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SHORT COMMUNICATION

The exoproteomes of clonally related *Staphylococcus aureus* strains are diverse

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Abstract Several studies have shown that protein expression patterns vary in unrelated bacterial strains due to genomic plasticity and gene regulation, resulting in enhanced heterogeneity in the infection potential. However, exoprotein expression patterns of closely related clonal strains have not been well characterized. Here, we used medium-range (pH 4–7) immobilized pH gradient–two-dimensional gel electrophoresis to investigate the exoproteome from closely related *Staphylococcus aureus* clonal isolates. Interestingly, we found that, under identical in vitro experimental conditions, a number of protein spots were uniquely present in samples from each clonal isolate irregardless of the similarity of the genotype and the same virulence gene profile. Only a few abundant invariant proteins were found among identical genotypic isolates. Our results clearly shown that heterogeneity in the exoproteome was present even among clonally related strains. We suggest that this heterogeneity may contribute to the degree of virulence even within one clonal genotype. The heterogeneity in the exoproteome of closely related *S. aureus* strains observed in the current study postulates that pre-existing antibodies are not very protective during recurrent infection with the same strain. Therefore, our findings underscore the importance of taking all clonally related strains into account during proteome analyses.

Keywords *Staphylococcus aureus* · Exoprotein · 2-DGE · Closely related clonal

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Despite the large numbers of studies which have been carried out over the past 130 years on *Staphylococcus aureus*, the emergence of multidrug-resistant strains with a heterogeneous virulence potential is still on the rise (Sakoulas and Moellering 2008; Hongo et al. 2009; Ventura et al. 2010; Muthukrishnan et al. 2011; Alonzo III et al. 2012). Heterogeneity in exoprotein expression patterns has been observed for distinct genotypes of *S. aureus* (Bernardo et al. 2002; Ziebandt et al. 2010). This variation in exoproteins increases *S. aureus* fitness by enhancing its pathogenicity potential, including attachment and invasion/evasion of host defenses, as well as by promoting growth in different niches in the environments of the human host. However, the question of whether clonally related *S. aureus* strains [as defined by a pulsed-field gel electrophoresis (PFGE) profile with>80 % similarity and sharing of the same sequence type) display a homogeneous pattern at the protein level once subjected to medium-range immobilized pH gradient (IPG)–2-DGE remains unanswered. To address this question, we randomly selected six clinical isolates of three *S. aureus* clones, namely, ST1, ST30 and ST8 (2 isolates per clonal type), for 2-DGE comparison. PFGE patterns and virulence gene profiles for each isolate were also determined

(Fig. 1). This pilot study was not designed to identify the various spots on the gel due to the small number of clones tested, but rather to characterize the exoprotein expression patterns from closely related *S. aureus*strains through medium-range (pH 4–7) IPG–2-DGE and a modified silver staining technique for the clear visualization of protein variants.

Briefly, *S. aureus* culture (0.03–0.04 OD_{600}) was grown in Tryptic Soy Broth supplemented with 0.001 M 2,2′-dipyridyl (Sigma-Aldrich, St. Louis, MO) at 37 °C under constant agitation (150 rpm). The culture supernatant was precipitated using the ice-cold ethanol–TCA method when the growth reached the postexponential phase. Exoproteins isolated from a culture supernatant often comprise a wide variety of virulence factors. In our study, the exoproteins were subjected to 2-DGE with a pH 4–7 IPG strip (Bio-Rad, Hercules, CA) as described previously (Liew et al. 2013). The stained gels were scanned with a calibrated densitometer (model GS-800; Bio-Rad) and analyzed using PDQuest Advanced 8.0.1 2D Gel Analysis software (Bio-Rad), which determined the densities (volume by Gaussian integration) of each of the protein spots on the gels. Technical triplicates were performed for each sample. The correlation coefficients between technical replicates of gels ranged from 0.79 to 0.95, which is normal for 2-DGE analyses (Vydra et al. 2008). Mean values for spot intensity were subjected to analysis using the Student *t* test to determine whether there were any significant individual differences in exoprotein expression between paired isolates. A *P* value of$\leq$0.05 was considered to indicate statistical significance.

The abundant invariant protein spots were manually excised from the 2-DGE gels and digested with trypsin; the peptides were then extracted according to standard techniques. Electrospray ionization mass spectrometry analysis of peptides was carried out using the Ultimate 3000 nano HPLC system (Dionex Corp., Sunnyvale, CA) coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA). Briefly, tryptic peptides were loaded onto a C18 PepMap100, 3 μm column (LC Packings–Dionex Corp.) and separated with a linear gradient of water/acetonitrile/0.1 % formic acid (v/v). Spectra were analyzed to identify proteins of interest using Mascot sequence matching software (Matrix Science, London, UK) with the Ludwig NR database. Ludwig NR is a comprehensive, audited database which contains non-identical protein sequence information based on all major publicly available datasets. The following search parameters were applied for liquid chromatography–tandem mass (MS/MS) analysis on the 4000 QTRAP mass spectrometer: (1) oxidation (M) was set with variable modifications; a peptide mass tolerance of±1.2 Da and MS/MS tolerance of±06 Da were used; (2) a peptide charge state of +1+2 or +3 was accepted; (3) the maximum number of missed cleavages was set to 1. An individual ion score of >60 was considered to indicate significant identification of a protein spot, which corresponds to a *P*$\leq$0.05.

Interestingly, there was much more variation in exoprotein patterns than expected—even among the isolates that shared the same clonal lineage (Fig. 2). Isolate pairs showing a high similarity in the PFGE analysis did not necessarily show a homogeneous exoproteome pattern. Moreover, 40–50 % of protein spots within the similar exoprotein pattern showed more than a twofold variation in spot intensity. Only a few invariant proteins were abundantly expressed by same clonal lineage isolates, such as staphylococcal secretory antigen

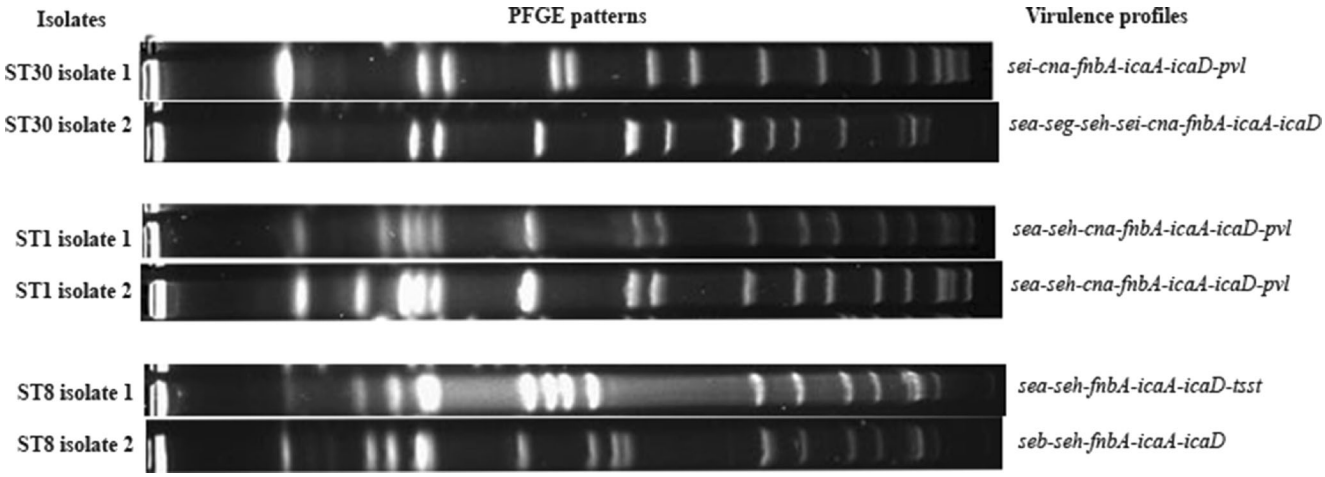

Fig. 1 Pulsed-field gel electrophoresis (PFGE) and virulence factor patterns. Most of the closely related isolates showed similar PFGE patterns after restriction with *Sma*I (Sigma-Aldrich, St. Louis, MO; Goering 2010; Alibayov et al. 2014). It should be noted that the PFGE pattern of ST1 isolates showed high similarity to an identical virulence profile

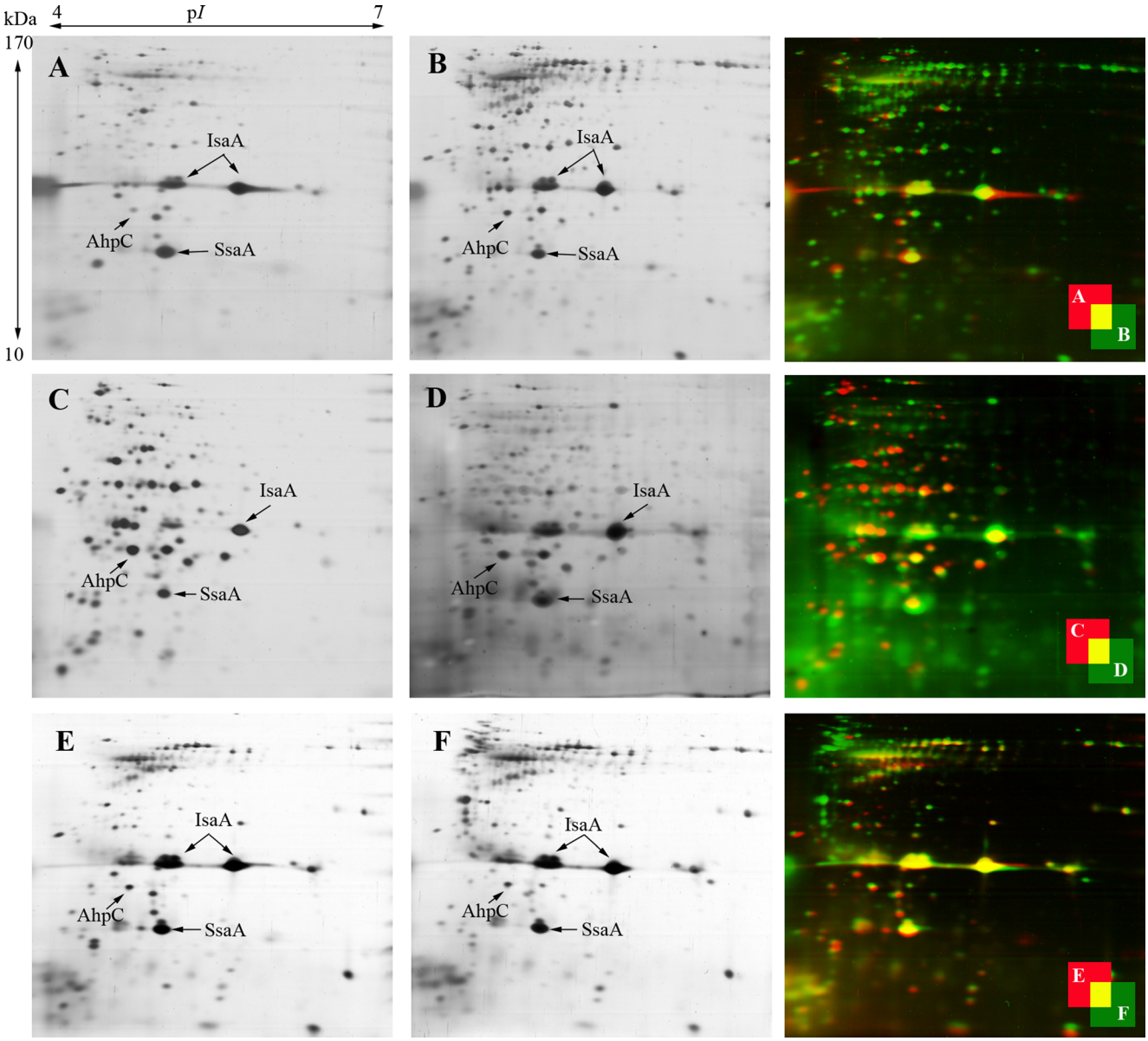

Fig. 2 Exoproteome of closely related isolates. Pairs *A/B*, *C/D* and *E/F*, respectively, were compared. Comparative pseudo-colored images of exoprotein expression between the closely related isolates of each pair is illustrated at the *right*. Both images were superimposed by PDQuest Advanced 8.0.1 2D Gel Analysis software (Bio-Rad, Hercules, CA). Protein spots whose intensity did not vary between closely related strains are indicated in *yellow*; *green* or *red* spots represent the exoproteome of closely related strains that were very heterogeneous. Exoprotein spot patterns varied in each comparison. In particular, a high level of heterogeneity in exoprotein expression can be seen in isolates of clone ST1 harboring the same virulence gene combination (**c**, **d**). The similarity between spot patterns was only as high as (approximately)50 % even when both isolates had the same clonal lineage. *Arrows* indicate abundant spots corresponding to the absence of any significantly differentially expressed proteins. **a**, **b** Exoprotein expression by clones of genotype ST30, **c**, **d** exoprotein expression by clones of genotype ST1, **e**, **f**, exoprotein expression by clones of genotype ST8. Only one of the three technical replicates is shown

(SsaA), immunodominant antigen A (IsaA) and alkyl hydroperoxidereductase subunit C (AhpC). Isolates with the same sequence type and same combination of virulence genes (for example, ST1 isolates with an identical virulence factor profile of *sea*, *seh*, *cna*, *fnb*A, *ica*A, *ica*D, *pvl*) also exhibited variation in their exoprotein expression pattern (Figs. 1, 2). While we do emphasize that our results are based on a relatively small number of strains, heterogeneity in the exoproteome was clearly present, even among clonally related strains.

Our results differ from those of earlier studies which reported that closely related strains exhibit highly similar exoproteome patterns (Nakano et al. 2002; Ziebandt et al. 2010). Ziebandt et al. (2010) demonstrated that *S. aureus*

Table 1 Identification of invariant and abundant proteins between closely related *Staphylococcus aureus* strains

Open reading frame name	Protein name	p*I*/kDa	Score	Sequence coverage (%)	Average protein intensity ratio within strain[a]		
					ST30	ST1	ST8
SAOUHSC_00365	Alkyl hydroperoxide reductase subunit C	4.88/20.963	403	70	2.5	1.4	0.9
SAO46_0518	Immunodominant antigen A	5.91/24.219	179	20	1.3	1.6	1.1
ST398NM01_2354	Staphylococcal secretory antigen	6.01/17.415	91	10	1.2	1.5	1.1

[a] The mean intensity change in the protein of a spot between two isolates per clonal type in terms of relative intensity to one another. The majority of these abundant protein spots showed non-significant differences of less than a twofold and more than a 0.5-fold ratio

isolates with the same sequence type (ST80) collected from different patients displayed an identical exoprotein pattern. Similarly, Nakano et al. (2002) showed that protein expression profiles were similar among strains of same coagulase type. However, most of these earlier studies used an IPG strip with a wide pH range (pH 3–10) instead of the relatively narrower pH range used in our study (pH 4–7); it must be noted that in these earlier studies most of the spots at pH 4–6 were poorly resolved and low-abundance proteins were not analyzed further. In contrast, in our study, the careful detection of low-abundance protein spots after the enrichment of spots and the enhancement of staining sensitivity by medium-range IPG, as well as the application of the modified silver staining method of Liew et al. (2013), clearly revealed variation in the exoproteome patterns. Similarly, our findings also differ from the results of more recent DNA microarray or PCR studies that revealed very similar virulence gene expression profiles in isolates with the same sequence type (Jamrozy et al. 2012; Shambat et al. 2012). Our results do agree with those of Munsky et al. (2012) and Le Maréchal et al. (2011) who found variable gene expression in identical environments even when the cells were genetically identical. One explanation of this variability is the possible accumulation of truncations and other nucleotide differences in certain genes. In our study, only three of the abundant conserved proteins showed no distinct difference in spot intensity between clonally related isolates such as SsaA and IsaA, which are responsible for *S. aureus* cell-wall metabolism, and AhpC, a stress response protein that efficiently provides resistance to hydrogen peroxide, cumene hydroperoxide, tert-butyl hydroperoxide, paraquat and peroxynitrite (Fig. 2; Table 1). Based on these results, we suggest that clonally related *S. aureus* strains are able to produce a variable proteome, which in turn causes developments in antibiotic therapy and immunotherapy to be more challenging and complicated, especially in the context of recurrent infection with the same strain.

In conclusion, we observed high heterogeneity in exoprotein expression that was irrespective of genotypic relatedness. These findings suggest that the comparison of exoproteomes from closely related *S. aureus* strains should not be excluded during any proteomic study as the results from such studies may contribute to our knowledge of the degree of virulence even within a clonal genotype. Along with genotype analysis (multilocus sequence typing and PFGE), the pattern of exoprotein spots provides a more discriminatory picture and facilitates further subdivisions of *S. aureus* strains. However, the 2-DGE approach, when used as a discriminatory tool, still needs to be refined for practical applications. Further investigations should address our lack of knowledge on protein expression patterns of *S. aureus* during the in vivo condition.

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Conflict of interest None.

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