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The Type VII Secretion System of *Staphylococcus*

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Staphylococcus, protein secretion, T7SS, virulence, bacterial antagonism

Abstract

The type VII protein secretion system (T7SS) of *Staphylococcus aureus* is encoded at the *ess* locus. T7 substrate recognition and protein transport are mediated by EssC, a membrane-bound multidomain ATPase. Four EssC sequence variants have been identified across *S. aureus* strains, each accompanied by a specific suite of substrate proteins. The *ess* genes are upregulated during persistent infection, and the secretion system contributes to virulence in disease models. It also plays a key role in intraspecies competition, secreting nuclease and membrane-depolarizing toxins that inhibit the growth of strains lacking neutralizing immunity proteins. A genomic survey indicates that the T7SS is widely conserved across staphylococci and is encoded in clusters that contain diverse arrays of toxin and immunity genes. The presence of genomic islands encoding multiple immunity proteins in species such as *Staphylococcus warneri* that lack the T7SS points to a major role for the secretion system in bacterial antagonism.

Contents

1. INTRODUCTION

The production of exotoxins is a key strategy that bacterial pathogens employ during infection (54, 107). Gram-negative bacteria have evolved numerous mechanisms to transport virulence factors across their double-membraned cell envelopes. Some of these pathways secrete proteins across the cell envelope in a single step, while others are two-step, with separate translocases moving proteins across the inner and outer membranes (42, 44, 85, 99). The cell envelopes of gram-positive bacteria are generally considered simpler, presenting a single hydrophobic barrier. The inner membrane protein translocases found in gram-negative bacteria, Sec (general secretory pathway) and Tat (twin arginine translocation), directly secrete proteins in gram-positive bacteria (12, 63, 100, 129). Some gram-positive pathogens, in particular from the *Streptococcus* and *Staphylococcus* genera, have an additional accessory Sec pathway that operates in parallel to the canonical Sec pathway and is largely dedicated to the secretion of surface glycoproteins (18, 22). A third general pathway, termed the type VII secretion system (T7SS), is also found in many gram-positive bacteria. Here we review the T7SS of *Staphylococcus*.

2. THE TYPE VIIa AND TYPE VIIb SECRETION SYSTEMS

The T7SS was first described in mycobacteria (65, 106, 121). *Mycobacterium tuberculosis* and its close pathogenic relatives secrete two small proteins, ESAT-6 (6-kDa early secretory antigenic target) and CFP-10 (10-kDa culture filtrate protein), that are potent T cell antigens. These proteins, subsequently renamed EsxA and EsxB, respectively, lack cleavable N-terminal signal peptides that are found on substrates of Sec and Tat pathways, pointing toward a different secretion route. A cluster of genes was identified at the region of difference 1 (RD-1), which was deleted in the vaccine strain of *Mycobacterium bovis*. Mutational analysis of strains with an intact RD-1 showed that several genes encoded at this locus are required for the secretion of EsxA and EsxB. This system was subsequently named ESAT-6 system 1 (ESX-1) (24, 65, 106, 121).

Genomic inspection indicates that slow-growing mycobacteria generally encode multiple ESX secretion systems. For example, *M. tuberculosis* encodes 5 (26), along with at least 23 EsxA/EsxB

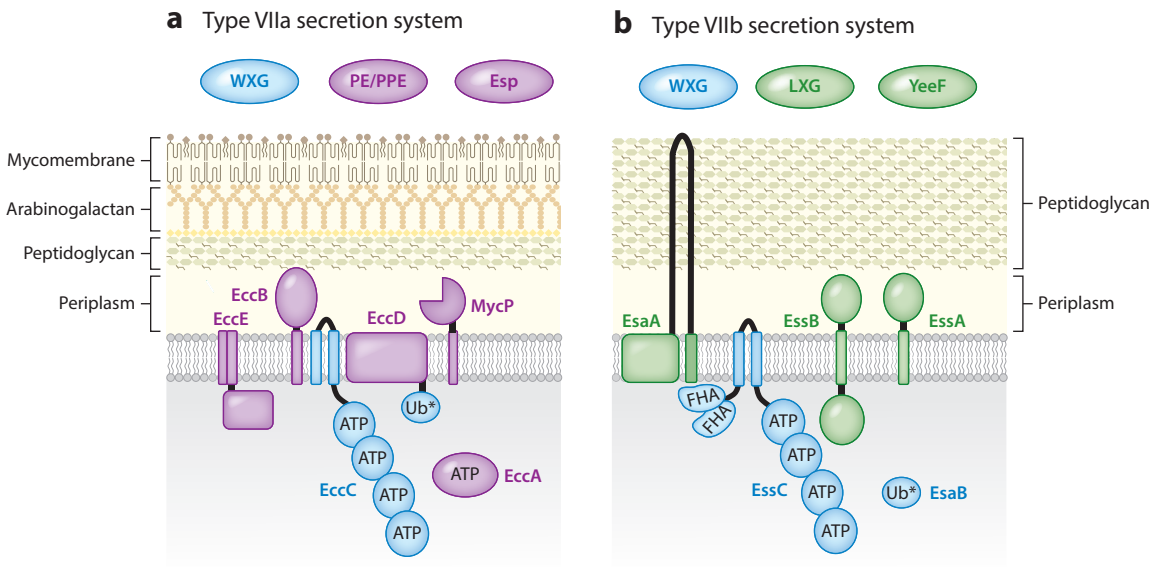


Figure 1

Schematic representation of the type VIIa (T7a) and T7b secretion systems. Components and substrates shared between the systems are blue. (*a*) The T7a system has been extensively characterized in mycobacteria but is also found in *Streptomyces* (5, 60) and other *Actinobacteria* (31). Components specific to T7a are purple. A cytoplasmic ATPase, EccA, is found in some but not all T7a systems. Its co-occurrence with EspG suggests it plays a role in dissociating this chaperone from substrate proteins prior to secretion (56). Substrates of the T7a system include proteins from the WXG100, PE/PPE, and Esp families. (*b*) The T7b system is found in bacteria of the *Firmicutes* phylum, including *Staphylococcus*, *Listeria*, and *Bacillus* (15, 30, 102, 143). EssC is related to EccC in sequence and structure, but it has two forkhead associated (FHA) domains at its N terminus that are not found in EccC (110, 127, 152). Components specific to the T7b system are green. The small cytosolic EsaB protein has a ubiquitin fold (Ub*) and is essential for T7b activity (80, 132). A similar domain is found at the C terminus of EccD in the T7a system (58, 104). Characterized substrates of the T7b system are from the WXG100, LXG, and YeeF protein families. Proteins are not drawn to scale.

paralogs [the exact number is strain dependent (24)]. The secretion machinery itself comprises the membrane proteins EccB, EccC, EccD, and EccE (16, 58, 64, 104), which assemble into a hexameric arrangement (16, 17, 27) (**Figure 1*a***). At the center of this complex is EccC, a multidomain ATPase of the FtsK/SpoIIIE family (16, 17, 27, 110). Multiple lines of evidence have shown that EccC recognizes substrate proteins through a C-terminal uncleaved signal sequence (28, 36, 48, 103, 110, 121, 139). A further essential component of the secretion machinery is mycosin (MycP), a subtilisin protease (43, 95, 119, 137). Surprisingly, the essentiality of MycP for ESX secretion is not linked to its protease activity; instead it seems to stabilize the assembled ESX complex (27, 95, 133).

Three families of T7 substrate proteins have been characterized in mycobacteria. The canonical substrates are EsxA/EsxB (136). They are members of the WXG100 family: small helical hairpin proteins of approximately 100 amino acids, with a conserved WXG motif situated at the hinge region (98, 109). These proteins are secreted as paired heterodimers, with the C-terminal signal sequence on the EsxB component (36, 48, 110, 139). The second family is the PE/PPE proteins (2, 3). This is a heavily expanded family in the slow-growing mycobacteria, forming up to 10% of the coding capacity of *M. tuberculosis* (13). Both proteins are extensively α-helical; the shorter PE partner forms a helical hairpin that dimerizes with a pair of long α helices in the PPE protein to form a four-helix bundle (122). A C-terminal secretion signal is present on the PE protein (48). PE/PPE complexes interact with a chaperone, EspG (39, 56, 82). This interaction stabilizes the complex and

also targets the PE/PPE pair to the cognate ESX secretion system (49, 82, 101). The third substrate family is the Esp proteins, which are substrates of the ESX-1 secretion system (53, 89). Structural analysis of EspB reveals that its N-terminal region forms a four-helix bundle that can be superimposed onto an EsxAB or PE/PPE dimer, suggesting EspB is secreted as a monomer (83, 120).

Mycobacteria and other relatives in the *Corynebacteriales* order have an unusual cell envelope that comprises, in addition to peptidoglycan, arabinogalactan and an outer mycomembrane containing mycolic acids (46). The mycomembrane to some extent mimics the outer membrane of gram-negative bacteria, and recently it was shown that an important function of PE/PPE proteins is in nutrient transport across this barrier (138). The complex envelope also acts as a barrier to secretion as well as import. The core ESX machinery resides in the inner membrane and does not span the envelope, so how substrate proteins reach the cell surface is unclear (16, 58, 110). Although numbering of protein secretion systems has generally been confined to those in true gram-negative bacteria, an argument was made that the ESX system should be named the T7SS (1). Despite some opposition (52), this nomenclature has now become established. Indeed, genomic analysis has revealed that components of the T7SS are encoded in some gram-negative bacteria, although they have yet to be functionally characterized (131).

Even before the T7SS had been described in mycobacteria, it was noted that homologs of ESAT-6/EsxA were encoded in the genomes of diverse gram-positive bacteria. Furthermore, these genes often clustered with a gene encoding a predicted AAA+ ATPase of the FtsK/SpoIIIE family (98). In commonly studied strains of *Staphylococcus aureus*, the EsxA homolog is encoded at a locus with a second WXG100 protein, EsxB. Burts et al. (30) showed that both of these proteins were secreted and that secretion was dependent on a functional FtsK/SpoIIIE ATPase, EssC. Further analysis of genes at the *esxA* cluster revealed that *esaA*, *esaB*, *essA*, and *essB* were all essential for EsxA secretion in addition to *essC* (30, 80) (**Figure 1*b***). Studies with *Bacillus subtilis* confirmed that these five genes were also required for the secretion of YukE, the only WXG100 protein encoded in that organism (15, 66). Given the limited similarity between the mycobacterial and *Firmicutes* T7SSs, they have been designated T7a and T7b, respectively (1).

Sequence comparisons indicate that apart from the ATPase EssC/EccC, there is little similarity between the membrane components mediating T7 secretion in the T7a system and those in the T7b system. Even the ATPases differ at their N termini; prior to the transmembrane segments, the T7b ATPase, EssC, has two forkhead associated (FHA) domains that are lacking in EccC (127, 152) (**Figure 1**). FHA domains have diverse functions, sometimes interacting with phosphothreonine-containing peptides (145), although residues required for recognition and binding of phosphothreonine are not conserved among EssC proteins (127, 152). A further difference can be found with the WXG100 proteins: While EsxAB heterodimers are seen for T7a, in the T7b systems, EsxA and EsxB (where present) instead form homodimers (109, 124, 125). Structural information is lacking on T7b complexes; there are structural details for the individual components EsaA (78, 114), EssB (128, 151, 153), and EssC (92, 110, 152), but little information about how these proteins interact with one another. Intriguingly, the extracellular domain of EsaA reveals an extremely elongated dimer capable of spanning the cell wall (78, 114), which may act to facilitate the transport of substrates to the bacterial surface. While homo-oligomeric interactions of EsaA, EssB, and EssC have been detected (7, 72, 91, 128), including hexamers of EssC (92, 152), complexes containing mixtures of these proteins have been purified only in small amounts (7, 91), and at present the overall architecture of the T7b system is not known.

Despite the differences in machinery components, it is likely that the mechanism of protein secretion is similar between T7a and T7b systems. At least some substrates of T7b have a C-terminal signal sequence similar to that found on T7a substrates (8, 125), and substrate recognition in T7b is also at the level of the EssC ATPase (10, 33, 71, 92). Experiments using irreversibly cross-linked

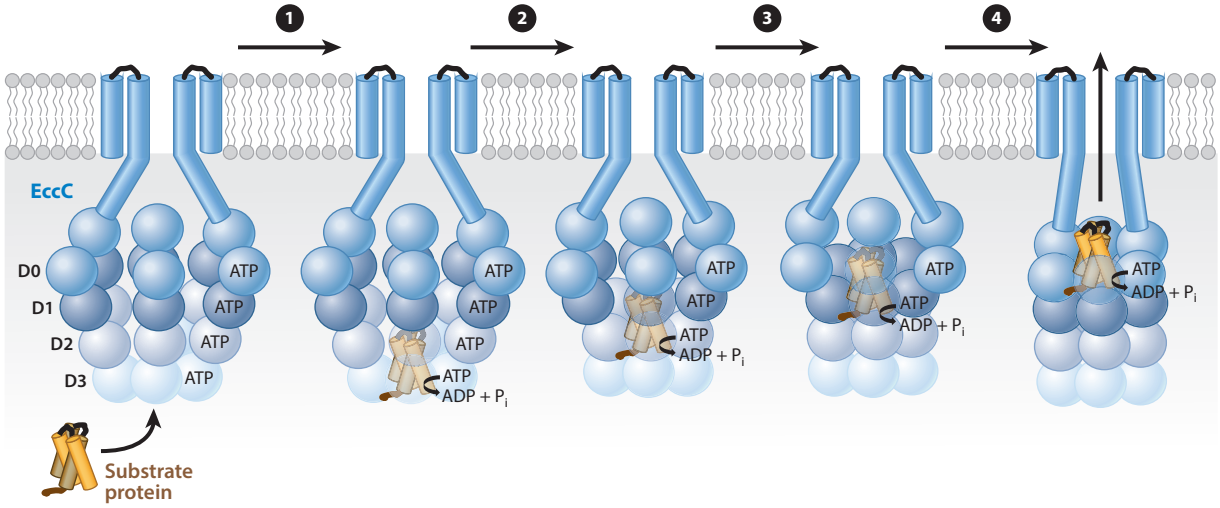


Figure 2

Model for protein secretion by the type VII secretion system (T7SS). For clarity, only EccC is shown. (❶) A substrate protein (shown as a WXG heterodimer) interacts with the most C-terminal ATPase (D3) of hexameric EccC. (❷) ATP hydrolysis at D3 causes a conformational change that liberates the signal sequence binding cleft of D2, which becomes occupied by the substrate. (❸) ATP hydrolysis at D2 results in a similar conformational change at D1 and substrate occupation of the liberated binding cleft. (❹) ATP hydrolysis at D1 passes the substrate to the D0 domain. ATP hydrolysis at D0 results in conformational changes at the linker domain and opening of the pore for passage of substrate across the membrane. Figure adapted from Reference 58. Abbreviation: P_i, inorganic phosphate.

forms of *B. subtilis* EsxA have shown that the protein is exported as a folded dimer, and it is probable that the heterodimeric EsxAB proteins from the T7a systems are also secreted as a complex (e.g., 48, 109). Structural analysis of an EccB/EccC/EccD/EccE protomer of mycobacterial ESX-3, and docking into the lower-resolution structure of the assembled ESX-5 complex, suggests that the translocation pore is formed primarily from the transmembrane helices of hexameric EccC, with an estimated pore size of 25 Å, sufficient to accommodate a pair of folded WXG100 proteins (58). This has recently been confirmed with reports of the high-resolution cryo–electron microscopy structure of hexameric ESX-5, where EccC was shown to form the central pore (17, 27). In the reported structure, transmembrane helix 2 of EccC occludes the pore and represents a closed state. However, the lower electron density associated with this helix is consistent with some conformational flexibility, which is presumably necessary for regulated opening of the secretion channel (17).

The EccC/EssC ATPase clearly lies at the heart of the secretion mechanism. The protein has an extensive C-terminal cytoplasmic region. It was initially anticipated by sequence analysis to have three P-loop ATPase domains, but structural studies have now revealed a fourth, which was originally assigned as a domain of unknown function (D0 in **Figure 2**) (17, 27, 58, 104). ATP binding and hydrolysis by each of these domains appear to be essential for secretion activity (58, 92, 108, 110, 152). X-ray crystallographic analysis of a fragment of EccC containing D1–D3 has shown that each of these domains has a surface cleft. For D1 and D2, the cleft is occupied by a linker from the neighboring domain, which allosterically regulates ATPase activity (110). D3, which is at the very end of the C terminus of the protein, has an unoccupied cleft that interacts with the C-terminal signal sequence of a WXG100 substrate protein (110, 139). Experiments in *S. aureus* have also implicated D3 in substrate recognition (71), but they have shown that in vitro, D2 can also bind a substrate protein (92). Taken together, these data suggest a model whereby

substrate binding to D3 modulates the conformation and catalytic activity of the ATPase domains, freeing up the substrate binding pockets on D2 and D1. Sequential interaction of a substrate with D2 and D1 would funnel it toward the translocation pore (**Figure 2**). A stalk domain that bridges transmembrane helix 2 and ATPase domain D0 contacts the N-terminal helical region of EccB and could link conformational changes in EccC to pore opening (58) (**Figure 2**). The analogous partner to EccB in the T7b system is not clear, but it could be EssB (which displays a similar topology to EccB, although it has a much larger cytoplasmic domain) (**Figure 1**).

The recent structural description of assembled T7a complexes has paved the way for a detailed understanding of protein secretion by this pathway (26), but several open questions remain. For example, there is growing evidence that the secretion signal is bipartite, with the positioning and sequence of the WXG motif (or variant thereof) important for substrate recognition (e.g., 23, 120, 125). At present, it is unclear how this is recognized and whether it is critical for protein secretion. Likewise, all characterized substrates have two α helices that flank the WXG motif. Structural predictions indicate that an N-terminal helical hairpin is a common substrate feature, but it is not clear why this should be a necessary structural feature for transport by this pathway. Are all substrates of this pathway transported in a folded state? There is good evidence that the small helical hairpin complexes of EsxA/EsxB are exported as folded dimers, but some substrates are much larger and have C-terminal globular domains (e.g., 146) that are larger than the estimated pore size of the secretion pathway. Do these globular domains unfold, and if so how? If not, does the transport channel open up further to allow the passage of larger folded proteins? And perhaps most pressing, just how structurally (and mechanistically) similar are the T7a and T7b systems?

3. T7SS DIVERSITY IN *STAPHYLOCOCCUS*

The T7SS of *S. aureus* is encoded at the *ess* locus (**Figure 3*a***). The 5′ end of the gene cluster harbors genes that encode the highly conserved core components of the T7 secretion machinery: *esxA*, *esaA*, *essA*, *esaB*, and *essB*. Most of the *essC* gene is also highly conserved across *S. aureus* strains, but there is significant variability toward the 3′ end of *essC* and in the genes found downstream, which is discussed further in Section 4.

Genetic regulation of the *ess* locus is mediated through the alternative sigma factor, SigB, in a complex and indirect manner (115). Transcription of *esxA* is activated by the two-component system ArlRS, probably through the global regulator MgrA, and by the DNA-binding protein SpoVG (45, 115). Transcription of both *arlR* and *spoVG* is under positive control by SigB (74, 90). The Agr quorum-sensing system also positively regulates *esxA* (55, 115), whereas the DNA-binding protein SarA represses *esxA* expression (115). Unlike *arlR* and *spoVG*, *sarA* is negatively regulated by SigB, meaning that the sigma factor has intricate control of T7 gene expression (19). Outside of the SigB circuit, the SaeRS two-component system, a major factor in the regulation of exoprotein production, also regulates production of the T7 components (8, 73). Clinical and laboratory strains frequently have mutations in these regulatory pathways (e.g., 75, 118). For example, strains Newman, RN6390/NCTC8325–4, and COL, which are commonly used for the study of T7 secretion, have mutations in the SaeRS, SigB, and Agr pathways, respectively (4, 61, 94). This leads to variable levels of T7 protein production across different strains (8, 35, 80).

A longitudinal study of an isogenic pair of *S. aureus* isolates from the lungs of a cystic fibrosis patient followed over 13 years showed that the *ess* genes were highly upregulated (approximately tenfold) over this time (148). They are also upregulated during persistent colonization in a mouse vaginal model (51). Other studies have shown that fatty acids present in pulmonary surfactant and serum induce expression of T7 genes (69, 88). The active component of serum is *cis*–unsaturated fatty acids that require further bacterial metabolism through fatty acid kinase to exert their

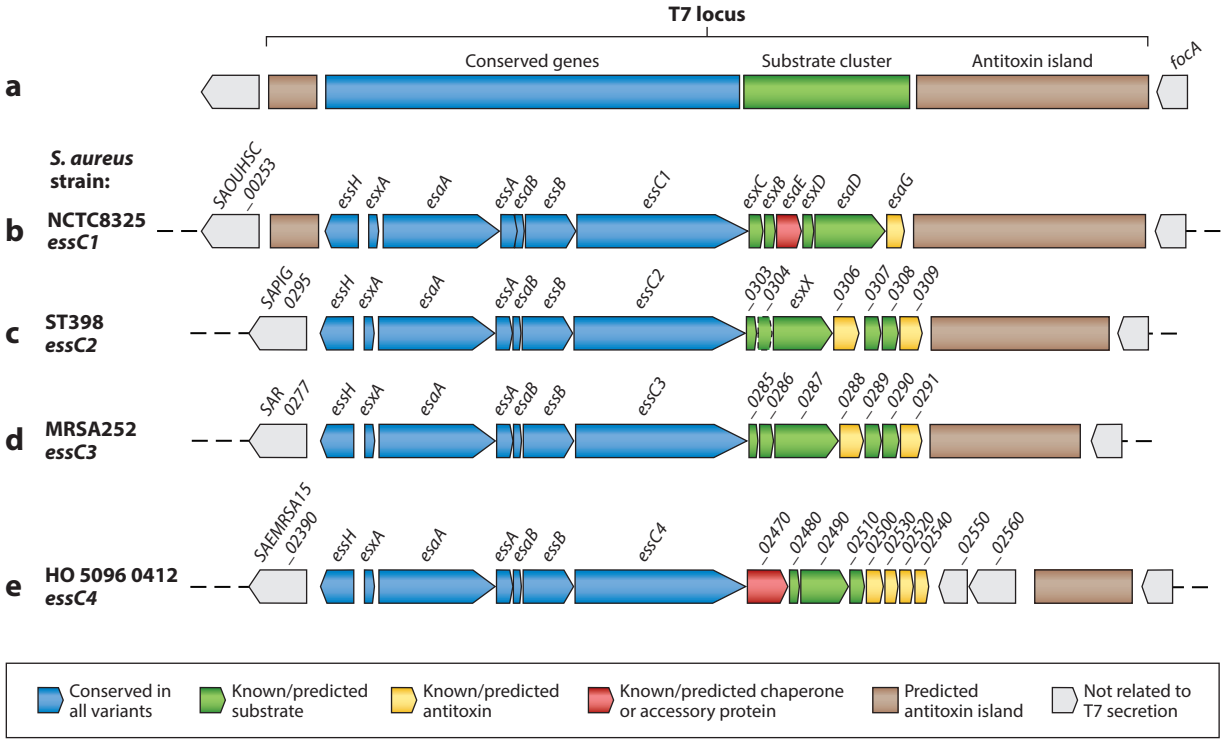


Figure 3

The diverse genetic organization of the *Staphylococcus aureus* type VII (T7) secretion system locus. (*a*) Schematic of the T7 locus. The locus is flanked by genes encoding a hypothetical protein and the formate/nitrite transporter FocA. (*b–e*) The T7 loci of selected strains representing *essC1*, *essC2*, *essC3*, and *essC4* variants. Genomic neighborhood comparisons were generated using FlaGs.py (113).

regulatory effect. At least part of this effect was linked to decreased membrane fluidity resulting from *cis*–unsaturated fatty acid incorporation (88). The fatty acid–mediated induction of *ess* gene expression was independent of ArlRS, SaeRS, and Agr (69, 88), but in the case of pulmonary surfactant–derived fatty acids it still required SigB (69).

Despite the extensive regulation mediated by SigB, expression of *esxA* is controlled solely through a SigA-dependent promoter, mapped to 74 base pairs upstream of the start codon of *esxA* (80, 115). Initial work found that *esxA* is monocistronic in strain Newman and not cotranscribed with the other *ess* genes. A putative Rho-independent terminator is located just after the *esxA* stop codon (115). Kneuper et al. (80) extended these observations by examining transcriptional organization of the *ess* cluster in several strains within the same clonal complex. Surprisingly, even though there is 100% sequence identity across the *esxA* region in these strains, *esxA* was found to be monocistronic in RN6390 and Newman, but coexpressed with the downstream *ess* genes in COL, USA300, and SA113. A transcriptional start site was mapped within the *esxA–esaA* intergenic region of RN6390 that is presumably also present in Newman, but it was not found in USA300 (80). Although the terminator sequence was present for all of the strains studied, there must be some readthrough in strains where an *esaA* promoter was not detected. Given the sequence conservation at this region, it is not clear why the *esaA* promoter should be cryptic in some strains, nor how termination readthrough is mediated. Studies have shown that *esxA* is very highly expressed relative to the other *ess* genes, with *esxA* transcript abundance approximately 100 times higher than that of *essC* across multiple strains (80, 141).

A further gene, expressed divergently from *esxA* on the opposite strand, is also conserved across staphylococci and encodes EssH, a nonlytic peptidoglycan hydrolase (20) (**Figure 3*a***). EssH is essential for secretion of the T7 substrates EsxA and EsxC in USA300 (20). It should be noted that outside of the staphylococci, homologs of EssH are not encoded at the T7 clusters, or indeed elsewhere on the chromosome. However, the cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain found within EssH is present in a number of proteins encoded by diverse gram-positive bacteria. At present it is unclear whether any of these could substitute for the role of EssH in these organisms.

Several open questions remain regarding the regulation of T7 gene expression. Apart from SigA, it is not clear which transcription factors directly interact with the *esxA* promoter region, nor how the expression of *essH* is controlled. It is likely, however, that there is additional regulatory complexity at this locus. For example, it was recently reported that the small noncoding RNA RsaI interacts with *esxA* mRNA and upregulates its expression and/or stabilizes its mRNA. RsaI expression is controlled by cellular glucose levels and appears to link gene expression with carbon source availability (25). It will be interesting to see how these factors interlink to fine-tune control of the T7 secretion machinery.

4. T7SS VARIABILITY IN *S. AUREUS*

4.1. The *essC1* Variant Cluster

Comparative genomic analysis revealed that there is extensive variability at the 3′ region of the *ess* locus (141). Sequence divergence starts approximately three-quarters of the way through *essC*, resulting in four EssC variants across *S. aureus* strains (**Figure 3**). Each *essC* variant is associated with its own suite of genes directly downstream. The variant found most commonly is termed *essC1* (**Figure 3*b***) and is the most extensively studied to date, with strains Newman, USA300, and COL being among those that possess the *essC1* arrangement (141). To the immediate 3′ of *essC1* are a number of genes known to encode T7 substrates: *esxC*, *esxB*, *esxD*, and *esaD* (9, 29, 30, 33, 80, 96). EsxB, like EsxA, is a protein of the WXG100 family. However, unlike the case in mycobacteria, where EsxA and EsxB heterodimerize, the two *S. aureus* proteins do not interact with one another (124). Moreover, *esxB* mRNA is at least a hundredfold less abundant than *esxA*, indicating that these two proteins are probably present at very different stoichiometries (80, 141). Instead, EsxB was shown to interact with EsxD, another small substrate protein, suggesting that they may be exported as a pair. Like EsxD, EsxC is a small secreted protein distinct from the variant 1 T7SS. EsxC and EsxD are not classified as members of the WXG100 protein family, but structural predictions indicate that both are predominantly α-helical, similar to other characterized T7SS substrates. The biological functions of EsxB, EsxC, and EsxD are yet to be fully elucidated.

EsaD (also called EssD), at 614 amino acids in length, is the largest T7 substrate identified in *S. aureus*. It is encoded only in strains that have the *essC1* variant (141). The N-terminal half of the protein encompasses a YeeF domain; this domain is predicted to be extensively helical and is found on related proteins such as *B. subtilis* YeeF and candidate T7 substrates in *Listeria monocytogenes* (21, 76). The C-terminal approximately 150 amino acids of EsaD share an endonuclease fold and have DNase activity (33, 96). EsaD is highly toxic and producing strains are protected through coproduction of an antitoxin, EsaG, which interacts specifically with the nuclease domain (33, 96). EsaE, also encoded at the *ess* locus in variant 1 strains, interacts with the N-terminal domain of EsaD (10, 33). EsaE appears to act as a chaperone because it stabilizes the full-length form of EsaD; it also interacts with EssC1 and therefore serves to target the EsaDG complex to the secretion machinery (10, 33). In this respect it is analogous to the EspG chaperone found in some of the T7a

systems, although the two proteins do not share any significant sequence similarity. Interestingly, pull-down experiments have shown that EsaE also interacts with EsxC, but not with EsxB or EsxD (10). This raises the possibility that, like the situation in mycobacteria, some substrates are targeted to the EssC1 secretion system through interaction with a chaperone, whereas others are chaperone independent.

4.2. The *essC2* and *essC3* Variant Clusters

EssC1 is the most dissimilar of the four *S. aureus* EssC variants, diverging from the other three ATPase sequences partway through the D2 ATPase domain. The EssC2, EssC3, and EssC4 variants share 100% sequence identity throughout D2 but differ in sequence in the final ATPase domain. Furthermore, none of these other strain variants encode an EsaE-like chaperone anywhere in the genome. The *essC2* and *essC3* clusters are most similar to one another (141), and several genes immediately downstream of *essC* in these clusters also share limited sequence homology (between 30% and 50% identity; **Figure 3*c*,*d***). The first two genes encode small proteins that are predicted to be predominantly α-helical. SAPIG0303/SAR0285 are members of the DUF5344 protein superfamily. Modeling suggests they are likely to have the same overall fold as EsxA and EsxB, although they lack the WXG motif. SAPIG0304 and SAR0286 fall into the DUF5082 family. Nothing is known about these proteins, but given their small size and predicted helical structure, they are strong candidates for T7 substrate proteins.

SAPIG0305 and SAR0287 are larger proteins that have an LXG domain at their N terminus, marking them as T7 substrates. SAPIG0305, also named EsxX, has been investigated in strain ST398 and shown to be secreted by the T7SS, although its precise biological function remains obscure (47). SAPIG0306 and SAR0288 have no detectable sequence similarity but are both members of the DUF5079 family and are highly hydrophobic, with six predicted transmembrane segments. All LXG proteins characterized to date are toxins and are coproduced with a cognate antitoxin that is usually encoded immediately downstream (79, 130, 146, 149). It is therefore likely that SAPIG0306 and SAR0288 are antitoxins for the LXG proteins to which they are adjacently encoded. A close homolog of SAPIG0306, SAPIG0312, with which it shares 90% sequence identity, is also encoded at the *ess* locus in strain ST398 (141).

The SAPIG0307 and SAPIG0308 proteins, like SAR0289 and SAR0290, are members of the DUF5085 family. This family comprises small proteins, less than 150 amino acids in length, about which nothing is currently known. Structural prediction suggests they are unrelated to other small T7SS substrate proteins and have mixed α-helical/β-sheet secondary structure. While SAPIG0307 and SAR0289 share 50% sequence identity, the similarity between SAPIG0308 and SAR0290 is lower (28%), and neither SAPIG0307 and SAPIG0308 nor SAR0289 and SAR0290 have significant similarity with one another. It is not clear whether they are secreted substrates of the T7SS, but if they are, they would constitute a new exoprotein family.

4.3. The *essC4* Variant Cluster

EssC4 is the least common of the four EssC types, being found in only approximately 5% of sequenced *S. aureus* strains (140, 137). As with *essC2* and *essC3*, there is no WXG100-family protein encoded downstream of *essC4* (**Figure 3*e***). However, both SAEMRSA15_02480 and SAEMRSA15_02500 are small all-helical proteins that can be structurally modelled on EsxA, suggesting that they are likely substrates of the EssC4 secretion system. The encoding genes flank *SAEMRSA15_02490*, which codes for an LXG protein and is therefore also a probable EssC4 substrate. Of the other genes in the *essC4* 3′ region, *SAEMRSA15_02470* encodes a 359–amino acid protein that is predicted to have mixed α-helical/β-sheet secondary structure and is likely

cytoplasmic, whereas *SAEMRSA15_02510*, *SAEMRSA15_02530*, and *SAEMRSA15_02540* encode small polytopic membrane proteins from unrelated families. The other gene in this region, *SAEMRSA15_02520*, is a predicted lipoprotein with a cystatin-like fold that presumably has an extracellular function.

5. T7 SUBSTRATE PROTEINS ENCODED OUTSIDE OF THE *ess* GENE CLUSTER

All strains of *S. aureus* encode a conserved LXG family protein outside the *ess* locus (**Figure 4*a***). This protein, named TspA, is exported by the T7SS and remains surface attached, with a C-terminal toxin domain exposed close to the cell surface. Immediately downstream of *tspA* is *tsaI*, encoding a membrane-bound antitoxin of the DUF443 protein family (130). Unlike most other LXG proteins, no WXG100-family protein is encoded in the vicinity of *tspA*.

A conserved locus encoding a protein of the DUF5344 family is also present across *S. aureus* strains (SAOUHSC_02907; **Figure 4*b***). This DUF family is also annotated as

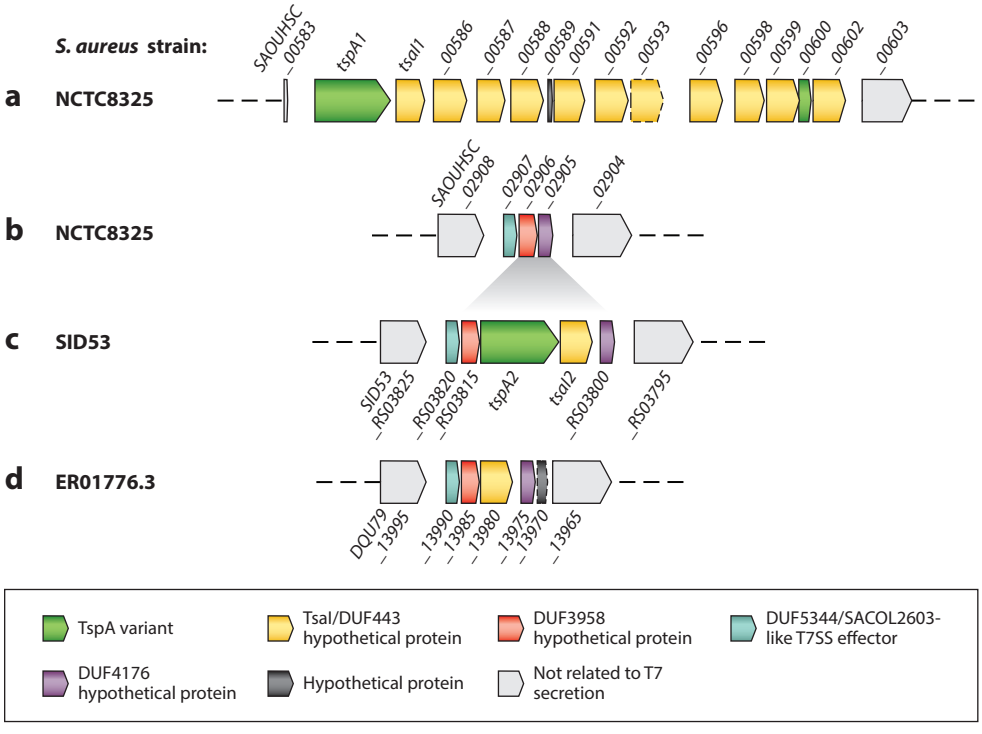


Figure 4

Predicted type VII secretion system (T7SS) substrates encoded outside of the *ess* locus. (*a*) TspA, a membrane-depolarizing toxin, is encoded in a gene cluster with a variable number of genes encoding DUF443 proteins and is found in all *Staphylococcus aureus* strains. The DUF443 protein encoded immediately downstream of *tspA* has been renamed TsaI and is a TspA immunity protein (130). Toward the end of the cluster is gene *SAOUHSC_00600*, which encodes the TspA C-terminal region lacking the LXG domain. (*b*) The locus encoding the DUF5344-family protein is found in all *S. aureus* strains. Some *S. aureus* strains possess an insertion at this locus encoding either (*c*) a TspA/TsaI pair or (*d*) an orphan DUF443 protein. Pseudogenes are shown with dashed outlines. Genomic neighborhood comparisons were generated using FlaGs.py (113).

TIGR04197:T7SS_SACOL2603, and both SAPIG0303 and SAR0285 (encoded at the *ess* locus in variant 2 and variant 3 strains, respectively) are family members. TIGR04197:T7SS_SACOL2603 has been linked to the T7SS because these proteins are restricted to species that encode a T7SS, and they share remote sequence similarity to WXG100 proteins. Also encoded at this locus are two small cytoplasmic proteins, SAOUHSC_02906 and SAOUHSC_02905. SAOUHSC_02906 is a member of the DUF3958 family, about which nothing is known. Structural predictions, however, suggest that it is largely α-helical, so it could potentially represent a novel T7SS substrate. SAOUHSC_02905 is a DUF4176 protein and predicted to have a primarily β-sheet structure. Intriguingly, in a few *S. aureus* strains there is an insertion in this region, between the DUF3958- and DUF4176-encoding genes (**Figure 4*c*,*d***). In some strains, this insertion introduces two genes: One encodes a TspA homolog, TspA2, sharing 48% identity with the canonical TspA from RN6390, and the second encodes a DUF443-family protein related to TsaI (**Figure 4*c***). A few other *S. aureus* strains lack *tspA2* but carry the DUF443-encoding gene (**Figure 4*d***).

6. T7SS ACTIVITY IN *S. AUREUS*

It has been noted that there is mutually dependent substrate secretion in the mycobacterial T7SS, where several substrates are contingent on one another for export (e.g., 59). At present, it is unclear whether the secretion of different T7SS substrates is coordinated in *S. aureus*. It has been reported that EsxB, EsxD, and EsaD are each essential for the secretion of EsxA and EsxC in USA300 (8), but this is not the case in RN6390 (33, 80). Whitney et al. (146) demonstrated that LXG proteins in *Streptococcus intermedius* have a cognate WXG100 protein partner that interacts specifically with the LXG domain and mediates export of the toxin. Not all LXG proteins in *S. aureus* are encoded with candidate WXG protein partners, although in most cases they are found in gene clusters that also encode small proteins with related folds. It is feasible that some of these act as LXG partner proteins.

EssC is clearly a main specificity determinant for substrate recognition. It was shown that secretion of EsxC is supported only by EssC1, and not by EssC2, EssC3, or EssC4 (71). This is in accord with EsxC being encoded only in clusters alongside *essC1*, and it implies that the EssC variable region mediates substrate selectivity. Although EsxA is a secreted substrate of the T7SS, it may also be considered a structural/functional component of the secretion system. Deletion of *esxA* abolishes secretion of other substrates, and overproduction of the EsaD substrate also results in much enhanced EsxA secretion (33). Interestingly, the EsxA sequence is almost invariant across *S. aureus* strains, and it is secreted by all four EssC subtypes (71). By implication, it would be expected that EsxA should interact with an invariant region of EssC (or potentially another T7SS component). The exact role of EsxA in T7 secretion is not known, but it may be involved in facilitating the active assembly of the machinery.

T7 secretion activity is relatively inefficient for a number of *S. aureus* strains when they are cultured in laboratory growth media, and it varies considerably between *S. aureus* strains even within the same clonal complex. For example, production and secretion of EsxA in RN6390 and COL were observed at all cell densities, whereas the onset of EsxA secretion was delayed in USA300 and SA113. Production of EsxA in Newman was detected only at late exponential/stationary phase (80). Furthermore, in all strains examined, the vast majority of EsxA and EsxC was found in the cellular fraction, with supernatants requiring extensive concentration to detect these proteins in the secretome (35, 80).

Cross-linking and blue native PAGE analysis of solubilized membrane fractions have indicated that in RN6390, the T7 membrane proteins do not form detectable heteromeric complexes (72). This ties in with the low secretion efficiency and may suggest that assembly of the active T7SS is transient, or that it requires some form of posttranslational modification. The membrane

chaperone flotillin has been implicated in promoting assembly of the *S. aureus* T7SS (91), and supplementation of the growth media with hemin also causes T7 hypersecretion in some strains (35). A further candidate for the posttranslational regulation of T7 secretion is EsaB. This small cytoplasmic protein, which is essential for T7 activity, has a ubiquitin fold but lacks the ability to be conjugated to other proteins (15, 34, 66, 80, 132). Of note, a domain with the same fold is found at the C terminus of the polytopic EccD component of the T7a system, where it acts as a scaffold for the cytoplasmic domains of the other T7a membrane proteins (58). It could plausibly play a similar role in the T7b system.

7. THE STAPHYLOCOCCAL T7SS HAS ROLES IN VIRULENCE AND INTERBACTERIAL COMPETITION

7.1. The T7SS Contributes to *S. aureus* Pathogenesis

S. aureus produces a plethora of secreted toxins and virulence factors (116, 126), and initial characterization of the T7SS focused on establishing whether the secretion system was required for pathogenicity (29, 30). Inoculation of *S. aureus* into the bloodstream of mice seeds abscess formation in the peripheral tissues such as those of the kidney and liver (40). Mutant strains of Newman lacking the core components *esxA*, *esaB*, and *essC* had a reduced ability to form abscesses in this infection model, and it was concluded that the T7SS supports bacterial persistence (29, 30). A similar reduction in abscess formation was noted when any of the substrate-encoding genes *esxB*, *esxC*, and *esaD* was deleted in the Newman background (9, 29, 30).

These findings have been supported by studies using strain USA300, where a reduction in abscess numbers was observed for an *esaE* (*essE*) mutant. Further experiments measuring cytokine levels in the bloodstream of infected mice showed that production of interleukin-12, an immune modulator, was stimulated upon infection with USA300, but not to the same extent if *esaD* (*essD*) or *esaE* (*essE*) was deleted (10, 96). An *essB* mutant of strain ST398 also yielded smaller abscesses in a murine skin infection model and fewer kidney abscesses in a bacteremia model (10, 140). Deletion of *esxX*, which encodes a T7 substrate of ST398, appeared to mirror loss of *essB*, suggesting that EsxX may be required for abscess persistence in EssC2 strains (47).

Neutrophils are key players in the clearance of *S. aureus* infections (50). Histology of skin abscesses formed during infection with ST398 showed that there was increased neutrophil infiltration when the T7SS was functional. During in vitro experiments, researchers noted reduced neutrophil lysis by strains with *essB* or *esxX* deleted, accompanied by reduced bacterial survival of the mutant strains compared with wild-type ST398 (47, 140). Experiments using a lung epithelial line have shown that USA300, an EssC1 variant, can be readily internalized. Although no significant difference in internalization was observed when either *esxA* or *esxB* was deleted, an increase in apoptosis was seen when cells were infected with the *esxA* mutant strain, suggesting that EsxA or another substrate of the variant 1 T7SS acts as an antiapoptotic factor (81).

A further virulence model animal that is increasingly used for studies of staphylococcal infection is the zebrafish (e.g., 62, 105). The larval stage lacks adaptive immunity and therefore provides a system to investigate interaction with the innate immune system. T7 mutants of RN6390 and COL showed a reduction in virulence in this model and were more rapidly cleared by the larvae. A mutant of RN6390 lacking *esaD* was as virulent as the wild type, whereas a strain with *tspA* deleted was partially reduced in virulence and had increased clearance in vivo (130). Substantial recruitment of both neutrophils and macrophages to the site of infection was observed, but this was not significantly different between the wild type and any of the T7 mutant strains. Likewise, the larval proinflammatory markers interleukin-8 and interleukin-1β were induced during infection by the T7 mutant strains and the wild type to a similar extent (130).

Taken together, these findings make it clear that strains lacking a functional T7SS have a defect in virulence in murine and zebrafish models of infection, and some strains are also impaired in intracellular survival. However, the underlying mechanism behind these observations is not fully apparent. The interdependence of substrate secretion and stability in some strains is an added complication, as is the pleiotropy seen with mutations in T7 structural genes, which lead to altered expression of many other genes, including those for cell surface components (34, 35). Further studies are required to dissect the precise roles of the T7SS and its secreted substrates in pathogenesis.

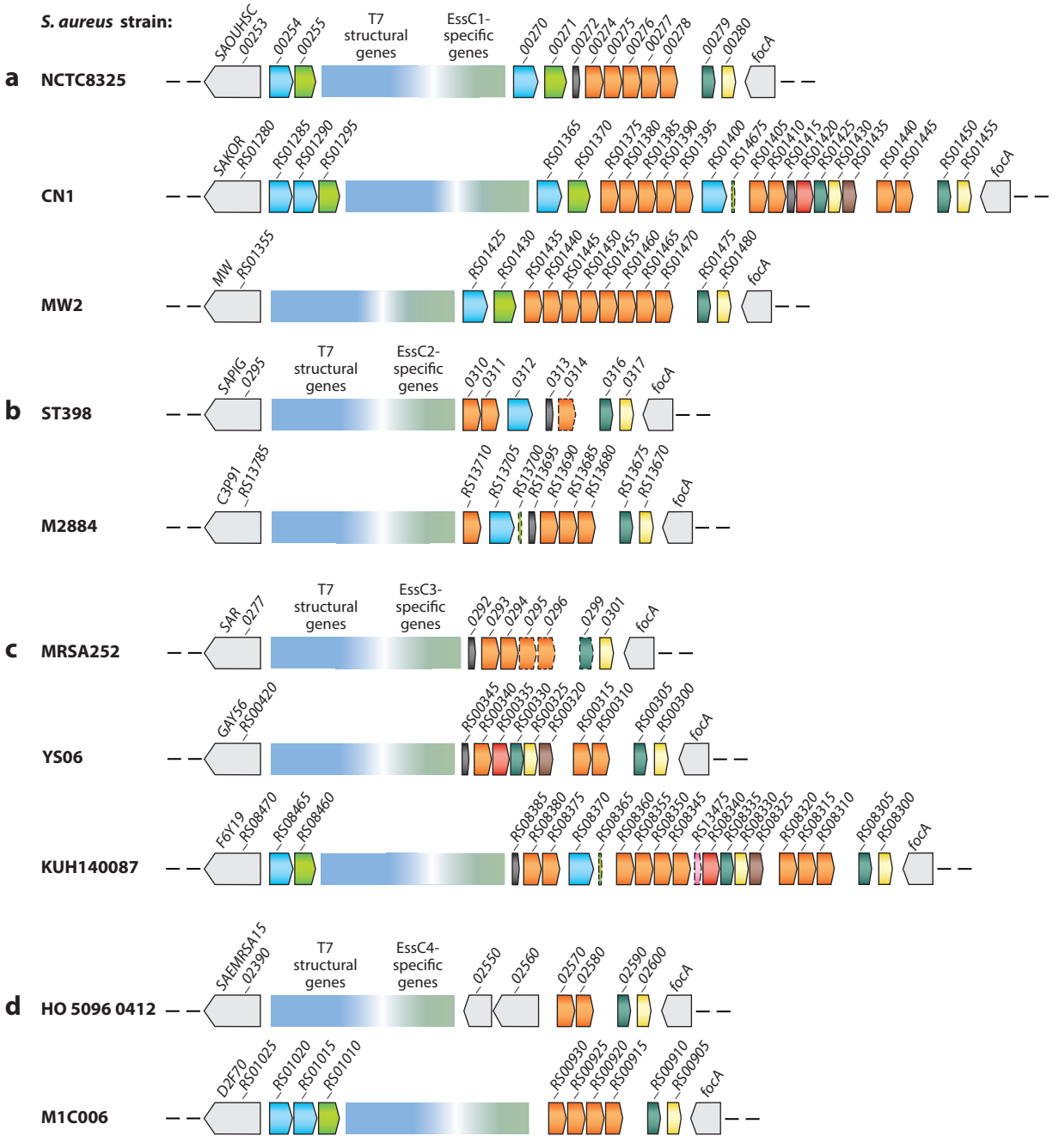
7.2. The T7SS Likely Plays a Major Role in Intraspecies Competition

S. aureus is a frequent colonizer of the nasal cavity, and approximately 30% of humans are carriers (86). This body site is a competitive environment, and numerous mechanisms among the microbiota modulate *S. aureus* colonization (70, 84, 97, 117, 150). *S. aureus* is also an earlier colonizer of the lungs of children with cystic fibrosis, where it generally persists until adulthood (68). It is therefore likely that *S. aureus* employs competitive mechanisms to colonize such polymicrobial niches, and the T7SS may be an important factor in this process. Indeed, the T7SS is conditionally essential for *S. aureus* survival during polymicrobial infection (67).

The first indication that the T7SS is associated with antibacterial activity came from studies on EsaD. This nuclease substrate is encoded at the T7SS locus of *essC1* strains, where it is coproduced with an antitoxin, EsaG, a member of the DUF600 protein family, which protects the producer from nuclease activity prior to secretion (33, 96). It was noted that although *essC2*, *essC3*, and *essC4* strains do not encode EsaD, they all encode copies of DUF600 proteins at their T7 loci (33, 141) (**Figure 5**). The inference was that the orphan EsaG homologs protect these *S. aureus* strains from nuclease attack by variant 1 strains and point to a role for the nuclease in intraspecies competition. The low T7 secretion activity of *S. aureus* strains when cultured in laboratory media has hampered studies to examine the role of the T7SS in interbacterial warfare. However, when EsaDG was overproduced in strain COL, growth prevention of a target strain, RN6390, was observed. Growth inhibition was absolutely dependent on both an active T7SS in the attacker strain and functional nuclease activity. Increased protection was afforded to the target strain if it was provided with extra copies of *esaG* on a plasmid (33).

Interestingly, further DUF600 protein–encoding genes are also found just downstream of *esaG* in *essC1* strains. For example, there are five of these in NCTC8325 (14, 141) (**Figure 5*a***). The proteins encoded by these genes share 66–87% identity with EsaG but do not appear to interact as tightly with EsaD as the cognate antitoxin. Moreover, they do not offer protection from self-killing by EsaD because even though these genes are present on the chromosome, *esaG* cannot be deleted in the presence of a functional *esaD* gene (33). Further analysis of EsaD indicates that it is a polymorphic toxin—the encoding gene is a hot spot for recombination within the 3′ region encoding the toxin domain (141). Polymorphic toxins are modular proteins involved in interbacterial competition. They can be split into classes depending on which N-terminal sequence is present to facilitate protein export. The toxic cargo of these proteins is found at the C terminus but can differ significantly (112). Numerous EsaD variants are found across *essC1* strains, and it is highly likely that accumulated EsaG homologs offer protection from EsaD variants secreted by competing strains. In support of this, it was noted that two polymorphic variants of the EsaD nuclease domain from variant 1 strains A8819 and SAU060112 showed different patterns of interaction with the six DUF600 proteins from NCTC8325 (32).

Recently it was shown that TspA is a second *S. aureus* T7 toxin with antibacterial activity. It has a C-terminal domain that is toxic when heterologously targeted to the periplasm of *Escherichia coli*,



(*Caption appears on following page*)

Figure 5 (*Figure appears on preceding page*)

Highly variable antitoxin islands are located at the *Staphylococcus aureus* type VII (T7) gene cluster. Genomic analysis reveals a diverse array of candidate antitoxin-protein-encoding genes found at the *ess* locus of *S. aureus* strains; a few examples are shown. (*a*) *essC1* variant clusters. (*b*) *essC2* variant clusters. (*c*) *essC3* variant clusters. (*d*) *essC4* variant clusters. Pseudogenes are shown with dashed outlines. Genomic neighborhood comparisons were generated using FlaGs.py (113).

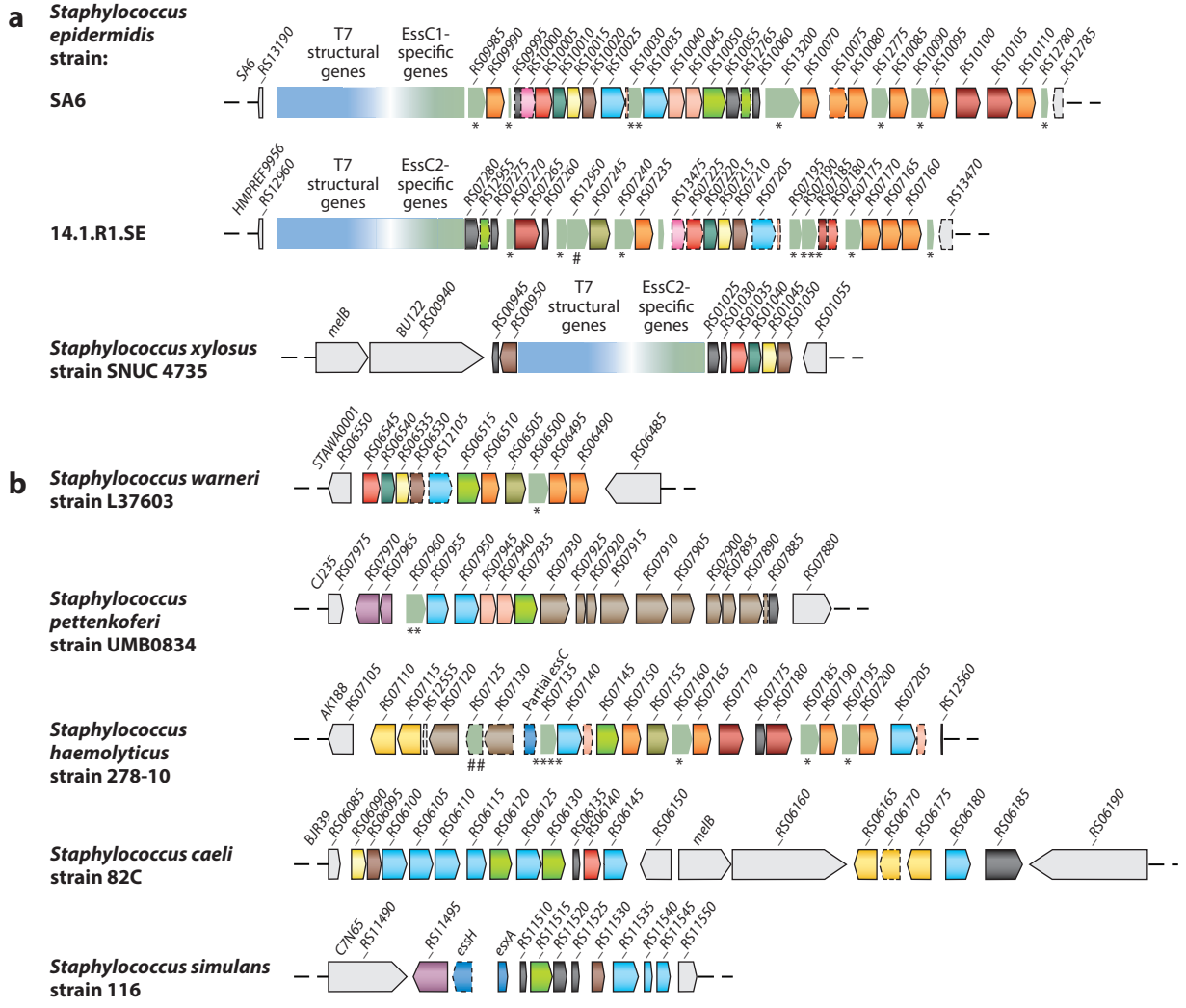
where it depolarizes the membrane. Protection from TspA activity is afforded by the membrane protein TsaI, a member of the DUF443 protein family. Across *S. aureus* strains, the C terminus of TspA is polymorphic. Strains accumulate multiple DUF443 protein–encoding genes at the *tspA* locus, presumably as protection from TspA sequence variants that are secreted by competing strains (**Figure 4*a***). Interestingly, many strains contain a second, truncated *tspA* gene at the *tspA* locus that codes for a variant C-terminal domain (**Figure 4*a***). It is plausible that through genetic recombination this allows strains to produce different TspA variants to increase competitiveness.

The zebrafish infection model has recently been adapted to explore interbacterial competition in vivo (147). Pairs of bacteria are co-injected into the hindbrain, which is an otherwise sterile compartment, allowing their interaction to be monitored. The advantage of this system is that the T7 is clearly active when *S. aureus* is in this environment, and therefore it avoids the requirement to overproduce candidate toxins from plasmids. Using this model, it was shown that strain COL is able to mediate killing of RN6390 in a T7SS-dependent manner, provided that the genes coding for antitoxins to EsaD and TspA are deleted from the RN6390 chromosome. Through use of attacker strains lacking either *esaD* or *tspA* it was shown that both toxins contribute to intraspecies competition (130).

It is highly likely that there are additional antibacterial toxins across *S. aureus* strains; for example, the EssC variant 2, 3, and 4 strains each encode an LXG domain protein at their T7 loci. Strain variants 2 and 3 encode related LXG proteins that share 33% sequence identity [termed EsxX in variant 2 strains (47)]. In *essC2* and *essC3* strains, the encoding gene directly precedes a gene encoding a DUF5079 family protein that is a candidate antitoxin (SAPIG0306 and SAR0288 in variant 2 and 3 strains, respectively). Interestingly, a very close homolog of SAPIG0306 (98% sequence identity) is encoded in *essC1* strains. This orphan gene, *SAOUHSC_00270* in **Figure 5*a***, is found among the cluster of genes encoding DUF600/EsaG family antitoxins in what appears to be an antitoxin island. This genomic evidence strongly supports the hypothesis that EsxX has antistaphylococcal activity and plays a role in intraspecies competition. Likewise, a homolog of SAR0288, SAOUHSC_00254, is also encoded in *essC1* strains, but this time at the 5′ end of the *ess* locus in what appears to be a second antitoxin island that is present in some strains (**Figure 5**).

These antitoxin islands are regions of high variability across *S. aureus* strains, containing different numbers of probable antitoxins. Some examples are shown in **Figure 5**. In some respects, they appear analogous to the acquired interbacterial defense (AID) gene clusters found in *Bacteroidales*, which encode multiple antitoxins against gram-negative type VI secreted toxins (111). Within the 3′ antitoxin island of NCTC8325 (**Figure 5*a***), the encoded membrane protein SAOUHSC_00271, a member of the DUF5080 protein family, appears to be an orphan antitoxin that shares 98% identity with SAPIG0309 (**Figure 3**). It is not clear what the likely toxin might be, but given that it is encoded immediately downstream of the DUF5085 pair SAPIG0307 and SAPIG0308 it may be one or both of these proteins. SAR0291 (from variant 3 strains) is from the same DUF5080 family as SAPIG0309, and a close homolog of SAR0291 is also encoded in NCTC5325, but in the 5′ immunity island (SAOUHSC_00255; 85% sequence identity).

The LXG protein in *essC4* variants, SAEMRSA15_02490, has yet to be characterized. It is the least common of all *S. aureus* toxins, being found in only a few strains, primarily from clonal complex 22 (141). It is, however, encoded by other species, including environmental organisms such as



(*Caption appears on following page*)

Figure 6 (*Figure appears on preceding page*)

Type VII (T7)-related genes are found in a wide range of *Staphylococcus* species. (*a*) Genes encoding T7 structural components are present in a number of staphylococci, including *S. epidermidis* and *S. xylosus*. Furthermore, DNA sequences that share identity with the coding regions of known toxin domains present on LXG proteins are also found within these clusters (*green with no outline*). It is not clear whether these encode small toxins lacking an obvious T7-targeting motif or whether these gene fragments can be subject to recombination events to fuse the toxin with an LXG domain, therefore permitting secretion. Some of these also appear to be pseudogenes (with frameshifts or premature stop codons). (*b*) Clusters including toxin fragments and immunity-encoding genes are found in a wide range of staphylococci, where genes encoding components of the T7 machinery are missing from the genome. Pseudogenes are shown with a dashed outline. Genomic neighborhood comparisons were generated using FlaGs.py (113).

Sporosarcina pasteurii. The occurrence of this toxin in bacteria that are free-living nonpathogens is more likely to point to a role in bacterial competition rather than virulence.

Interbacterial competition therefore appears to be a major role of the T7SS in *S. aureus*. Indeed, based on growing evidence it is likely that this is also true for other *Firmicutes* (6, 37, 38, 77, 79, 128, 146). This raises many interesting questions that remain to be answered. For example, which organisms can *S. aureus* target using its T7 toxins? Work with *Streptococcus intermedius* has shown that it can use T7 secretion to inhibit the growth of other *Firmicutes* such as *Streptococcus pyogenes* and *Enterococcus faecalis*, but not gram-negative bacteria (146). What role does the T7SS play in *S. aureus* colonization of body sites and shaping the nasal microbiome? How do toxins access their sites of action in the target cell? Are they released from the surface of the producing cell, like the toxin domains of the Cdi contact-dependent toxins in gram-negative bacteria (144)? And could the polymorphic toxin domains play other roles, such as kin recognition and competitive exclusion, just as polymorphic toxins do in gram-negative bacteria (11, 134, 135)?

8. T7SSs OF OTHER *STAPHYLOCOCCUS* SPECIES

In contrast to the numerous T7SS studies in *S. aureus*, there is little information on this secretion system in other staphylococci. Genomic analysis does, however, indicate that the system is widely conserved across the genus. In study of *Staphylococcus lugdunensis*, the T7SS was found in two distinct organizations; one was closely related to *S. aureus* variant 2 and the other to variant 4 (87). Some strains of *Staphylococcus epidermidis* also have the T7SS, again found in two distinct organizations related to *S. aureus* variant 1 or variant 2 (41) (**Figure 6*a***). Interestingly, while *S. epidermidis* strains cluster into two major lineages (the A/C lineage and the B lineage), the T7SS is only found in lineage B strains, suggesting that the lineages employ distinct mechanisms to underpin their occupancy of the skin niche (57). Neighborhood analysis indicates that extensive and variable immunity islands are present at the 3′ end of the *S. epidermidis* T7 gene clusters, signifying strong selective pressure for the acquisition of immunity genes (**Figure 6*a***). Strains of *Staphylococcus caprae* and *Staphylococcus xylosus* also encode the T7SS, which is closely related to *S. aureus* variant 2 (93, 123, 142) (**Figure 6*a***).

Interestingly, some staphylococcal strains, such as those listed in **Figure 6*b***, do not encode the T7SS. Despite this, they contain genomic islands encoding multiple immunity genes against T7 toxins, along with fragments of toxin genes, again reminiscent of the gram-negative AID gene clusters that offer protection from T6SS-mediated competition (111). It is highly likely that these genes contribute to survival of staphylococci in competitive niches and point to a major role of the T7SS in intraspecies antagonism.

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