* contributes to an

increase in MICs of GM/ABK, but the effect on the MIC of ABK was slight. Earlier studies reported that AAC(6′)/APH(2″) modifies both gentamicin and arbekacin (19), but ABK was later found to be a poor substrate of AAC(6′)/APH(2″) (33). Barada et al. suggested that the presence of *aph*(*3′*)*-III* in addition to *aac*(*6′*)/*aph*(*2″*) is required for full resistance to ABK (27). This might explain the lack of correlation between ABK MIC and the presence or absence of *aac*(*6′*)/*aph*(*2″*) in clinical isolates.

The *msrA* and *mef* genes display inducible resistance to erythromycin by encoding an ATP-dependent efflux pump (23, 34). Our data, however, clearly indicated that *msrA* was not principally responsible for the macrolide resistance in ETB-producing *S. aureus* strains. Nakaminami et al. reported that the gene products of *ermA*, *ermB*, and *ermC* were major macrolide resistance traits in *S. aureus* strains causing impetigo/SSSS (32). These three genes (*ermA*, *ermB*, and *ermC*) display resistance to macrolides by methylation of the ribosomal target site (30, 35). Those authors also demonstrated the presence of *msrA* at a low frequency in *S. aureus* strains causing impetigo/SSSS (32). Our data support their observations.

A previous study suggested that there is an association between the ET serotype and the clinical severity of staphylococcal blistering diseases (29). ETB-producing *S. aureus* is more frequently isolated from SSSS or the severe form of impetigo than ETA-producing *S. aureus*. For the treatment of SSSS, β-lactams were a primary choice together with an ointment of GM. However, in recent years, it has become evident that ETB-producing *S. aureus* in Japan is almost 100% resistant to GM and the proportion of resistance to β-lactam and EM is significantly higher than those isolated before 1989 (Table 4). Our study suggests that the emergence of an ETB plasmid carrying multiple resistance genes partly contributes to an increase in multiple resistance of ETB-producing *S. aureus*. Most impetigo/SSSS patients are young children and neonates, and SSSS patients, especially newborns, require admission and general treatment. But quinolone and tetracycline are not first choices for treatment, and available antimicrobials are limited in the current situation. Thus, special caution may be necessary for the treatment of SSSS/severe impetigo caused by ETB-producing *S. aureus* strains in Japan.

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