ORIGINAL ARTICLE BACTERIOLOGY

Immune evasion cluster-positive bacteriophages are highly prevalent among human *Staphylococcus aureus* strains, but they are not essential in the first stages of nasal colonization

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Abstract

The *Staphylococcus aureus* immune evasion cluster (IEC), located on β-haemolysin-converting bacteriophages (βC-Φs), encodes the immune-modulating proteins chemotaxis inhibitory protein, staphylococcal complement inhibitor (SCIN), staphylococcal enterotoxin A and staphylokinase. Its precise role in *S. aureus* colonization is unclear. We studied the presence of the IEC-carrying bacteriophages in human and animal *S. aureus* isolates, using PCR for the gene encoding SCIN (*scn*). Human isolates were obtained by collecting serial nasal swabs from 21 persistent carriers. *S. aureus* strains from 19 (90%) persistent carriers contained an IEC that was present and indistinguishable in 95% of cases at all five sampling moments over a 3-month period. Of the 77 infectious animal strains included in the study, only 26 strains (34%) were IEC-positive. Integration of these IEC-positive strains into an amplified fragment length polymorphism genotype database showed that 24 of 53 (45%) strains were human-associated and only two of 24 (8%) were 'true' animal isolates ($p < 0.001$). The high prevalence and stability of IEC-carrying βC-Φs in human strains suggested a role for these βC-Φs in human nasal colonization. To test this hypothesis, 23 volunteers were colonized artificially with *S. aureus* strain NCTC 8325-4 with or without the IEC type B-carrying βC-Φ13. Intranasal survival was monitored for 28 days after inoculation. The strain harbouring βC-Φ13 was eliminated significantly faster (median 4 days; range 1–14 days) than the strain without βC-Φ13 (median 14 days; range 2–28 days; p 0.011). In conclusion, although IEC-carrying βC-Φs are highly prevalent among human colonizing *S. aureus* strains, they are not essential in the first stages of *S. aureus* nasal colonization.

Keywords: Artificial colonization, bacteriophages, human, immune evasion cluster, *Staphylococcus aureus*
Original Submission: 4 December 2009; **Revised Submission:** 15 February 2010; **Accepted:** 24 March 2010
Editor: J-M Rolain
Article published online: 25 May 2010
Clin Microbiol Infect 2011; **17:** 343–348
10.1111/j.1469-0691.2010.03227.x

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Introduction

Staphylococcus aureus is a human commensal, as well as an important pathogen that can cause infections ranging from mild to life-threatening [1]. *S. aureus* enters the body through breaches in the skin or mucous membranes, where it is immediately confronted by the innate immune system as the first line of defence. To counteract innate immunity, *S. aureus* expresses a number of immune-modulating proteins. One of the immune-modulating proteins is staphylococcal complement inhibitor (SCIN). It is an efficient complement inhibitor of the lectin, the classical and the alternative pathway. SCIN efficiently prevents opsonophagocytosis and killing of *S. aureus* by human neutrophils. Furthermore, SCIN prevents generation of C5a and neutrophil chemotaxis [2,3]. Recently, the gene encoding SCIN (*scn*) was found to be part of a so-called 'immune evasion cluster' (IEC). Seven IEC variants have been identified [4]. All IEC variants carry *scn* and a different combination of *sea, sak* and *chp*. These genes encode the human-specific immune modulators staphylococcal enterotoxin A (SEA), staphylokinase (SAK) and chemotaxis inhibitory protein of *S. aureus* (CHIPS). SEA is a well known superantigen [5], and is also involved in the down-regulation of chemokine receptors of monocytes [6]. SAK is a bacterial plasminogen activator. Plasmin formed by the conversion of plasminogen by SAK

leads to removal of important opsonic molecules, such as IgG and C3b, by cleaving these molecules. Therefore, SAK can efficiently prevent phagocytosis of staphylococci by human neutrophils [7]. SAK also inhibits the bactericidal effect of antimicrobial peptides, the α-defensins [8]. CHIPS blocks neutrophil chemotaxis by binding the formylated peptide receptor and the C5a receptor on neutrophils [4,9,10]. IECs are located on bacteriophages. Bacteriophages are mobile genetic elements that can be transferred between strains. The IEC-carrying bacteriophages are incorporated in the gene encoding β-haemolysin (*hlb*). Therefore, β-haemolysin is not produced when the IEC-carrying bacteriophage is present. Consequently, these bacteriophages are called β-haemolysin-converting bacteriophages (βC-Φs) [4]. It was shown that 90% of the human clinical *S. aureus* strains (isolated from blood, liquor, wounds, continuous ambulatory peritoneal dialysis fluid, pulmonary fluid, joint and pericardial fluid) contain an IEC-carrying βC-Φ; this is an exceptionally high percentage compared to other mobile elements carrying virulence factors in human *S. aureus* strains [4].

Little is known about the prevalence of IEC-carrying βC-Φs in animal *S. aureus* strains. It was shown that only 13 of 290 (4.5%) *S. aureus* strains isolated from cows are IEC-positive [11]. Other studies showed that β-haemolysin was produced or *hlb* was present in 66–92% of *S. aureus* strains isolated from cows [12–14]. This indicates that the prevalence of IEC-carrying βC-Φs in animal *S. aureus* isolates is low in contrast to the prevalence of these bacteriophages in human infectious *S. aureus* isolates. Because *S. aureus* infections are often endogenous [15,16], we hypothesized that IEC-encoded immune modulators play a role in colonization of *S. aureus* in humans, but not in animals. To test this hypothesis, we studied the presence and stability of IEC-carrying βC-Φs in nasal *S. aureus* isolates collected from healthy volunteers. Furthermore, we studied the prevalence of IEC-carrying βC-Φs in a well described collection of *S. aureus* animal isolates. Finally, we performed an artificial nasal inoculation study in human volunteers in which we compared survival between *S. aureus* strain NCTC 8325-4 with and without IEC-carrying βC-Φ.

Materials and Methods

Distribution and stability of IEC-carrying βC-Φs in human *S. aureus* strains

Human *S. aureus* strains were collected from nasal swab cultures from 21 healthy, adult persistent carriers who were positive for *S. aureus* at five culture moments over a time interval of 3 months. *S. aureus* was cultured quantitatively and identified as described previously [17]. For each culture, up to three colonies of each morphotype (colony morphology and haemolysis pattern) were stored at −80°C. Single-locus DNA sequencing of the repeat region of the protein A gene (*spa*) was used for comparative genotyping of the human *S. aureus* strains [18]. In all strains, *scn* was amplified to determine whether an IEC was present or absent [4]. Furthermore, *chp*, *sak*, *sea*, *sep* and *hlb* were amplified to determine the IEC type, using previously described PCR primers and amplification conditions [4]. Volunteers provided their written informed consent and the local Medical Ethics Committee of the Erasmus MC (Rotterdam, the Netherlands) approved the study (MEC-2007-106).

Prevalence of IEC-carrying βC-Φs in veterinary *S. aureus* strains

Seventy-seven *S. aureus* strains isolated from different infection sites in a variety of animal species were used. All strains were genotyped by amplified fragment length polymorphism (AFLP) analysis previously [19]. The AFLP fingerprints of these animal strains were introduced in an AFLP database comprising data of 829 nonclinical carriage strains isolated from healthy human individuals [20]. 'Typical animal strains' were defined as strains grouped in AFLP cluster IVa, as described by van Leeuwen *et al.* [19]. These strains show no clear AFLP integration with human carrier strains. 'Typical human strains' were defined as animal strains that show considerable integration with human carrier strains. They grouped in AFLP clusters I, II and III [19]. Chromosomal DNA was extracted from the animal strains and the *scn* gene was amplified [4].

Artificial nasal colonization with *S. aureus* strain NCTC 8325-4 with and without IEC-carrying βC-Φ

Study population. Twenty-three healthy individuals were included (eight males and 15 females, median age 29 years, range 19–58 years). The volunteers provided their written informed consent and they were notified of the fact that an infectious disease physician was on call for the entire study period. The study protocol was approved by the Medical Ethics Committee of Erasmus MC Rotterdam (MEC-2007-324).

S. aureus strains. C. Wolz provided *S. aureus* strains NCTC 8325-4 and 8325-4 with βC-Φ13 [21]. This strain has a defect in the *sigB* locus. Thus, although a strain from a natural carrier would have been preferred, we used NCTC 8325-4 because this strain has been approved for artificial colonization, is susceptible to all common antibiotics (e.g. flucloxacillin and vancomycin) and is devoid of staphylococcal toxins [22,23]. The volunteers were inoculated with either wild-type 8325-4 or 8325-4 with βC-Φ13 containing the IEC

[4]. We used βC-Φ13 because it has the most predominant IEC type (type B) in humans and this type has been completely sequenced [4,24]. Bacterial growth in tryptic soy broth was equal for both 8325-4 and 8325-4 with βC-Φ13 (data not shown).

Artificial inoculation protocol. The artificial inoculation protocol was as described previously [22,25]. In brief, before inoculation, the carrier state of the participants was defined on the basis of two nasal swabs. A volunteer was classified as a persistent carrier with two nasal swabs positive and as an intermittent or noncarrier with one or no swab positive. Furthermore, a serum sample was drawn for determination of the C-reactive protein (CRP) level, leukocyte number and anti-staphylococcal antibody levels. The antibody levels were determined using a bead-based flow-cytometry technique (xMAP; Luminex Corp, Austin, TX, USA) as described previously [26]. After taking the second swab, decolonization treatment was initiated for all participants (nasal mupirocin 2%; GlaxoSmithKline, Waltham, MA, USA) twice daily for 5 days in combination with once daily washing with chlorhexidine-containing soap (SSL Healthcare, London, UK). Five weeks after the treatment, nostrils were swabbed again to assess the colonization status and artificial inoculation was performed under medical supervision with either wild-type strain 8325-4 or 8325-4 with βC-Φ13. Inoculation was performed in a blinded fashion to prevent bias in reading the microbiological culture results. In both the left and right nostril, 1×10^7 CFU of the same strain were applied. Participants received hygiene advice and weekly medical checkups. Follow-up cultures were performed on days 1, 2, 3, 4, 7, 14, 21 and 28 after inoculation. At the end of the study, participants underwent their last medical examination and serum samples were drawn to again determine CRP levels, leukocyte numbers and anti-staphylococcal antibody levels. Furthermore, nasal, throat and perineum swabs were collected. Decolonization therapy was repeated for participants still carrying the inoculated strain at the end of follow-up.

Nasal swab cultures. Nasal swab cultures were performed as described previously [25,27]. Both the left and right anterior nares were swabbed. In short, the swabs were cultured quantitatively at 37°C on Columbia blood agar plates (Becton-Dickinson BV, Etten-Leur, the Netherlands) to visualize haemolysis patterns and were submerged in phenol red mannitol salt broth. For suspected colonies, a latex agglutination test (Slidex Staph Plus, bioMérieux, Marcy-l'Étoile, France) was performed. Three colonies of each morphotype were stored at −80°C. For all isolates, a *scn* and *spa* PCR was performed and isolates were typed by pulsed-field gel electrophoresis (PFGE) for identification and discrimination between inoculated and autologous *S. aureus* strains [4,28].

Statistical analysis

Statistical analyses were performed with SPSS software, version 15.0 (SPSS Inc., Chicago, IL, USA). A chi-squared test was used for comparing proportions or frequencies. After artificial colonization, the primary outcome was the survival time of *S. aureus* in the nose. The survival time was defined as the number of days until the final positive culture with the inoculated strain. Kaplan–Meier survival analysis (log-rank test) was used to compare survival between strain 8325-4 and 8325-4 with βC-Φ13. Participants still carrying *S. aureus* in the nose at the end of follow-up were censored in the analysis. To compare median number of CFUs and antibody levels between groups, the Mann–Whitney *U*-test was used; $p < 0.05$ was considered statistically significant.

Results

Distribution and stability of IEC-carrying βC-Φs in human *S. aureus* strains

spa-Typing showed that each of the 21 participants carried the same *S. aureus* strain at all five culture moments. Two of them incidentally carried an additional *S. aureus* strain.

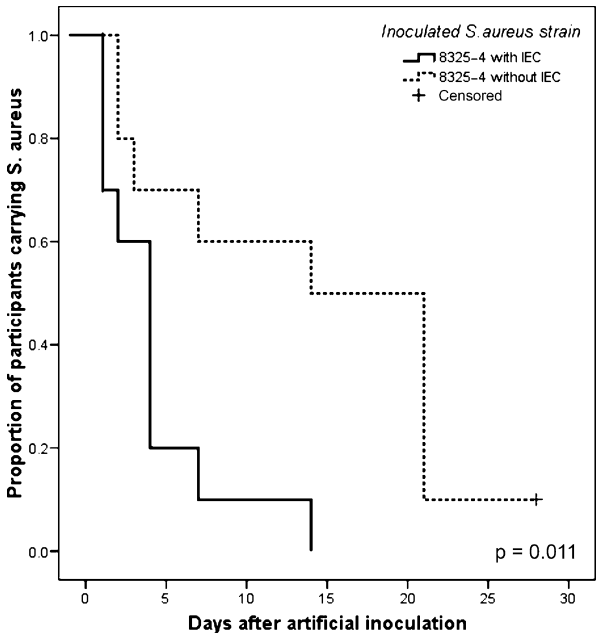

FIG. 1. Kaplan–Meier survival curves showing proportions of individuals with culture-positive nasal swab samples after artificial nasal inoculation of *Staphylococcus aureus* strain NCTC 8325-4 with or without immune evasion cluster (IEC)-carrying bacteriophage.

Isolates from 19 (90%) persistent carriers contained an IEC-carrying βC-Φ, as demonstrated by the presence of *scn*. The IEC in strains isolated from each individual was identical at all five culture moments in 18 out of 19 (95%) carriers. The predominant IEC variant was type B (*sak*, *chp* and *scn*), present in strains from 11 (58%) of 19 volunteers. Variant A (*sea*, *sak*, *chp* and *scn*), C (*chp* and *scn*), D (*sea*, *sak* and *scn*), E (*sak* and *scn*) and G (*sep*, *sak* and *scn*) were present in strains isolated from four (21%), two (11%), one (5%), zero and one (5%) of the nineteen carriers, respectively.

Prevalence of IEC-carrying βC-Φs in veterinary *S. aureus* strains

Twenty-six of the 77 (33.8%) veterinary isolates were *scn*-positive. Strains isolated from animals infected with a 'typical animal strain' were positive for *scn* in 8.3% (two of 24 strains). By contrast, strains isolated from animals infected with a 'typical human strain' were *scn*-positive in 24 of 53 (45.3%) isolates ($p < 0.001$). Animals infected with a 'typical human strain' were mostly pet animals in close contact with humans (cats, dogs, rabbits and horses). Human isolates ($n = 73$), sharing similar genetic backgrounds with the isolates obtained from animals infected with a 'typical human strain' (both integrated in almost the same position in the AFLP dendogram), were *scn*-positive in 83.6% of strains.

Artificial nasal colonization with *S. aureus* strain NCTC 8325-4 with and without IEC-carrying βC-Φ

Nine volunteers (39%) were classified as persistent carriers. Fourteen volunteers were classified as noncarriers (61%). Five persistent carriers and seven noncarriers were inoculated with strain 8325-4. The others were inoculated with strain 8325-4 with IEC-carrying βC-Φ. According to PFGE analysis and *spa*-PCR, in three carriers, including two carriers who still carried their own strain after mupirocin treatment, the follow-up cultures after inoculation contained their autologous strain or strains other than 8325-4 only. In none of these cultures was the inoculated strain detected, suggesting colonization resistance as a result of the enduring presence of their own strain. Therefore, these participants (one inoculated with the phage-positive strain and two with the phage-negative strain) were excluded from the analysis. For the remaining 20 participants, *S. aureus* survival and the number of CFUs per swab were determined. The Kaplan–Meier curves in Fig. 1 show the proportion of positive cultures during follow-up. The strain containing the IEC-carrying βC-Φ was eliminated significantly faster (median 4 days; range 1–14 days) than the strain without IEC-carrying βC-Φ (median 14 days; range 2–28 days; p 0.011). Antibody measurement showed that this was not a result of higher pre-existing levels of anti-CHIPS or anti-SCIN IgG in the group of volunteers who were inoculated with the IEC-positive strain (median level of anti-CHIPS IgG, median fluorescence intensity (MFI) 11604 vs. 12603; anti-SCIN IgG, MFI 8423 vs. 11580; $p > 0.05$). Although the bacterial count after inoculation appeared to be higher for the group of participants inoculated with IEC-containing 8325-4, this was not statistically significant (Fig. 2). No significant difference in survival and bacterial count of inoculated *S. aureus* was shown when noncarriers were compared with persistent carriers (median survival 4 days for both groups, range 1–28 days and 2–21 days, respectively), although this might be a result of the small number of persistent carriers. None of the volunteers experienced adverse effects and all adhered to the study protocol. At the end of the study, all participants were in good physical condition. Laboratory values indicated no signs of infection (CRP <1–8 mg/L, leukocytes 4.5–9.5 $\times$ 10^9/L) and anti-staphylococcal antibody levels showed no increase. All swabs were negative for the inoculated strain, except for one volunteer who was treated with mupirocin.

Discussion

It is known that bacteriophages are intrinsically unstable and that bacterial strains usually tend to loose their bacteriophages

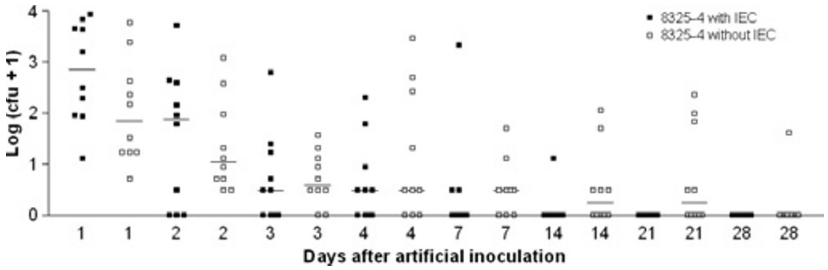

FIG. 2. Bacterial counts after artificial inoculation. Each dot represents the amount of CFUs in a nasal swab culture of a volunteer collected 1, 2, 3, 4, 7, 14, 21 and 28 days after artificial nasal inoculation of *Staphylococcus aureus* strain NCTC 8325-4 with or without immune evasion cluster (IEC)-carrying bacteriophage. Horizontal lines represent the median number of CFUs.

[29]. In the present study, we demonstrate that *S. aureus* strains of 90% of the persistent carriers contained an IEC-carrying bacteriophage that was present and undistinguishable in 95% of cases at all five culture moments over a 3-month period. This indicates that IEC-carrying bacteriophages are highly prevalent and stable over time in human *S. aureus* carriage isolates. The predominant IEC variant was type B, which is the predominant variant in human infectious isolates as well [4]. The prevalence of IEC-carrying βC-Φs in the veterinary *S. aureus* isolates was much lower (33.8%). However, the percentage of IEC-carrying βC-Φs in strains isolated from animals infected with human-related *S. aureus* strains (45.3%) was higher than in strains isolated from animals infected with animal-related strains (8.3%). Still, the percentage of IEC-carrying βC-Φs in strains isolated from humans was much higher. This indicates that human-related *S. aureus* strains in animals appear to lose their bacteriophages, whereas *S. aureus* strains in humans do not. This apparent advantage of IEC-carrying βC-Φs for human *S. aureus* strains, in combination with the high prevalence and stability, suggested a role for these bacteriophages in human *S. aureus* nasal colonization.

Using an artificial human inoculation study, we were unable to demonstrate an essential role for IEC-carrying bacteriophages in the first stages of colonization. The latter is not a result of the lack of expression of the IEC-encoded proteins. Inhibition ELISA assays showed that, under *in vitro* conditions, both SCIN and CHIPS are produced in *S. aureus* strain 8325-4 with IEC-carrying βCΦ13. The production of SCIN and CHIPS in this strain is comparable to the production of these proteins in strains isolated from eight of the persistent carriers included in the present study (data not shown). Perhaps IEC-carrying βC-Φs play a role in propagation and long-term (intracellular) survival, rather than adherence, of *S. aureus* in the nose. After all, these bacteriophages are present in almost all *S. aureus* carrier isolates [4] and, in all individuals tested so far, significant levels of antibodies directed against CHIPS and SCIN are detected, indicating wide spread *in vivo* expression [26,30]. This still does not explain why the phage-positive strain was cleared significantly faster than the phage-negative strain. A difference in growth rate *in vitro* was excluded. Furthermore, a difference in nose-picking behaviour between the volunteers inoculated with the phage-positive strain and phage-negative strain was excluded by analysing the questionnaires that were filled in after inoculation. Perhaps the introduction of IEC-carrying βC-Φs altered the regulation and composition of surface and excreted components of 8325-4 that are normally involved in colonization, and therefore the clearance of the phage-positive strain is faster. This question remains to be elucidated.

In conclusion, IEC-carrying βC-Φs are highly prevalent among *S. aureus* strains, although these bacteriophages are not essential in the first stages of nasal colonization. Further research into the role of IEC-carrying βC-Φs in human nasal colonization is necessary.

Acknowledgements

We thank C. Wölz for kindly supplying *S. aureus* strains NCTC 8325-4 and NCTC 8325-4 with βC-Φ13. We thank our volunteers for participating in this study.

Transparency Declaration

The authors declare that there is no source of funding and no potential conflict of interest.

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