Journal of Microbiological Methods 81 (2010) 56–60

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Development of pooled suppression subtractive hybridization to analyze the pangenome of *Staphylococcus aureus*

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ARTICLE INFO

Article history:
Received 26 October 2009
Received in revised form 26 January 2010
Accepted 28 January 2010
Available online 4 February 2010

Keywords:
Staphylococcus aureus
Pangenome
Subtractive hybridization

ABSTRACT

We describe the development and application of a Pooled Suppression Subtractive Hybridization (PSSH) method to describe differences between the genomic content of a pool of clinical *Staphylococcus aureus* isolates and a sequenced reference strain. In comparative bacterial genomics, Suppression Subtractive Hybridization (SSH) is normally utilized to compare genomic features or expression profiles of one strain versus another, which limits its ability to analyze communities of isolates. However, a PSSH approach theoretically enables the user to characterize the entirety of gene content unique to a related group of isolates in a single reaction. These unique fragments may then be linked to individual isolates through standard PCR. This method was applied to examine the genomic diversity found in pools of *S. aureus* isolates associated with complicated bacteremia infections leading to endocarditis and osteomyelitis. Across four pools of 10 isolates each, four hundred and twenty seven fragments not found in or significantly divergent from the *S. aureus* NCTC 8325 reference genome were detected. These fragments could be linked to individual strains within its pool by PCR. This is the first use of PSSH to examine the *S. aureus* pangenome. We propose that PSSH is a powerful tool for researchers interested in rapidly comparing the genomic content of multiple unstudied isolates.

1. Introduction

Staphylococcus aureus is an emerging bacterial pathogen and a leading cause of hospital- and community-acquired infections. In the United States alone, *S. aureus* is responsible for hundreds of thousands of infections and thousands of deaths each year (Klein et al., 2007). The severity of the disease caused by *S. aureus* is dependent upon both host susceptibility and the genomic background of the infecting *S. aureus* strain. The *S. aureus* genome encodes multiple virulence factors, including numerous toxins, surface adhesion proteins and proteolytic enzymes that synergistically contribute to virulence. Comparative genomic analysis of sequenced *S. aureus* genomes suggests that the number of virulence factors, as well as the presence and expression of specialized factors, such as the phenol soluble modulins (Diep and Otto, 2008) and ACME (Diep et al., 2006) can be linked to disease outcome. Many of the virulence factors are carried on mobile genetic elements (MGE), including pathogenicity islands, bacteriophages and plasmids; that are responsible for horizontal gene transfer between individual strains. Acquisition of known or novel virulence factors by mobilization may lead to the emergence of hypervirulent strains or strains linked to a specific disease outcome. However, the enhancement of virulence need not be solely due to novel sequence. Sequenced *S. aureus* genomes still have relatively large numbers of Open Reading Frames (ORFs) without functions attributed to them. Within the thirteen sequenced *S. aureus* genomes, over 40% of the ORFs on their chromosomes are annotated as Hypothetical, Conserved Hypothetical or Unknown Function. Thus, it is quite likely that many of these ORFs are responsible for virulence, despite having no clear assigned function.

Several techniques, including whole genome sequencing and microarray based methods (Dunman et al., 2004), have been used to associate genomic content with a pathogenic phenotype in *S. aureus*. While whole genome sequencing is the preferred method for identifying DNA polymorphisms and genomic rearrangements across strains, alternative array based methods such as array comparative genome hybridization (aCGH) is often used for detection of gene or operon size differences that may contribute to pathogenicity or fitness of the bacterium. However, a significant drawback of aCGH as well as other microarray based methods is their reliance on probes representing known genes and inability to discriminate

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0167-7012/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2010.01.022

between genes with significant sequence divergence or identify genes that are novel or are acquired by horizontal transfer from other strains or species (Snipen et al., 2006).

A more effective method for novel virulence ORF discovery would use a high-throughput DNA sequencing approach that surveys multiple genomes in a single experiment and targets novel regions of the genome or those potentially associated with virulence. One DNA sequencing based method, Suppression Subtractive Hybridization (SSH), has been used in the past as an effective method of comparing two bacterial genomes (Diatchenko et al., 1996) and identification of sequence level differences between a control or reference strain and a test strain (Agron et al., 2002; Dordet-Frisoni et al., 2007; Guo et al., 2006). Adaptation of SSH to examine an environmental metagenome by other investigators (Galbraith et al., 2004) led us to develop an approach in which genomic DNA from multiple *S. aureus* isolates was combined into a single pool that was hybridized to a reference strain. This approach, which we have named Pooled Suppression Subtractive Hybridization (PSSH), vastly increases the power and utility of SSH and has enabled us to identify multiple novel virulence factors among a collection of clinical *S. aureus* isolates.

We applied PSSH (Fig. 1) to survey a collection of 40 clinical *S. aureus* bacteremia isolates that were members of clonal complexes (CC) 5 and 30 associated with hematogenous seeding causing complicated endocarditis and bone and joint infections (Fowler et al., 2007). These isolates were pooled into four groups of ten isolates, based on CC (5 or 30) and complication type (endocarditis or bone and joint) and screened by PSSH using *S. aureus* NCTC 8325, a laboratory strain (Gillaspy et al., 2006) as the reference. We found that SSH can be scaled up to include pools of ten closely related microbial genomes. The application of PSSH to these pools quickly generates a library which allows for the detection of genomic differences of various sizes. We found PSSH has an exponential increase in genetic polymorphism detection power with the same efficiency and cost as a single strain comparison by SSH.

Overall, we describe a novel application, PSSH, which can be used in high-throughput screens of any bacterial genus/species to identify genomic level differences responsible for unique virulence phenotypes.

2. Methods

2.1. Strain isolation, characterization and general procedures

Strains (Table 1) were kindly provided by Vance G. Fowler from the *Staphylococcus aureus* Bacteremia Group (SABG) collected from patients at Duke University (Fowler et al., 2007). Genomic DNA was isolated as previously described in Fowler et al. (2007).

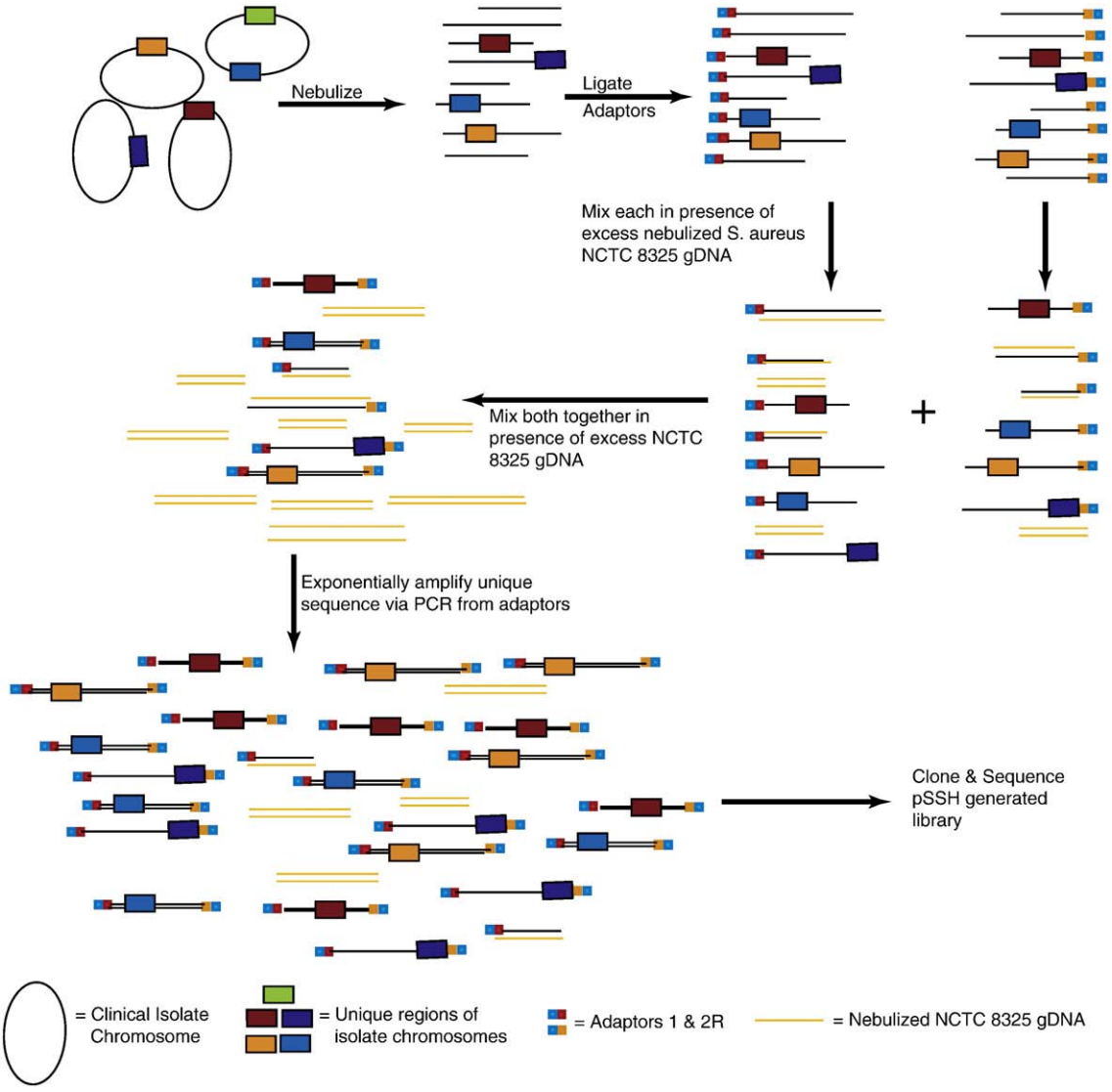

Fig. 1. Overview of the process of Pooled Suppression Subtractive Hybridization.