GUEST COMMENTARIES

Flexible Architecture of the *Streptococcus pyogenes* FCT Genome Region: Finally the Clue for Understanding Purulent Skin Diseases and Long-Term Persistence?[▽]

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Within a very short period, two papers in the *Journal of Bacteriology* highlighted what is probably the hottest current topic in research on the important human pathogen *Streptococcus pyogenes* (18, 22) by addressing the molecular epidemiology of the FCT genome region components and their contributions to *S. pyogenes* skin infections. There is a direct line from the publication of Bessen et al. (6) about the molecular epidemiology of the FCT region regulator genes back to the original paper of Bessen and Kalia (5), in which the principal structure of this genome region was defined and the term FCT (for fibronectin–collagen–*T*-antigen) region was coined. In fact, at that time, a good deal of sequence and functional data had been compiled by several groups, particularly concerning the fibronectin-binding proteins and global regulators encoded by the FCT region. However, Debra Bessen's group provided the "Columbus' egg" solution by combining these data, extending the sequence information, and giving new sense to studies on *S. pyogenes* adhesins.

Now, what is this mysterious genome region? The genomes of various *S. pyogenes* M-serotype strains contain 11 to 16 kb, flanked by highly conserved genes encoding a potential small heat shock protein and a protein with unknown function, that differ extensively between the individual strains in terms of the gene contents and the sequences of single genes. The region always contains at least one gene for what is currently understood to be a stand-alone regulator, *RofA* or *Nra*, and a surface-expressed, cell wall-anchored adhesin. In the overwhelming majority of strains tested, this adhesin is protein F1 or F2, and it binds to fibronectin. Many strains also encode a collagen type I-binding protein, *Cpa*, and an AraC-type regulator, *MsmR*, in their FCT regions. Based on the collection of *S. pyogenes* genome sequences predominantly contributed by Jim Musser's group (see reference 4 for a complete list of references), we now know that there are more than 20 additional genes for other functionally less understood or undefined proteins contained in various combinations in the FCT regions of diverse *S. pyogenes* serotype strains. Although the occurrence of various virulence factor genes within the FCT region, as well as the structural variability of this region between different *S.*

pyogenes strains, suggests a pathogenicity island status, there are several arguments against this classification. Thus far, the FCT region has been found in every *S. pyogenes* wild-type strain tested. As mentioned above, it is not flanked by, e.g., tRNA genes, as true pathogenicity islands often are in other gram-negative or -positive bacteria. Finally, consecutive in vitro passages of individual strains have not resulted in loss of the FCT region or even selected genes thereof.

The crucial roles of the fibronectin-binding proteins F1 (also termed SfbI) and F2 (also termed Pfbp or FbaB) in the pathogenesis of *S. pyogenes* infections have been demonstrated on several occasions, since isogenic *prtF1* or *prtF2* mutants attach with less than 10% efficiency to their epithelial target cells and are significantly attenuated in animal model infections, while decoration of beads with recombinant protein F1 or F2 is sufficient for their firm attachment to and eventual internalization into epithelial cells (17, 21, 22, 24, 33, 37, 39). However, the ubiquitous presence of the two genes in the *S. pyogenes* FCT regions does not explain the propensities of different *S. pyogenes* strains to infect their hosts predominantly either via the throat or the skin route and subsequently to cause various complications in a strain-specific manner or to invade epithelial cells and persist therein. In addressing this important issue, the functional characterization of all FCT region factors and the determination of structural variability within FCT regions and the relationship to all aspects of *S. pyogenes* infections have become equally significant.

In spite of the uniform binding to fibronectin, a critical role of protein F1 or F2 in targeting specific host tissue types cannot be ruled out. In both proteins, the two fibronectin-binding domains are located close to the C terminus and adjacent to the cell wall-anchoring sites of the mature proteins. However, both protein species comprise several hundred N-terminal amino acid residues with a high degree of sequence variation between individual strains and, so far, no attributed function (32, 40). Therefore, these sections of protein F1 or F2 could contribute to the specific tissue tropism.

Similarly, the collagen type I-binding domain of *Cpa* has been located in the central regions of molecules from serotype M1 and M49 strains (20) with highly variable and functionally unannotated sequences upstream and downstream of the binding region. *Cpa* proteins from other serotype strains—roughly two-thirds carry a *cpa* gene, according to Kratovac et al. (18)—may bind other types of collagen at their specific sites, and still

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other host factors could interact with the variable N and C termini of these molecules.

Since one-third of the serotype strains tested by Kratovac et al. (18) harbor Cpa and protein F2 genes and another third have an additional protein F1 gene in their FCT regions, the mere combination of sequence variants of these two or three genes could also sufficiently explain the tissue tropism of a given *S. pyogenes* isolate. The in silico-predicted van Willebrand factor binding capacity of the FctX protein (B. Kreikemeyer, unpublished results), one of the less frequent and functionally undefined proteins encoded by the FCT region, would direct a strain's attachment to still other types of host target structures.

One of the most remarkable discoveries in the last 2 years was the demonstration of pilus-like appendages formed by several FCT region-encoded proteins (27). The responsible genes could be part of a five-gene operon, as in FCT types 2, 3, and 4 described by Kratovac et al. (18), or they could be transcribed, probably into monocistronic messages, as was found in the type 1 FCT region. In the FCT-2, -3, and -4 regions, the operon contains genes for a potential signal peptidase (*lepA* or *sipA*) and a specialized sortase (*srtC*) (1, 2; M. Nakata, C. Riani, L. Jonas, D. A. Ribardo, K. S. McIver, S. Kawabata, A. Podbielski, and B. Kreikemeyer, unpublished data), in addition to *cpa* plus the *fctA* and *fctB* genes. The FctA protein most probably forms the backbone of the pilus, while FctB and Cpa are ancillary proteins added to circumscribed sections of the pilus, but they may also be directly inserted into the cell wall. In contrast, the serotype M6 pili formed by the prototypical type 1 FCT region-encoded proteins contain the T-antigen backbone protein and only one ancillary protein, FctX.

An exciting aspect of the pilus story relies on the fact that similar structures have been described in *Streptococcus agalactiae* and *Streptococcus pneumoniae* (recently reviewed in references 34 and 38). As demonstrated by the genome sequences presented by Beres et al. (4) and the results of Kratovac et al. (18), the block of responsible genes has potentially been exchanged among the three species. Since protective immune responses to serotype M1 *S. pyogenes* pilus proteins were evoked in a mouse model infection (27), the utilization of the proteins for a vaccine potentially directed against all three important bacterial pathogens is conceivable (8).

However, several general problems need to be addressed first. As impressively shown by Kratovac et al. (18) in *S. pyogenes* strains, the structural and potential sequence diversity between the pilus loci is immense and may by itself prevent the generation of a broadly applicable vaccine. Also, the function of the pili and their true impact on the pathogenesis processes are unclear. According to the study by Lizano et al. (22), deletion of the Cpa gene attenuated serotype M53 bacteria grown to log phase in a humanized mouse skin model, while mutation of the FctA backbone protein had no effect. The expression of these two proteins was irrelevant for an infection caused by stationary-phase bacteria. An ex vivo *S. pyogenes* transcriptome analysis performed on skin samples from mice infected with a serotype M1 strain failed to detect transcripts from the Cpa operon (15). When we inactivated the Cpa operon-encoded sortase in a serotype M49 strain and thus prevented pilus formation, but not Cpa cell wall insertion, as evidenced by

Western immunoblots and electron microscopy, we observed larger skin lesions in a mouse model infection (Nakata et al., unpublished).

Finally, the expression regulation of the Cpa operon or the corresponding pilus genes is complex and, again, probably strain specific. First, pili have been demonstrated with unequal frequencies on the surfaces of various serotype strains. For strains with a Cpa operon, it is unclear how they are generally able to form pili. The Cpa operon does not contain obvious attenuators. Therefore, if the bacteria produce large amounts of FctA to form the pilus backbone, what happens to the processing and ancillary proteins that are transcribed at equal levels but are needed at much lower levels? Is the polycistronic message selectively processed, or are the proteins degraded or used at different sites?

Furthermore, there is huge variation among cognate regulators in terms of presence and precise function. In most strains with a Cpa operon, the FCT region also harbors a combination of either a RofA or Nra regulator plus an MsmR regulator. Nra and MsmR have been demonstrated to exert an antagonistic negative-positive control of Cpa operon transcription (28, 31). As opposed to Nra, RofA acts as a positive regulator for at least one FCT region gene, *prtF1* (13). In some strains with a Cpa operon, an additional RALP4 regulator harbored in a pathogenicity islet outside the FCT region is superimposed on the Nra-MsmR regulatory axis (B. Kreikemeyer, M. Nakata, T. Köller, H. Hildisch, V. Kourakos, G. Beyer-Sehlmeyer, K. Standar, M. Glocker, and A. Podbielski, submitted for publication). In all strains without the Cpa operon, the FCT region exclusively contains a RofA regulator. However, in a serotype M2 and M6 strain, RofA was shown to display opposite regulatory effects on corresponding virulence factors (19). Overall, the regulation of FCT region factors, in terms of the regulators involved and the activities of single regulators, appears to be at least serotype specific and could even be strain specific.

Even in single, thoroughly studied strains, the analysis of Cpa operon regulation is far from complete. Thus, in our serotype M49 isolate, the operon is much more strongly expressed when the isolate is incubated at 30°C, a temperature consistent with typical skin conditions (Nakata et al., unpublished). However, the responsible regulatory mechanism is unknown. Overall, the abundance of *cpa* message isolated from all bacteria of a culture is relatively low, but at present, we do not know the *cpa* expression levels within single cells.

When individual cells were inspected by immunofluorescence or electron microscopy, only a small subset expressed Cpa exclusively at the older cell poles (20; Nakata et al., unpublished). While the localized deposition could be a consequence of the interplay of the specific signal peptide of Cpa and the operon-encoded signal peptidase (9), the expression pattern of Cpa, restricted to a few cells, resembles the bistability phenotype in other gram-positive bacteria (11) and could be due to an open-circuit type of regulation (3), which is so far unknown in streptococci. Since the proportions of Cpa operon-expressing bacteria may vary between different strains, the bistability phenotype could explain the variable impacts of pilus interference strategies when different strains are used for superficial animal infections. If bistability prepares a subset of bacteria within the large local population for quick adaptation

to environmental changes, what are the conditions that require the presence or absence of pilus structures? And how promising, in terms of preventing a disease or eradicating persistent bacteria, would a vaccine directed against only a subset of the bacterial target population be?

Some answers may appear when one looks at the molecular epidemiology of the FCT region genes and the relationship to the presence of other virulence factors or the particular virulence properties of the studied strains. Screening for the presence of the protein F1 gene, and later also for the protein F2 and Cpa genes, in the genomes of strains from (inter)national collections or isolated from patients with defined diseases or complications has been performed for 12 years with results varying by up to 10-fold (14, 19, 26, 29, 42). In some, but not all, studies of *S. pyogenes* treatment failures, an association with the presence of protein F1 was observed (7, 12, 16, 30, 35, 36), which was explained by an increased bacterial capacity to internalize into the protective intracellular spaces of host cells due to protein F1 functions. Data on the impact of the presence of protein F1 or F2 on the tissue specificity or invasiveness of clinical *S. pyogenes* isolates were contradictory (10, 23, 25, 26).

A simple explanation for some ambiguous results in the earlier studies lies in the quality of the probes used for the molecular screening. Only in the last few years have a sufficient number of *S. pyogenes* genome sequences become available to generate probes with predictable sensitivity and specificity. Another explanation could be the very small number of markers that were examined in the above-mentioned studies, causing us to look at the bacteria at too narrow an angle. By listing all FCT region genes in terms of presence or absence in a large number of genetically well-defined strains, Kratovac et al. (18) took an important step in the right direction. Together with the group's previous study of the presence of Mga and RofA/Nra regulators in a large strain collection (6), the attempt to elucidate the association between special sets of virulence genes responsible for eukaryotic-cell attachment and internalization, as well as prevention of phagocytosis and the clinical circumstances under which a given *S. pyogenes* strain was isolated, probably made much better sense than any approach in previous studies. Based on their results, Kratovac et al. (18) could predict that Cpa or protein F1 primes/facilitates skin or throat infections, respectively, although the presence of some skin or throat strains without the *cpa* or *prtF1* genes indicated the existence of alternative infection strategies.

Because the primary aim of the study was to analyze genetic linkage, the strains of Kratovac et al. (18) were chosen by maximizing their genetic distance. The clinical background as throat or skin strains had been recorded for only about 40% of the isolates. Another study is needed in which only strains with a defined clinical background are included. In addition, groups of several strains from a few epidemiologically important M serotypes should be tested. This should help to decide which combination of markers shows the strongest association to the clinical impact of the *S. pyogenes* isolates. In turn, this determination would promote clinical epidemiology analyses and support the choice of strains best suited for tedious molecular characterizations of complex virulence factor interactions or regulatory networks—not only for *S. pyogenes*, but also for *S. dysgalactiae*, which shares the *emm* region and at least some factors of the FCT region with *S. pyogenes* (6, 41). The results

of Kratovac et al. (18) provide optimism that these solutions will soon be feasible.

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