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Two highly divergent lineages of exfoliative toxin B-encoding plasmids revealed in impetigo strains of *Staphylococcus aureus*

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ABSTRACT

Exfoliative toxin B (ETB) encoded by some large plasmids plays a crucial role in epidermolytic diseases caused by *Staphylococcus aureus*. We have found as yet unknown types of *etb* gene-positive plasmids isolated from a set of impetigo strains implicated in outbreaks of pemphigus neonatorum in Czech maternity hospitals. Plasmids from the strains of clonal complex CC121 were related to archetypal plasmid $pETB_{TY4}$. Sharing a 33-kb core sequence including virulence genes for ETB, EDIN C, and lantibiotics, they were assigned to a stand-alone lineage, named $pETB_{TY4}$-based plasmids. Differing from each other in the content of variable DNA regions, they formed four sequence types. In addition to them, a novel unique plasmid pETB608 isolated from a strain of ST130 was described. Carrying conjugative cluster genes, as well as new variants of *etb* and *edinA* genes, pETB608 could be regarded as a source of a new lineage of ETB plasmids. We have designed a helpful detection assay, which facilitates the precise identification of the all described types of ETB plasmids.

1. Introduction

Exfoliative toxin B (ETB), similarly to exfoliative toxin A (ETA), is the active serine protease produced by some strains of *Staphylococcus aureus*. They are closely associated with toxin-mediated epidermolytic skin disorders in humans (Ladhani et al., 1999). While ETA is encoded on a prophage (Yamaguchi et al., 2000; Holochová et al., 2010; Botka et al., 2015), ETB is encoded on a large penicillinase-type plasmid (Yamaguchi et al., 2001). Both the ETs are characterized by splitting the target skin substrate, cadherin-like transmembrane glycoprotein desmoglein 1 (Amagai et al., 2000; Hanakawa et al., 2002). Cleavage of the desmosomal cadherins in the superficial layers of the skin is directly responsible for the clinical manifestation of the blistering skin disease pemphigus neonatorum and/or generalized staphylococcal scalded skin syndrome (SSSS) in neonates, and bullous impetigo in young children and adults (Ladhani et al., 1999).

Multiple reports have shown that ETs are produced approximately by 5% of *S. aureus* strains (reviewed in Bukowski et al., 2010). In a previous study, we have described a set of the 127 impetigo strains of *S. aureus* isolated in 23 maternity hospitals in the Czech Republic (Růžičková et al., 2012). ETB alone was produced by 4.4% of strains, and 17% of strains secreted both ETA and ETB. The *etb* gene was located on the 38–41-kb plasmids, hereinafter termed ETB plasmids, which were characterized only by restriction endonuclease analysis (REAP).

The early studies concerned with the ETB-producing strains described a large *etb* gene-positive plasmid pRW001 (Jackson and Iandolo, 1986), and the complete sequences of the $pETB_{TY4}$ (Yamaguchi et al., 2001) and $pETB_{TY825}$ (Hisatsune et al., 2013) have been determined. However, recent data on the structural diversity and evolutional course of ETB plasmids are not available.

In this study, we present a molecular characterization of 13 *etb* gene-positive clinical strains of *S. aureus* isolated in Czech maternity hospitals from 1999 to 2015. Their plasmids were analyzed and compared with the already published ETB plasmids to determine their similarity and elucidate their phylogeny and impact on the pathogenicity of the host bacteria. To differentiate between ETB plasmids, a helpful PCR method was designed.

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Table 1
Characteristics of 13 ETB-producing impetigo strains of *Staphylococcus aureus*.

Strain[a]	Hospital no.[b]	Clinical origin	Year of isolation	MLST	*spa* type	*agr* type	Plasmids		Prophages[c]	*etb* gene	*eta* gene	ETA-B-phage type	SAgs genes[d]
							No.	pETB type					
791*	9	Child, blister	2013	ST121	t159	IV	1	I	B, Fb	+	+	B5	*seg, sei*
445	2	Nurse, nose	2013	ST121	t159	IV	2	II	A, B, Fb	+	+	B1	*sei*
507	25	Child, blister	2010	ST121	t159	IV	1	III	Fb	+	−	−	*seg, sei*
586	9	Child, blister	2014	ST121	t169	IV	3	I	B, Fb	+	+	B5	*seg, sei*
013	26	Child, blister	2014	ST121	t169	IV	1	II	−	+	−	−	*sei*
459*	17	Child, blister	2003	ST121	t169	IV	1	III	Fb	+	−	−	*seg, sei*
049*	14	Child, SSSS	1999	ST121	t916	IV	1	II	A, B, Fb	+	+	B1	*seg, sea, sei*
797*	1	Child, blister	2008	ST121	t916	IV	1	II	A, B, Fb	+	+	B1	*seg, sei*
497	3	Child, SSSS	2011	ST121	t272	IV	1	II	−	+	−	−	*sei*
151	18	Child, blister	2008	ST121	t645	IV	1	II	Fb	+	−	−	*sei*
618	15	Child, nose	2015	ST123	t1688	IV	2	II	Fb	+	−	−	*seg, sei*
420	27	Child, blister	2013	ST2276	t876	IV	1	II	B, Fb	+	+	B1	*seg, sei*
608*	26	Child, blister	2011	ST130	t843	III	1	V	−	+	−	−	−

[a] Host strains of fully-sequenced ETB plasmids are marked with asterisks.
[b] Czech hospitals are numbered according to Růžičková et al. (2012).
[c] Prophage serogroups were classified according to Pantůček et al. (2004).
[d] Genes *seg* and *sei* were detected according to Banks et al. (2003), and Becker et al. (2003) respectively.

2. Materials and methods

2.1. Bacterial strains

Thirteen ETB-producing *S. aureus* strains were isolated from skin blisters of the Czech pediatric patients between 1999 and 2015. Two of them originated from skin lesions of pediatric patients diagnosed with SSSS and one from a nurse (Table 1). Seven of these strains and other fourteen ETB-producing collection strains, tested in the multiplex PCR assays of all ETB plasmid types, were previously published by Růžičková et al. (2012). The production of exfoliative toxin serotypes A and B was verified by using the Reverse Passive Latex Agglutination Kit (Denka Seiken for Unipath, Tokyo, Japan).

2.2. Isolation and restriction endonuclease profiling of plasmids

Plasmid DNA for whole genome sequencing and restriction analysis was isolated from bacterial cells by the High Pure Plasmid Isolation Kit (Roche Diagnostics, Germany) according to the manufacturer's protocol with minute modifications as described previously (Varga et al., 2012). The plasmid DNA was digested with EcoRI, HindIII, PstI, and XbaI restriction endonuclease (Roche Diagnostics, Germany) according to the manufacturer's protocol and electrophoresed as described previously (Doškař et al., 2000). Restriction endonuclease analysis of plasmids (REAP) was used for plasmid typing.

2.3. PCR genotyping

Bacterial DNA was isolated by the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) according to the manufacturer's protocol. Multilocus sequence typing (MLST) of *S. aureus* strains was carried out as described previously (Enright et al., 2000), *spa* typing was performed according to the Ridom StaphType standard protocol (www.ridom.org), and the Ridom StaphType software package (Ridom, Germany) was used for assignment. For the detection of *agr* type and staphylococcal genes *mecA*, *eta*, *etb*, *etd*, *sea*, *sej*, *see*, *sec*, *seh*, *seb*, *seg*, *sed*, *sei*, *tst*, and Panton Valentine leukocidin (PVL) genes *lukS-PV* and *lukF-PV*, the polymerase chain reactions were carried out as described previously (Jarraud et al., 2002; Murakami et al., 1991; Růžičková et al., 2005; Monday and Bohach, 1999; Banks et al., 2003; Becker et al., 2003 and Lina et al., 1999). Prophage serogroups and ETA-B types were identified as described previously (Pantůček et al., 2004; Holochová et al., 2010). PCR products for MLST and *spa* typing were sequenced by Eurofins MWG Operon (Germany).

2.4. Identification of ETB plasmids

To distinguish between $pETB_{TY4}$-based plasmids and pETB608, a novel multiplex PCR assay mapping plasmid sequence structure was designed. Primers targeting typical sites of the core sequence of $pETB_{TY4}$-based plasmids and the common part of the *etb* genes of both types were used. The cycling scheme was as follows: 95 °C for 10 min, followed by 30 cycles at 94 °C for 90 s, 58 °C for 70 s, 72 °C for 60 s, and finally 72 °C for 3 min. Another multiplex PCR assay was developed for classifying ETB plasmids into five pETB types. Primers, targeting a common part of the core sequence from one side and the variable DNA sequence from the other side, were designed to maintain the specificity to ETB plasmids. The cycling scheme was as follows: 95 °C for 10 min, followed by 30 cycles at 94 °C for 80 s, 58 °C for 50 s, 72 °C for 60 s, and finally 72 °C for 5 min. Both assays were verified using plasmid DNA as well as whole bacterial genome DNA. Primers used in both assays are shown in Table S1. The amplicon spectra are illustrated in Fig. S1.

2.5. DNA sequencing and analysis software

Sequencing libraries were prepared from 1 ng of plasmid DNA using the Nextera XT DNA Sample Preparation kit (Illumina, USA). Sequencing reactions were performed using the MiSeq Reagent Kit v3 in the MiSEQ system (Illumina). Raw sequencing reads were trimmed using the sliding window model in Trimmomatic (Version 0.32; Bolger et al., 2014) in order to remove low-quality reads. Trimmed paired-end reads were assembled by *de novo* assembler IDBA_UD (Version 1.1.1; Peng et al., 2012) with a modification to the default settings; k-mer sizes ranged from 20 to 120 with an increment of 10. Contigs were assembled using the CLC Genomics Workbench software (CLC, Inc., Denmark). Sequence gaps were closed using PCR and the corresponding designed primers. Amplified PCR products were purified using the QIAquick PCR Purification kit (Qiagen) and sequenced by Eurofins MWG Operon (Germany). The sequences have been deposited in the GenBank database under accession nos.: pETB049 (KY436021), pETB459 (KY436022), pETB608 (KY436023), pETB791 (KY436024), and pETB797 (KY436025).

Open reading frames were identified by GeneMark.hmm with Heuristic models (http://exon.gtech.edu) and annotated by BLAST (http://blast.ncbi.nlm.nih.gov), InterPro (http://www.ebi.ac.uk/interpro), Phobius (http://phobius.sbc.su.se), and IS Finder database (Siguier et al., 2006). BPROM (Solovyev and Salamov, 2011) was applied to promoter prediction. Only the promoters with an overall score above 3.5 and located in the close vicinity of ORF were