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The Innate Immune Modulators Staphylococcal Complement Inhibitor and Chemotaxis Inhibitory Protein of *Staphylococcus aureus* Are Located on β-Hemolysin-Converting Bacteriophages

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Two newly discovered immune modulators, chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS) and staphylococcal complement inhibitor (SCIN), cluster on the conserved 3′ end of β-hemolysin (*hlb*)-converting bacteriophages (βC-φs). Since these βC-φs also carry the genes for the immune evasion molecules staphylokinase (*sak*) and enterotoxin A (*sea*), this 8-kb region at the 3′ end of βC-φ represents an innate immune evasion cluster (IEC). By PCR and Southern analyses of 85 clinical *Staphylococcus aureus* strains and 5 classical laboratory strains, we show that 90% of *S. aureus* strains carry a βC-φ with an IEC. Seven IEC variants were discovered, carrying different combinations of *chp*, *sak*, or *sea* (or *sep*), always in the same 5′-to-3′ orientation and on the 3′ end of a βC-φ. From most IEC variants we could isolate active bacteriophages by mitomycin C treatment, of which lysogens were generated in *S. aureus* R5 (broad phage host). All IEC-carrying bacteriophages integrated into *hlb*, as was measured by Southern blotting of R5 lysogens. Large quantities of the different bacteriophages were obtained by mitomycin C treatment of the lysogens, and bacteriophages were collected and used to reinfect all lysogenic R5 strains. In total, five lytic families were found. Furthermore, phage DNA was isolated and digested with EcoR1, revealing that one IEC variant can be found on different βI-φs. In conclusion, the four human-specific innate immune modulators SCIN, CHIPS, SAK, and SEA form an IEC that is easily transferred among *S. aureus* strains by a diverse group of β-hemolysin-converting bacteriophages.

Recently, we described two new innate immune modulators of *Staphylococcus aureus*: staphylococcal complement inhibitor (SCIN) and chemotaxis inhibitory protein of *S. aureus* (CHIPS). SCIN is a C3 convertase inhibitor, blocking the formation of C3b on the surface of the bacterium and the ability of human neutrophils to phagocytose *S. aureus* (25). CHIPS is a bacterial chemokine receptor modulator that specifically binds two chemokine receptors. CHIPS attenuates the response of the C5a receptor (C5aR) as well as the formylated peptide receptor of human neutrophils. This results in inhibition of neutrophil chemotaxis and activation in response to C5a and formylated peptides (6, 10, 20, 21). Both SCIN and CHIPS are important virulence factors that protect *S. aureus* from innate immune defense systems.

The SCIN (*scn*) and CHIPS (*chp*) genes are found in 6/7 and 3/7 sequenced *S. aureus* strains, respectively. Analyses of these sequenced genomes showed that both *scn* and *chp* are carried by β-hemolysin (*hlb*)-converting bacteriophages (βC-φs), formerly known as double- and triple-converting phages. Double-converting bacteriophages were earlier described to negatively convert β-hemolysin expression but simultaneously introduce the staphylokinase (SAK) gene. Triple conversion leads to β-hemolysin-negative and SAK- and staphylococcal enterotoxin A (SEA)-positive strains. φ13 and φ42 are examples of, respectively, double- and triple-converting bacteriophages. Both φ13 and φ42 incorporate in the *S. aureus* genome via an orientation- and site-specific recombination by targeting a 14-bp core sequence present in the β-hemolysin gene (*attB*) and the phage genome (*attP*) (2–5). Besides SCIN and CHIPS, the two other molecules that are encoded adjacent to the CHIPS gene are also involved in opposing the innate immune system. SEA is a superantigen but also has the capability to modulate the function of chemokine receptors like CCR1, CCR2, and CCR5 (23). SAK has recently been described as a modulator of different parts of the innate immune system: Tarkowski's group showed that SAK directly destroys defensins (14), and we described that SAK has antiopsonic activities (24). Interestingly, all four immune evasion molecules on βC-φs display an extreme human specificity.

In this paper we describe that the SCIN and CHIPS genes are located on an 8-kb region at the conserved 3′ end of β-hemolysin-converting bacteriophages. This 8-kb region at the conserved 3′ end also encodes the genes for SAK, SEA, or SEP, thereby forming an immune evasion cluster (IEC). Since IECs are bacteriophage encoded, we investigated the distribution and diversity among clinical *S. aureus* isolates.

MATERIALS AND METHODS

Bacterial strains. In this study, the classical laboratory strains COL, Smith diffuse, Wood 46, Cowan, and Newman were used, next to 85 clinical *S. aureus* strains. Of these 85 strains, 18 methicillin-resistant *Staphylococcus aureus* isolates, 10 blood isolates, 10 continuous ambulatory peritoneal dialysis isolates, 5 liquor isolates, 4 pulmonary fluid isolates, 1 pericardial venal isolate, and 3 joint isolates were obtained within the UMC Utrecht. Furthermore, 15 wound isolates

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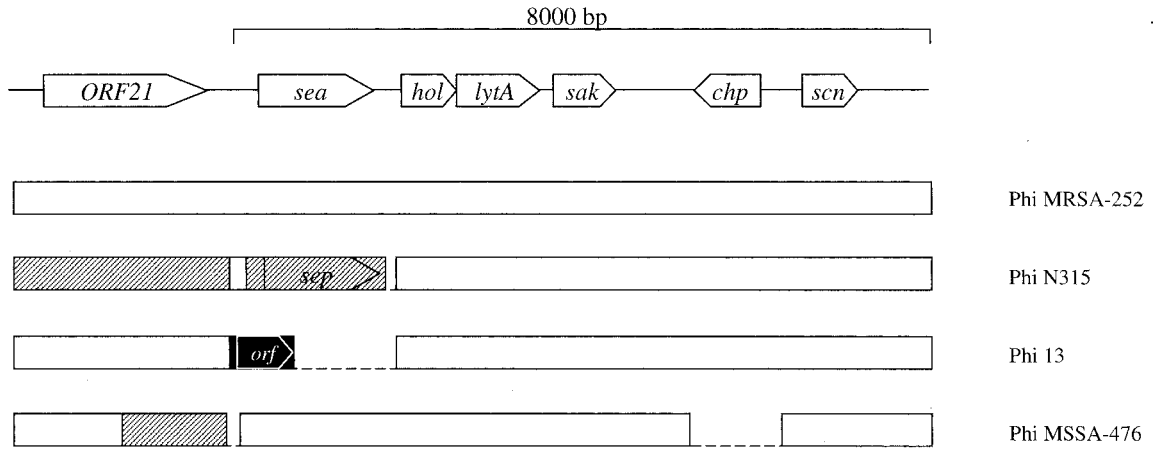


FIG. 1. Comparison of the genomic organization of the conserved 3′ ends of four representative βC-φs obtained from *S. aureus* strains φ-MRSA-252, φ-N315, φ13, and φ-MSSA-476. White bars represent homologous nucleotide regions (>99%), hatched areas show a homology between 75% and 85%, black bars show no significant homology, and dashed lines indicate deletions in the corresponding sequence.

were obtained from the Diakonesse Hospital Utrecht, 10 blood isolates came from the Wilhelmina Children's Hospital Utrecht, and 9 blood isolates came from the RIVM (National Institute for Public Health and the Environment, Bilthoven, The Neverlands). *S. aureus* R5, known for its susceptibility to a broad range of *S. aureus* bacteriophages, was kindly provided by the RIVM for transduction experiments. This study used sequence data of MRSA-252 (11), MSSA-476 (11), N315 (16), Mu50 (16), MW2 (1), NCTC 8325 (NC002954), φ13 (12), and φ42e (17).

DNA extraction. Bacteria were cultured overnight (ON) at 37°C on blood agar (Becton Dickinson, Sparks, MD). Chromosomal DNA was isolated using the High Pure PCR template preparation kit (Roche) according to the manufacturer's protocol, with the exception that bacteria were lysed using 50 μg/μl lysostaphin (Sigma).

PCR analyses. Primers were designed specific for *chp*, *sak*, *sea*, *sep*, *scn*, and *hlb* (Chp-1, TTTACTTTTGAACCGTTTCCTAC; Chp-2, CGTCCTGAATTCT TAGTATGCATATTCATTAG; Sak-1, AAGGCGATGACGCGAGTTAT; Sak-2, GCGCTTGGATCTAATTCAAC; Sea-1, AGATCATTCGTGGTATA ACG; Sea-2, TTAACCGAAGGTTCTGTAGA; Sep-1, AATCATAACCAACC GAATCA; Sep-2, TCATAATGGAAGTGCTATAA; Scn-1, AGCACAAGCTT GCCAACATCG; Scn-2, TTAATATTTACTTTTTAGTGC; Hlb-1, GTTGGTG CTCTTACTGACAA; and Hlb-2, TGTGTACCGATAACGTGAAC).

Amplification was carried out on a PE 9600 thermocycler (Perkin-Elmer Corp., Norwalk, Conn.) under the following conditions: 30 cycles of 30-s denaturation at 94°C, 30-s annealing at 50°C, and elongation at 72°C depending on the fragment length (1 kb/min). Amplification products were electrophoresed on a 1% agarose gel containing ethidium bromide and visualized by transillumination under UV.

Molecular characterization. Southern blot analyses of the conserved 3′ ends were performed on EcoRI/XhoI-digested chromosomal DNA using digoxigenin-labeled specific probes for *chp*, *sak*, *sea*, and *sep* as described previously (27). Pulsed-field gel electrophoresis (PFGE) typing by SmaI macrorestriction was performed as described by Struelens et al. (26). For identification of *agr* alleles, an *agr* group-specific multiplex PCR was performed on chromosomal *S. aureus* DNA according to the methods described in reference 9. Amplification products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualized by transillumination under UV.

Bacteriophage induction and DNA isolation. Bacteriophages were induced and isolated according to the methods described by Kaneko et al. (15). In short, bacteria were grown to exponential growth phase and treated with 1 μg/ml mitomycin C (Sigma) for 3 h at 30°C. Lysates were centrifuged twice at 15,000 × *g* for 20 min. After addition of polyethylene glycol 6000 (10%) and NaCl (0.7 M), mixtures were incubated for 12 h at 4°C. Finally, bacteriophages were isolated by centrifugation at 3,000 × *g* and suspended in phosphate-buffered saline. Chromosomal bacteriophage DNA was isolated with the High Pure PCR template preparation kit (Roche) using the manufacturer's instructions on isolation of nucleic acids from bacteria/yeast.

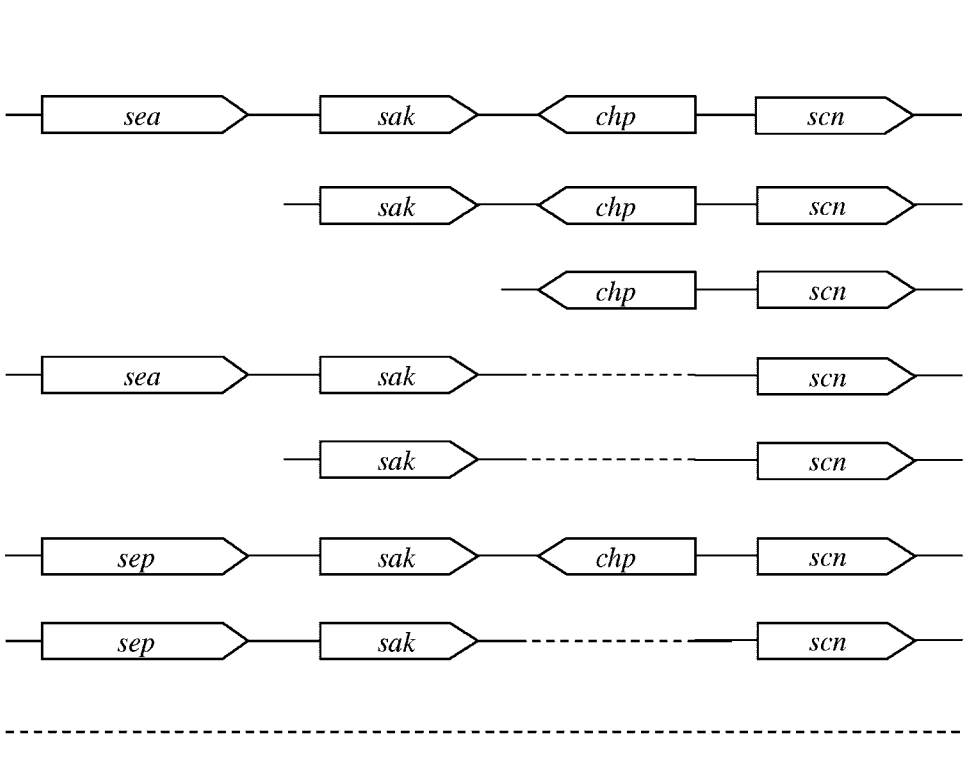
Lysogen generation. Bacteriophages obtained from the clinical *S. aureus* strains were serially diluted and mixed with *S. aureus* R5 and soft LB agar (LBA). Mixtures were plated on LBA containing 0.5 mM $CaCl_2$ (LBA-Ca) and incubated ON at 37°C. Loose plaques were picked and used to inoculate fresh *S. aureus* R5 in LB. After ON incubation at 37°C, bacteria were plated on LBA and single colonies were tested for lysogeny using PCR specific for conserved 3′ ends and Southern blotting.

Lytic assay. Serial dilutions of bacteriophages were spotted on lysogenic strains plated on LBA-Ca and incubated ON at 37°C. A lysogenic strain was designated resistant when, compared to *S. aureus* R5, an over-fourfold amount of bacteriophage was needed to induce lysis.

CHIPS capture enzyme-linked immunosorbent assay. *S. aureus* lysogenic strains were cultured on blood agar for 18 h. One CFU was used to inoculate 3 ml Todd-Hewitt medium and cultured overnight at 37°C under constant agitation. The optical density was measured at 650 nm, and bacteria were pelleted by centrifugation. Supernatants were stored at −20°C for analysis. CHIPS was quantified as described earlier (10).

RESULTS

***scn* and *chp* are part of a bacteriophage.** Database analyses revealed that *scn* and *chp* are both located on βC-φs. From databases we obtained the DNA sequences of seven bacteriophages that were incorporated in the β-hemolysin gene. One is published as an individual bacteriophage (φ13), whereas the others are part of the genomes of six different *S. aureus* strains: MRSA-252, MRSA-476, Mu50, MW2, N135, and NCTC 8325. Since the βC-φ from NCTC 8325 is φ13, only φ13 was included in the analyses. The regions of βC-φs encoding phage packing, head, and tail proteins and modules for lysogeny are rather different. Despite these differences, all bacteriophages are (as can be expected) strictly homologous in the first 1,000 bp at the 5′ end, encoding the integrase and *attP*. In addition, the 3′ ends of βC-φs show an interesting similarity. In the prototype φ, MRSA-252, the genes encoding SEA, SAK, CHIPS, and SCIN are located at the final 8 kb of the 3′ end. In almost all strains this region is strictly homologous except for a 1.8-kb *sea* cassette, which is missing in φ-N315 and φ13. Interestingly, in φ-N315 the 1.8-kb *sea* cassette was replaced by a 1.75-kb *sep* cassette. Furthermore, a 0.78-kb *chp* cassette could not be found in φ-Mu50, φ-MW2, and φ-MSSA-476 (Fig. 1). Since the 8-kb region at the 3′ end of βC-φs encodes up to four different immune evasion molecules, we named this region an IEC.



IEC type	n (%)
A	11 (12.2%)
B	24 (26.7%)
C	12 (13.3%)
D	14 (15.6%)
E	13 (14.4%)
F	4 (4.4%)
G	3 (3.3%)
-	9 (10%)

FIG. 2. Schematic representation of the different IEC types and their incidence found among 85 clinical *S. aureus* strains and 5 classical laboratory strains. Arrow boxes indicate the location and orientation of *sea*, *sak*, *chp*, and *scn*, and dashed lines represent the absence of one or more of these genes. Data are based on Southern and PCR analyses.

Distribution of βC-bacteriophages and their immune evasion modulators. DNA was isolated from 85 randomly selected clinical strains and 5 classical lab strains to assess the frequency distribution of βC-ϕs. PCRs were performed using primer pairs specific for *sea*, *sep*, *sak*, *chp*, *scn*, and *hlb* in order to identify the presence of the genes. To obtain information on the organization of these genes, PCRs were performed using relevant combinations of the individual primers. In addition, Southern blot analyses were performed on EcoR1- or XhoI-digested chromosomal DNA of each strain using digoxigenin-labeled *chp*, *sak*, or *sea* probes. Figure 2 shows that βC-ϕs were found in 80 (88.9%) of the *S. aureus* strains, containing seven different IEC types named A through G. Type B (*sak-chp-scn*) showed the highest prevalence and was found in 24 (26.7%) strains. Concerning the virulence factors, CHIPS was present in 51 (56.6%) of these strains, SAK was in 69 (76.6%), and the superantigens SEA and SEP were in 25 (27.8%) and 7 (7.8%), respectively. Noteworthy is that SCIN was present in all βC-ϕ-containing strains. All IEC types contained up to four virulence factors located in the same order, 5′-*sea*/*sep*-*sak*-*chp*-*scn*-3′, and were always found at the 3′ end of βC-ϕs.

Diversity of bacterial strains containing IEC types A to G. To analyze the diversity of the collected clinical strains, we performed PFGE on SmaI-digested chromosomal DNA of all 90 strains. The obtained patterns were analyzed using Gel Compare (BioNumerics 4.0; Applied Maths, Sint-Martens-Latem, Belgium), and data were correlated with the data on IEC types A to G. Using a 55% cutoff value, the 90 strains could be divided into six PFGE super groups: IEC type A was found in three of six PFGE supergroups, B was found in four, C was found in one, D was found in four, E was found in five, F was found in one, and G was found in one. Using a 65% cutoff value, the 90 strains could be divided into 20 groups. In this case, IEC type A was found in 3 of 20 PFGE groups, type B was in 9, type C was in 3, type D was in 7, type E was in 11, type F was in 2, and type G was in 2. In particular, IEC types B, D, and E were found in multiple different PFGE variants. Although PFGE patterns were rather diverse, one group of 15 identical PFGE type strains contained 8 (53.3%) IEC type A, 3 (20%) type B, 1 (6.7%) type D, 2 (13.3%) type E, and in 1 (6.7%) for which no variant was found (Fig. 3).

Finally, we also typed our 90 strains for the global regulation system *agr*. We found *agr* groups 1, 2, 3, and 4 in, respectively, 46 (51.1%), 25 (27.8%), 18 (20.0%), and 1 (1.1%) of the strains. In *agr* groups 1 and 2, all IEC variants were found, except type F was not found in *agr* group 1. In *agr* group 3, IEC variants A, B, D, and E were found. The only a*gr* group 4-positive strains carried IEC variant E (Table 1).

In conclusion, βC-ϕs with the same IEC types can be found in different PFGE and *agr* groups. Moreover, related *S. aureus* strains can contain βC-ϕs with different IEC variants. These data strongly suggest that the βC-ϕs carrying IECs are mobile elements that move around in the population.

βC-ϕs are active prophages. First, we tested whether we could induce, isolate, and transfer βC-ϕs from clinical strains into *S. aureus* R5 (the broad phage acceptor strain); therefore, *S. aureus* strain C555, carrying a βC-ϕ with IEC B, was treated with mitomycin C. Collected bacteriophages contained IEC

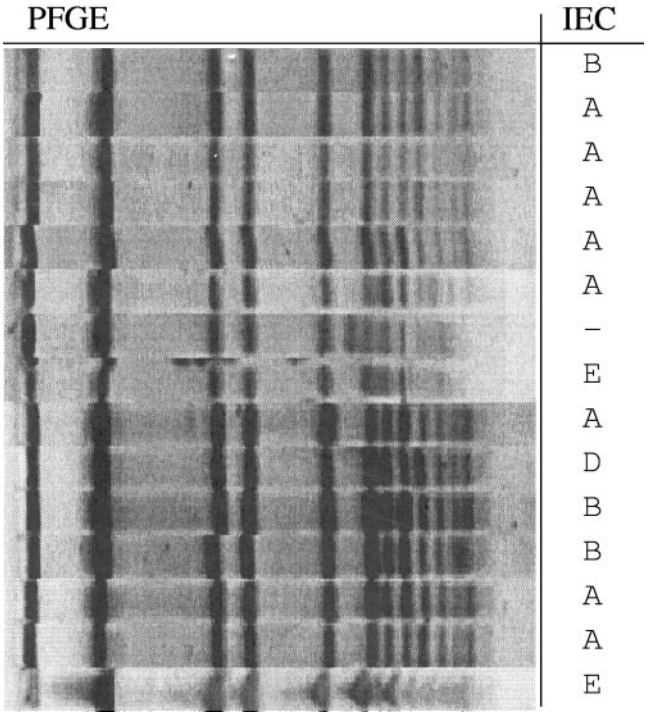


FIG. 3. Related *S. aureus* strains harbor different IEC types. The PFGE results of 15 very related *S. aureus* strains are shown in combination with their IEC types.

with *chp* as shown by PCR analysis. Subsequent incubation of *S. aureus* R5 with these *chp*-carrying βC-ϕs resulted in lysogenic strains that carried *chp*-positive βC-ϕs. These strains also produced and excreted CHIPS as measured in the supernatants by enzyme-linked immunosorbent assay. Then, a subset of 46 *S. aureus* strains equally distributed among the IEC variants and representing the major PFGE groups were analyzed as well. A total of 33 (71.7%) strains contained mitomycin C-inducible phages, for plaques were found on *S. aureus* R5. Among these inducible bacteriophages, 17 were βC-ϕs and all IEC variants were found except for type C. These data clearly show that βC-ϕs carrying the different IEC variants are active prophages. On the other hand, in 29 (63.0%) of the *S. aureus* strains no mobile characteristics of the βC-ϕs could be detected. This may be explained by loss of phage mobility or R5 host restriction.

Finally, 14 R5 lysogens were obtained with stable integrated βC-ϕs which contained the IEC variant A, B, D, or E. To determine the βC-ϕ incorporation site in these lysogens, we

TABLE 1. Distribution of IEC types among *S. aureus agr* types 1 to 4

agr type	No. (%) with *agr* type	No. with IEC variant							
		A	B	C	D	E	F	G	No IEC
1	46 (51.1)	3	17	1	11	7		2	5
2	25 (27.8)	1	2	11	2	3	4	1	1
3	18 (20.0)	8	5		1	2			2
4	1 (1.1)					1			

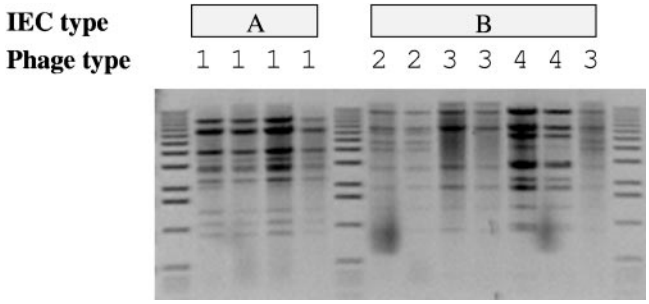


FIG. 4. One IEC variant can be carried by different βC-ϕs. Chromosomal DNA of βC-ϕs containing IEC A and B were digested with EcoR1 and analyzed on 1% agarose.

analyzed SmaI-digested chromosomal DNA by PFGE and Southern blotting using digoxigenin-labeled *chp*, *sak*, and *sea* probes (depending on the IEC type) and *hlb* probes. All IEC-containing bacteriophages were incorporated into *hlb.* All lysogens harbored one copy of the prophage, except for one where we found two copies (which was excluded from further analyses).

IECs are carried by different βC-ϕs. It is well known that lysogenic bacteria are resistant to infection with homologous bacteriophages. In addition, a recent study by Dempsey et al. (7) showed that ϕ42 introduces a restriction modification system into its lysogen, making the bacterium resistant to all international basic *S. aureus* typing phages. Using these properties we studied whether the IEC variants were carried by related or different βC-ϕs. Therefore, we took the 13 lysogens carrying one of the βC-ϕs with an IEC variant and induced bacteriophages from each individual strain using mitomycin C. All lysogens were assayed for lysis by reinfection with the whole set of IEC-containing bacteriophages. Lysis patterns indicated the presence of five lytic groups among IEC-containing βC-ϕs. Lysogens of one of the lytic groups showed resistance to all of the tested bacteriophages, which suggests that these βC-ϕs are ϕ42 or related bacteriophages. These data indicate that IECs can be carried by different βC-ϕs.

In addition, chromosomal DNA of all bacteriophages was isolated, digested with EcoR1, and analyzed on agarose. If βC-ϕs within one IEC group differed by three or more bands from each other, they were considered different. In the cases of IEC variants B and E, three different βC-ϕs were found and, in the case of variants A and D, only one was found (Fig. 4). This means that the same IEC variant can be located on different βC-ϕs.

DISCUSSION

Here we describe a staphylococcal IEC that is located on the 3′ end of β-hemolysin-converting bacteriophages, better known as double- and triple-converting bacteriophages. These phages were named for their capacity to delete the presence of β-hemolysin and add either *sak* or *sea* to the genome of the infected staphylococcus. Now that we know that SCIN and CHIPS are also present on these phages, we could rename these into triple-, quadruple-, and quintuple-converting phages. Bacterial virulence factors are frequently found on the 3′ ends of bacteriophages (28). Examples in *S. aureus* include

Panton-Valentine leukocidin (PVL; LukF-PV/LukS-PV) of ϕ-PVL and ϕ-SLT (15, 19), a PVL variant [LukF-PV(P83)/LukM] of ϕ-PV83 (30), and exfoliative toxin A (ETA) of ϕ-ETA (8) and ϕZM-1 (29). Interestingly, these bacteriophages carry only one virulence factor at their 3′ end. βC-ϕs are an exception, as these phages can encode four virulence factors clustered on their 3′ end. Furthermore, so far we identified seven different variants of IEC to be carried by several different βC-ϕs.

In 90% of strains from a genetically diverse clinical *S. aureus* strain collection, a βC-ϕ was found that carries one of the seven IEC variants. The high incidence of IEC-carrying βC-ϕs in human *S. aureus* strains is a unique feature among staphylococcal mobile elements carrying virulence factors. In a recent study (13) a collection of 198 clinical *S. aureus* strains was tested for a vast amount of virulence factors. *eta*, *lukS-PV*/*lukF-PV*, and *lukM*, which are all carried by bacteriophages, were found in only 18.7%, 18.2%, and 0.0% of the strains, respectively. In another study, PVL was found with an incidence of only 2% (22). Also, *S. aureus* pathogenicity island 1 encoding toxic shock syndrome toxin 1 can be mobilized by bacteriophages (18) and was found in only 19.7% of strains. All these data point in one direction: βC-ϕs carrying IECs are an exception. In comparison with other mobile virulence factors, IEC-encoding genes exhibit an exceptional high incidence among clinical *S. aureus* strains. One important reason for this is probably the fact that IEC can be carried by several different phages, allowing them to cover a huge host range. Furthermore, one can predict that diversity in the lysogenic module may facilitate the spread of βC-ϕs. If, for instance, a lysogenic strain (containing a βC-ϕ) were infected by a βC-ϕ with another lysogenic module, the lysogenic pathway would be blocked, while on the contrary the lytic pathway would not be. Lysogenic strains (containing a βC-ϕ) can therefore be strongholds for other βC-ϕs and allow them to spread. In addition, superinfections presumably facilitate recombination events, thereby promoting the development of the different variants of IEC.

The most striking feature of the collection of virulence factors in IEC is that they all, in one way or another, seem to affect certain elements of the human innate immune system. Next to its role as a superantigen, SEA has been described to modulate the function of chemokine receptors like CCR1, CCR2, and CCR5 (23). CHIPS blocks two other G-protein-coupled receptors involved in chemotaxis and phagocyte activation, the C5a receptor and the formylated peptide receptor, by direct binding to these receptors (6, 20). SAK was recently described to inhibit human α-defensins (14). In our own group we recently described that SAK is antiopsonic by degradation of both immunoglobulin G as well as C3b/C3bi on the surface of staphylococci that is mediated via plasmin (24). SCIN is a highly specific antiopsonic molecule as well, aimed at the key component of the complement system, the C3 convertase (25). This large collection of human-specific anti-innate immunity factors is a likely explanation for the high incidence of βC-ϕs among human *S. aureus* isolates.

The large variety in both IECs and βC-ϕs carrying this cluster show that the IEC is a very dynamic DNA element. It has spread successfully through the *S. aureus* population and will continue to do so. This provides *S. aureus* with a unique mechanism to adapt to, and counteract, the human host.

Elucidation of the mechanisms involved in IEC generation, acquisition, and exchange and the role in virulence will enhance our understanding of the niche-dependent evolutionary potentials of *S. aureus*.

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