The 1928 Bundaberg disaster is one of the greatest vaccine tragedies in history. Of 21 children immunized with a diphtheria toxin–antitoxin preparation contaminated with *Staphylococcus aureus*, 18 developed life-threatening disease and 12 died within 48 h. Historically, the deaths have been attributed to α-toxin, a secreted cytotoxin produced by most *S. aureus* strains, yet the ability of the Bundaberg contaminant microbe to produce the toxin has never been verified. For the first time, the ability of the original strain to produce α-toxin and other virulence factors is investigated. The study investigates the genetic and regulatory loci mediating α-toxin expression by PCR and assesses production of the cytotoxin *in vitro* using an erythrocyte haemolysis assay. This analysis is extended to other secreted virulence factors produced by the strain, and their sufficiency to cause lethality in New Zealand white rabbits is determined. Although the strain possesses a wild-type allele for α-toxin, it must have a defective regulatory system, which is responsible for the strain’s minimal α-toxin production. The strain encodes and produces staphylococcal superantigens, including toxic shock syndrome toxin-1 (TSST-1), which is sufficient to cause lethality in patients. The findings cast doubt on the belief that α-toxin is the major virulence factor responsible for the Bundaberg fatalities and point to the superantigen TSST-1 as the cause of the disaster.

**INTRODUCTION**

In Bundaberg, Australia, in 1928, 21 children (aged 1–10 years) were accidentally immunized with diphtheria toxin–antitoxin contaminated with *Staphylococcus aureus*. Within 24 h, 18 of the children fell seriously ill. Despite medical intervention, 12 died within 48 h, and all survivors developed abscesses at the injection sites, earning its designation as one of the most significant vaccine-related tragedies in history (Anon., 1928). Investigation into the contaminant strain by Sir F. Macfarlane Burnet, commissioned by the Commonwealth of Australia, revealed that the organism produced an exotoxin capable of pathophysiological activity: intradermal injection produced skin lesions and subcutaneous injection caused lethality in rabbits (Burnet, 1929). The exotoxin, he concluded, probably mediated the illness in the children.

Later work by Glenny & Stevens (1935) following the Bundaberg fatalities led to the identification of two immunologically distinct exotoxins, α-toxin and β-toxin, differentiated by their species-specific haemolytic activity; rabbit cells were sensitive only to α-toxin. This discovery, in the context of Burnet’s observation on the sensitivity of rabbits to the Bundaberg exotoxin, implicated α-toxin in the Bundaberg fatalities.

Over the century since its discovery and attribution to human disease, great advances have been made in clarifying α-toxin’s genetics, biochemistry and pathological properties (Berube & Bubeck Wardenburg, 2013). α-Toxin is a heat-labile cytotoxin encoded in the core genome of *S. aureus* at the *hla* locus. It is secreted as a hydrophilic 33 kDa monomer and oligomerizes to form a heptamer β-barrel upon insertion into eukaryotic cell membranes. Although it has also been reported as a signal disrupter in nucleated cells, particularly through the ADAM10 pathway, the hallmark activity of the holotoxin is erythrocyte lysis. This property is best observed on rabbit blood agar, where α-toxin-producing strains confer a zone of beta-haemolysis, allowing differentiation from β-toxin-producing isolates, which produce a zone of incomplete lysis.

When we recently plated the Bundaberg strain (National Collection of Type Cultures; NCTC 2669) on rabbit or sheep blood agar, the organism showed no direct haemolysis, rendering the α-toxin–contaminant strain of *S. aureus* differentiated by its pathophysiological activity as an isolated variant. We established that this variant is not a distinct strain but rather a wild-type strain of *S. aureus* in which a chromosomal mutation has rendered the strain incapable of producing α-toxin to levels sufficient to cause lethality in rabbits. This finding raises doubts about the strain’s ability to secrete functional α-toxin. Furthermore, literature searches revealed that although α-toxin is widely accepted as the cause of...
Table 1. Primers used in this study

<table>
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<th>Sequence</th>
<th>Size (bp)</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>wholehla_Fwd</td>
<td>GACATATTGATTATGTGTTTCCTC</td>
<td>1080</td>
<td>This study</td>
</tr>
<tr>
<td>wholehla_Rv</td>
<td>CATTTCTGAAGTTATCGGCATTA</td>
<td></td>
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<td>AGTGTGATAGTAAGTGGAATCA</td>
<td>1326</td>
<td>This study</td>
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<tr>
<td>AgrC_R</td>
<td>ATACATTCCATCCCTTATGCGC</td>
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the Bundaberg fatalities (Bhakdi & Tranum-Jensen, 1991; Berube & Bubeck Wardenburg, 2013), the original biochemical properties of α-toxin identified by Burnet, Glenny and Stevens, including heat sensitivity and rabbit cell haemolysis, were never ascribed to the Bundaberg isolate, but rather to a panel of other S. aureus isolates (Burnet, 1929; Glenny & Stevens, 1935). To our knowledge, there have been no investigations into the organism’s propensity to produce α-toxin or other more recently described virulence factors.

To address this, we sought to determine the genetic and biochemical potential of the Bundaberg (2669) strain to produce α-toxin in vitro. Here, we find that although NCTC 2669 may encode wild-type α-toxin, it does not produce the cytotoxin at quantifiable levels under conditions that favour production. We suggest that this in part may be due to a dysfunctional agr or other regulatory locus. Genetic analysis reveals that NCTC 2669 harbours other clinically relevant exotoxin genes, including the superantigen (SAg) toxic shock syndrome toxin-1 (TSST-1). In vitro data indicate that TSST-1 is produced at high levels, and purified toxin is sufficient to cause lethality in rabbits. In summary, our findings challenge the dogma that α-toxin is the primary virulence factor in the NCTC 2669 strain and strongly implicate TSST-1 in the Bundaberg fatalities.

METHODS

Bacteria. S. aureus strain NCTC 2669 was generously provided by the late Dr Margaret M. Peel, Senior Bacteriologist, Department of Microbiology, Microbiological Diagnostic Unit, University of Melbourne, Parkville, Victoria, Australia, in 1984. Because of the NCTC designation, the strain is maintained by Public Health England, and our laboratory shared the strains with Dr Richard P. Novick’s laboratory (New York University). Investigators wishing to perform our laboratory shared the strains with Dr Richard P. Novick’s lab-designation, the strain is maintained by Public Health England, and late Dr Margaret M. Peel, Senior Bacteriologist, Department of Temperatures were monitored rectally for 4 h. At 4 h, endotoxin (Schlievert, 1982). New Zealand white rabbits, of either sex, were injected intravenously in their marginal ear veins with TSST-1 at 5 µg ml⁻¹ kg⁻¹ of or with an equivalent volume of PBS. Temperatures were monitored rectally for 4 h. At 4 h, endotoxin (purified by hot phenol extraction from Salmonella enteritica serovar Typhimurium; 1 µg ml⁻¹ kg⁻¹) was injected intravenously into the ear veins (Westphal et al., 1952). Rabbits were monitored for survival over 24 h (Priest et al., 1989).

Synergistic haemolysis test. RN4220 was streaked across the centre of sheep blood agar plates (BD), and stains of interest were streaked perpendicularly. Plates were incubated at 37 °C for 24 h before analysis. Synergistic haemolysis was determined visually (Geisinger et al., 2012).

Gene amplification and sequencing. DNA was extracted by colony lysis from NCTC 2669 grown overnight on 1.5 % TH agar. The primers used to detect SAg genes by PCR have been published (Salgado-Pabón et al., 2014, Vu et al., 2014). Primers used for detection of agrC and hla are listed in Table 1. Amplifications were performed with Taq polymerase (Qiagen), excluding sel-α, which was amplified using Phusion polymerase (New England Biolabs). Sanger sequencing was conducted on amplicons, after purification of PCR products (Qiagen).

SAg immunoblot. To assay for TSST-1 production, bacteria were grown for 24 h at 37 °C with high aeration, and supernatants were collected and concentrated with 4 volumes of ethanol. Protein concentrates were centrifuged at 4000 g for 10 min and resolubilized with distilled water at 10 % original volume. For strains grown in horse serum, ethanol precipitation was not possible due high levels of albumin in the samples. Instead, supernatants were filtered (0.22 µm filter). Western immunoblots were conducted with polyclonal rabbit antiserum against TSST-1 (Blake et al., 1984; Schlievert, 1988). Purified TSST-1 of known concentration was used to generate a standard curve for toxin quantification; this was done with the use of ImageJ software.

TSST-1 purification. TSST-1 was purified by isoelectric focusing (Blomster-Hautamaa et al., 1986; Blomster-Hautamaa & Schlievert, 1988). NCTC 2669 was cultured in BH medium for 48 h. Cultures were treated with four volumes of absolute ethanol. Precipitates were reconstituted in distilled water and TSST-1 was purified by isoelectric focusing. The product was electrophoresed on an SDS-PAGE gel and stained with Coomassie brilliant blue R250 (Bio-Rad) to assess purity; quantification was conducted using the Bradford reagent (Bio-Rad).

Rabbit TSS model. Animal experimentation was performed under an approved University of Iowa IACUC protocol (4071100). Rabbits are highly susceptible to fevers induced by SAgS and develop accelerated TSS when administered SAg and then 4 h later a sublethal dose of endotoxin (Schlievert, 1982). New Zealand white rabbits, of either sex, were injected intravenously in their marginal ear veins with TSST-1 at 5 µg ml⁻¹ kg⁻¹ of or with an equivalent volume of PBS.
Statistics. Student’s t-test and Fisher’s exact test were used to compare differences in animal temperature and survival, respectively.

RESULTS

S. aureus NCTC 2669 produces minimal levels of α-toxin in vitro

α-Toxin production by S. aureus is characterized by beta-haemolysis on rabbit blood agar. When NCTC 2669 was plated on commercial rabbit blood agar and incubated overnight at 37 °C, no characteristic zone of α-toxin-induced clearing was observed (Fig. 1c); interestingly, the lack of haemolysis was also noted by Burnet (1929). For comparison, the CA-MRSA isolate LAC, a high α-toxin producer, and the menstrual TSS isolate MN8, a low α-toxin producer, were also plated (Fig. 1a, b), where haemolysis was observed.

α-Toxin has biological activity at low concentrations: for example, it is lethal to Dutch-belted rabbits when given intravenously at concentrations as low as 1 μg kg⁻¹ (Wiseman, 1975; Dinges et al., 2000). To assay for haemolysis with increased sensitivity, filtered NCTC 2669 supernatants from overnight cultures were added 1:3 with 3 % heparinized rabbit erythrocytes and incubated statically at room temperature for 1 h. Haemolysin concentration was calculated by comparing absorbance readings with purified α-toxin standards of known concentration, relative to 3 % Triton X-100 as a lysis control. Multiple growth conditions were tested: rich medium (TH), exotoxin-promoting medium (BH) and dilutions of horse serum in TH, included to mimic growth conditions of strain 2669 in the toxin–antitoxin preparation. Negligible lysis was observed for all conditions (Fig. 2). Quantification of total haemolysin produced by S. aureus NCTC 2669 indicated amounts below the lower limit detection (5 ng ml⁻¹) for all growth conditions. There was also no detectable erythrocyte lysis from lysates of strain NCTC 2669, indicating that α-toxin was not sequestered within bacterial cells. These results suggest S. aureus NCTC 2669 does not produce detectable haemolysin, including α-toxin, in vitro.

Dysfunctional agr locus may contribute to low haemolysin production

Expression of hla varies across clinical isolates. One mechanism known to attenuate α-toxin production is the presence of a premature stop codon in hla in the USA200 menstrual TSS isolate MN8, characterized as a low α-toxin producer.

Fig. 1. NCTC 2669 lacks haemolytic phenotypes indicative of α-toxin expression and agr induction. Haemolysis phenotype of S. aureus strains LAC (a), MN8 (b) and NCTC 2669 (c) on trypticase soy agar with 5% rabbit blood plates. (d) NCTC 2669 and control strains were streaked perpendicularly to RN4220 and examined for the development of synergistic haemolysis after overnight incubation.
To determine if such a mutation reduced α-toxin production in NCTC 2669, the hla locus was amplified, sequenced and compared against the BLAST database. NCTC 2669 did not encode a premature stop codon, but instead shared identity with over 60 complete coding sequences for hla, including well-characterized clinical isolates recognized to produce the functional product (Fig. S1A, available in the online Supplementary Material).

Global regulators are also important in cytotoxin gene expression in S. aureus. The most well-studied regulator of α-toxin production is the agr quorum-sensing system (Thoendel et al., 2011). When a threshold cell density is reached, the system is activated, inducing the expression of an effector RNA molecule, RNAIII. RNAIII directly/indirectly regulates downstream expression of virulence genes, including secreted and cell-surface-associated factors. α-Toxin expression increases with agr, and consequently RNAIII, induction.

To examine the autoinduction of the agr system of S. aureus NCTC 2669, a synergistic haemolysis (CAMP) test was conducted. δ-Toxin, a small peptide haemolysin, transcribed from RNAIII upon agr induction, can be used to measure agr functionality; its production can be assessed visually by the toxin’s ability to synergize with β-toxin to produce complete clearing on blood agar. To a lesser extent, phenol-soluble modulins may also synergize with β-toxin to produce a similar phenotype (Cheung et al., 2012). NCTC 2669 was streaked perpendicularly to laboratory strain RN4200 (β-toxin+/δ-toxin−) and examined for haemolysis phenotype after 24 h of incubation. No zone of clearing was observed, in contrast to MN8, which displayed synergistic haemolysis in the presence of β-toxin, and LAC, which demonstrated direct erythrocyte haemolysis (Fig. 1d).

Induction dynamics of the agr regulon have been correlated with the agr type of the strain. We amplified and sequenced agrC and examined the hypervariable region (base pairs 344–472) of the gene to determine the agr type of NCTC 2669. Alignment with strains of known agr types identified NCTC 2669 as an agr III strain (Fig. S1B), the group possessing the weakest and slowest agr induction and lowest α-toxin production of the four agr types (Geisinger et al., 2012; Mairpady Shambat et al., 2014). These findings suggest a possible genetic basis for the low levels of α-toxin observed.

**S. aureus NCTC 2669 encodes SAg genes**

We identified other secreted factors of NCTC 2669 that may have functioned in the Bundaberg tragedy. Review of patient symptoms, documented in the Royal Commission report on the Bundaberg fatalities, are...
strikingly similar to the clinical features of TSS; the children experienced vomiting, convulsions, loss of consciousness, cyanosis, hypotension and pyrexia (Anon, 1928; Todd et al., 1978). TSS is classically defined by: hypotension, pyrexia, erythematous (scarlet-fever-like) rash and three multi-organ components, which may include the gastrointestinal, central nervous, liver, mucous membrane, renal and muscular systems (Todd et al., 1978; Davis et al., 1980; Shands et al., 1980; John et al., 2009; Spaulding et al., 2013).

This syndrome is dependent on production and systemic dispersion of SAgs (Spaulding et al., 2013), small immunomodulatory exotoxins produced by all pathogenic S. aureus. They include TSST-1, the staphylococcal enterotoxins (SEs) and the SE-like molecules (Lina et al., 2004; Spaulding et al., 2013). To determine whether S. aureus 2669 had the potential to produce SAgs, which may have contributed to the Bundaberg children’s infections, we examined NCTC 2669 for SAg genes.

NCTC 2669 encoded eight of the 22 known SAg genes: tstH, seg, sei, sel-m, sel-n, sel-o, sel-q and sel-u (Table 2). Of these, seg, sei, sel-m, sel-n, sel-o and sel-u compose the full-length enterotoxin gene cluster (egc), which has recently been implicated in local tissue effects (Nowrouzian et al., 2015). To our knowledge, sel-q has yet to be associated with human disease. The gene tstH, which encodes TSST-1, causes TSS, and contributes to pneumonia, infective endocarditis and sepsis (Spaulding et al., 2013). TSST-1, SEB, and SEC are the major SAgs that cause TSS (Spaulding et al., 2013). The remainder of our investigation focuses on TSST-1 as the likely candidate exotoxin responsible for the Bundaberg fatalities.

**S. aureus NCTC 2669 produces TSST-1**

Supernatant from NCTC 2669 was collected after growth overnight in TH media, concentrated by ethanol precipitation and blotted with polyclonal sera raised against TSST-1. The SAg was produced at concentrations of $32.7 \pm 1.5 \mu g \, ml^{-1}$ (Fig. S2A). For reference, nearly all strains encoding tstH produce 3–20 $\mu g$ TSST-1 ml$^{-1}$ in vitro in comparable broth cultures (Spaulding et al., 2013).

When NCTC 2669 was grown for 24 h in HS, TSST-1 could be detected by immunoblot for HS dilutions at ≤50% (Fig. 3). We hypothesize that toxin was not detected in the 100% sample because the strain did reach the post-exponential phase of growth, where TSST-1 is produced (Spaulding et al., 2013). Unlike in TH broth, quantification of TSST-1 production was not possible due to the high concentration of HS proteins, which distorts lane widths.

**Purified TSST-1 causes lethality in rabbits**

We next examined the ability of purified TSST-1 from NCTC 2669 to cause lethality in an endotoxin enhancement

![Fig. 3. NCTC 2669 expresses TSST-1 in vitro. NCTC 2669 was inoculated at $10^8$ c.f.u. ml$^{-1}$ into 25 ml broth cultures containing dilutions of horse serum in TH broth and grown for 24 h at 37°C with high aeration (225 r.p.m.). Bacterial burden in the flasks was determined by serial dilution and plating. The limit of detection was 10 c.f.u. ml$^{-1}$ or log of 1. Toxin production was detected by immunoblot of filter-sterilized supernatants of bacterial cultures with rabbit polyclonal antiserum raised against TSST-1. Purified TSST-1 was also run as a control. The dashed line represents the starting inoculum. Error bars represent SD.](http://mic.microbiologyresearch.org)
DISCUSSION

For nearly a century, z-toxin has been the accepted and cited cause of the Bundaberg fatalities, contributing to its designation as one of the most important virulence factors of *S. aureus* (Berube & Bubeck Wardenburg, 2013). The present study, however, reveals that the causative strain, NCTC 2669, lacks the ability to produce z-toxin *in vitro* under favourable conditions. Investigation into the regulatory network of z-toxin provides a possible genetic basis for this finding: (1) we first demonstrate that NCTC 2669 encodes an intact z-toxin gene, unlike the majority of TSST-1-producing *S. aureus* (and thus a premature stop codon does not account for deficient production); and (2) additionally, NCTC 2669 belongs to the agr type III system, which typically has the lowest levels of autoinduction and, in corollary, the lowest levels of z-toxin production (Geisinger et al., 2012; Mairpady Shambat et al., 2014). Future studies should examine the functionality of srr, sar, sae and other global regulators of *S. aureus* exotoxin production for their contribution to the deficient z-toxin production (Pragman & Schlievert, 2004).

Review of the original case studies revealed that symptoms and progression of the children's illness fits TSS, not described until 1978 (Todd et al., 1978). Therefore, we examined the strain for its repertoire of SAgs, which are known to mediate TSS (Spaulding et al., 2013). NCTC 2669 encodes eight SAgs, including TSST-1, the toxin responsible for 75% of all TSS cases (Spaulding et al., 2013). Strain NCTC 2669 produces TSST-1 in high concentrations, and purified toxin is sufficient for pyrogenicity and lethality in rabbits. Collectively, our findings support a model in which TSST-1, not z-toxin, mediated the Bundaberg fatalities.

Interestingly, our findings and conclusions are corroborated by historical documentation in the original investigation of the Bundaberg isolate. Burnet, the investigator commissioned by the Australian Commonwealth, noted not only that *S. aureus* NCTC 2669 demonstrated no direct haemolytic properties, but also that the Bundaberg staphylococcus was capable of producing a 'positive and specific skin test in a dilution of 1 to 2000' when grown for 8 h *in vitro* (Burnet, 1929). Based on our quantification of total cytotoxin produced by NCTC 2669 in undiluted samples, which was ≤5 ng ml⁻¹ under all conditions tested, it is highly unlikely that z-toxin is the exotoxin. In contrast, we showed that 2669 produces TSST-1 in concentrations exceeding 32 μg ml⁻¹; thus, even when diluted 2000-fold, the toxin exceeds by 10-fold the lowest concentration with pathophysiological activity (10 pg ml⁻¹) (Stich et al., 2010).

Auxiliary to our purpose but of interest is that, to our knowledge, NCTC 2669 is the oldest tested strain to have the genes for both TSST-1 and all EGC proteins. We have observed that this SAg profile (stsH/egc⁺) is indicative of the modern USA200 clonal group. TSST-1-producing isolates emerged as the dominant population...
in this clonal group by 1972, such that they account for nearly 100% of isolates today; however, they represented as few as 5% in the clonal group from 1940 up to 1972 (Spaulding et al., 2013). Our results demonstrate that tstH strains were present in the population as early as 1928. Furthermore, the discovery of the intact ege, regarded as the genetic nursery from which staphylococcal enterotoxins are derived, suggests that the genetic diversity necessary to evolve the modern repertoire of distinct SAGs pre-dates 1928 (Jarraud et al., 2001). Overall, these findings question the population dynamics of the S. aureus clonal groups and the evolution and acquisition of SAg genes over time.

In summary, this study contributes to a growing body of literature underlining the importance of SAGs in eliciting diseases (Spaulding et al., 2013). In other studies, we have demonstrated that immunizing rabbits against SAGs protects from lethal challenge (Spaulding et al., 2012).

REFERENCES


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