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Original article

Auxotrophic mutant of *Staphylococcus aureus* interferes with nasal colonization by the wild type

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Abstract

Staphylococcus aureus nasal carriage is a risk factor for infection in humans, particularly in the hospital setting. Bacterial interference was used as an alternative strategy for the prevention of upper respiratory, urogenital and gastrointestinal tract infections. This study was designed to assess if the administration of a live-attenuated *aroA* mutant of *S. aureus* is useful as a potential approach to prevent transient staphylococcal nasal carriage by virulent strains. We constructed an *aroA* mutant of *S. aureus* Newman strain by homologous recombination. The auxotrophic NK41 mutant was attenuated as determined by the increase of the LD_{50} after intraperitoneal challenge. In mice, previous nasal colonization with the NK41 mutant significantly reduced the number of CFU of *S. aureus* (HU-71 and Hde288) clinical isolates and the parental Newman strain. The NK41 mutant was unable to induce a pro-inflammatory response and to damage the invaded human respiratory epithelial cells. Moreover, the cells previously or simultaneously infected with the NK41 mutant were invaded by virulent strains in a significantly lower degree than those of the control group. In conclusion, the attenuated NK41 mutant interfered with the colonization and establishment of pathogenic strains of *S. aureus*, which produce severe infections.
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Keywords: *Staphylococcus aureus*; Bacterial interference; Nasal carriage

1. Introduction

Staphylococcus aureus is a common cause of hospital and community-acquired infections worldwide. A key risk factor for these infections is *S. aureus* anterior nasal carriage [1]. In general, patients and hospital personnel show higher *S. aureus* nasal carrier rates compared with individuals of the general community [2]. Furthermore, individuals belonging in risk populations such as patients with diabetes mellitus [3], patients undergoing hemodialysis [4] and patients with HIV infection [5] have shown higher rates of *S. aureus* nasal carriage compared with health workers and individuals of the healthy general population [6]. Therefore these patients have an increased risk of acquiring infections from endogenous origin. The control of *S. aureus* infections has been deeply hampered by frequent isolation of methicillin-resistant *S. aureus* (MRSA) not only from hospitalized patients but also from individuals with community-acquired infections [7]. The situation has only gotten worse by the increasing prevalence of clinically relevant isolates with reduced susceptibility to vancomycin [8]. Besides, the discovery of *S. aureus* with high levels of resistance to vancomycin is worrying [9].

Several approaches have been utilized to eliminate *S. aureus* nasal carriage to reduce the risk of endogenous staphylococcal infection [6]. Local application of mupirocin has shown to be effective to eliminate the *S. aureus* nasal

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carriage. Resistance to mupirocin, however, is increasing [10]. Both systemic administration of antibiotics and vaccination has rendered disappointing results [11]. Bacterial interference refers to interactions between species of microorganisms that interfere with each other with regard to their growth and niche ecological location [12]. In this regard, several studies showed the potential application of avirulent microorganisms for the prevention and/or treatment of infections in the upper respiratory, urogenital and gastrointestinal tracts [13–15]. Shinefield et al. [16] have observed in newborns that colonization of the nasal mucosa with *S. aureus* interfered with the acquisition of a second strain of *S. aureus*. Active colonization of the upper respiratory tract was achieved with the 502A strain of *S. aureus*. This strain exhibited minimal pathogenic features and prevented colonization by more virulent strains through competition for binding sites in the nasal epithelium. Unfortunately, the therapeutic use of this strain was impeded because its pathogenic potential was later demonstrated [17]. The purpose of the present investigation was to construct a stable live-attenuated mutant of *S. aureus* in order to test in mouse and cell culture models whether administration of an *aroA* attenuated mutant is a valid approach to prevent or reduce nasal carriage in individuals at risk. Our laboratory has previously demonstrated the feasibility of attenuating *S. aureus* virulence by mutation of the *aro*A gene of the aromatic amino acid biosynthesis pathway [18].

2. Material and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *Escherichia coli* were grown in Luria–Bertani medium supplemented with ampicillin (Amp) (50 or 100 μg/ml), kanamycin (Ka) (50 μg/ml), isopropyl-beta-D-1-thiogalactopyranoside (IPTG) (0.5 mM) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) (20 mg/ml) as required for the preservation of the plasmids. *S. aureus* strains were grown in trypticase soy broth (TSB) or trypticase soy agar (TSA). For selection of chromosomal markers or preservation of plasmids in *S. aureus*, the following compounds and concentrations were used: chloramphenicol (Cm), 10 μg/ml; erythromycin (Em), 5 μg/ml; Ka, 2.5 μg/ml; and X-Gal; 40 mg/ml. For phenotype characterization assays, colonies were replicated onto defined minimum medium (DMM) agar plates for *S. aureus* as described elsewhere [19] with or without addition of tryptophan (Trp) (0.05 mM), phenylalanine (Phe) (0.24 mM), tyrosine (Tyr) (0.28 mM), *p*-aminobenzoic acid (PABA) (0.05 mg/l), and 2,3 dihydrobenzoic acid (DHB) (10 mg/l). *S. aureus* Newman wild type and *aro*A mutant strains were grown in TSB (supplemented with Ka 2.5 μg/ml for the *aro*A mutant) to exponential phase, extensively washed with physiologic saline solution (PSS), and suspended in PSS to the desired density for inoculation to mice.

2.2. Nucleic acid techniques and sequencing

Chromosomal DNA was purified from *S. aureus* strains RN6390, Newman or the auxotrophic mutant NK41 (obtained in this study), after bacterial lysis with lysostaphin (5 mg/ml) and lysozyme (10 mg/ml) by the method of Pitcher et al. [20]. Restriction enzymes, T4 DNA ligase, Taq DNA polymerase were used as recommended by the manufacturer. The 3.5 kb fragments obtained by PCR to screen *aro*A::Ka^R mutants were sequenced. The BLAST software package was used to determine sequence homologies in the GenBank databases (http://www.ncbi.nlm.nih.gov/BLAST/). The challenging *S. aureus* strain Newman and clinical isolates HU-71 and Hde288 were electroporated with the pALC1743 vector which carries the gfp_{uvrs} reporter gene under the control of the *agr* P_3 promotor to be utilized in bacterial interference assays. In this way, it was possible to perform the differential counts between challenging strains (Newman$_{gfp+}$, HU-71$_{gfp+}$, Hde288$_{gfp+}$) and the NK41 mutant by UV light transillumination on the TSA plates. Clinical isolates HU-71 and Hde288 belonged in the *agr* II type, whereas, the Newman strain presented the *agr* I type as assessed by the multiplex PCR described by Gilot et al. [21].

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Reference
Newman	*S. aureus* polysaccharide capsular serotype 5 (CP5)	[49]
RN6390	*agr*$^+$ laboratory strain related to 8325-4	[50]
RN4220	Mutant strain of *S. aureus* 8325-4 that accepts foreign DNA	[50]
Newman$_{gfp+}$	Newman strain with plasmid pALC1743	This study
HU-71$_{gfp+}$	MRSA Cordobes clone with plasmid pALC1743	This study
Hde288$_{gfp+}$	MRSA Pediatric clone with plasmid pALC1743	This study
NK41	Newman *aroA*::Ka^R	This study
DH5α	*Escherichia coli* host cloning vector	Invitrogen
JM109	*Escherichia coli* host cloning vector	Promega
pMAD	*E. coli–S.aureus* (*Listeria*) shuttle vector with the *bgaB* gene encoding a β-galactosidase. Amp^r/Em^r	[22]
pMAD-1	pMAD plasmid contained the mutant allele for insertion with Ka^r of the *aroA* gene	This study
pGEM-T Easy	Amp^r, *lacZ'*, f1 *ori*, MCS, Mob$^-$, cloning T vector	Promega
pGEM-aro-Ka	pGEM-T Easy plasmid contained the mutant for insertion with Ka^{R}of the *aroA* gene	This study
pCR2.1-TOPO	Amp^r, ka^r, *lacZ'*, f1 *ori*, MCS, Mob$^-$, cloning vector	Invitrogen
pALC1743	pSK236 (gfp_{uvr} with *agr* P3 promoter)	[24]

2.3. Insertion of the Ka^R gene into the aroA gene (aroA::Ka^R) by allelic replacement mutagenesis and screening for auxotrophic mutants

The *S. aureus* Newman *aro*A mutant was constructed by homologous recombination. Briefly, the Ka^R gene was amplified by PCR from pCR2.1-TOPO with specific primers: 5′-CTC AAG CTT CAA CAC TCA ACC CTA-3′ and 5′-CTC AAG CTT ATA AGG GCG ACA CGG AA-3′. A restriction site for *Hind*III (underlined) was introduced into the fragment extremes. The PCR product was restricted and ligated into the *Hind*III site of *aro*A gene cloned previously in pGEM vector (to produce pGEM-aro-Ka). Then, the pGEM-aro-Ka vector was digested with *Bam*HI to release the *aro*A gene with the Ka^R gene inserted. The 3.5 kb fragment was gel-purified and ligated into the temperature-sensitive shuttle plasmid pMAD. The resulting plasmid (named pMAD-1) was electroporated into RN4220 *S. aureus* to generate transformants. Electrocompetent organisms of the Newman strain were subsequently transformed with pMAD-1 isolated from RN4220. pMAD contains a temperature-sensitive origin of replication and an Em resistance gene [22]. Homologous recombination experiments were performed as previously described [23]. Briefly, Newman harboring pMAD-1 were grown in TSB with Em and Ka at 30 °C (permissive temperature for the Gram-positive replication origin), diluted 1:1000 in fresh medium, and propagated through several cycles of alternating 30 and 42 °C (non-permissive temperature). Em-sensitive (Em^S) and Ka^R white colonies, representing possible double-crossover events and which no longer contained the pMAD plasmid were selected. The Ka^R insertion in the *aro*A gene was screened by PCR of the *aro*A gene of selected strains with specific primers: 5′-CTC GGA TCC ACA TTA CAA CAT GCA TGT GAA C-3′ and 5′-CTC GGA TCC CAT CGC CGT GTT CTA TTT CC-3′. Fragments of 3.5 kb length were sequenced. *S. aureus* Newman strains with the desired fragment of PCR product were replicated onto DMM agar plates without Trp, Phe, PABA and DHB to check the aromatic amino acid auxotrophic phenotype.

2.4. Complementation

A 1.4 kb fragment encompassing the *aro*A gene from *S. aureus* RN6390 was amplified by PCR using primers 5′-CTC TCT AGA ACA TTA CAA CAT GCA TGT GAA C-3′ and 5′-ACG CGT CGA CTG CGT CAT CGT TGT CAG TAG T-3′. Restriction sites for *Xba*I and *Sal*I (underlined) were introduced into the fragment at the 5′ and 3′ ends, respectively. The PCR fragment was restricted and ligated into the vector pALC1743 after deletion of the gfp_{uv} gene and then transformed into *E. coli* DH5α [24]. Restriction analysis and DNA sequencing confirmed the orientation and authenticity of the cloned gene. The recombinant plasmid was electroporated into the NK41 mutant, and Cm resistant (Cm^R) colonies were selected. Transformants were tested for restoration of the wild type phenotype.

2.5. Determination of virulence for mice

CF-1 outbred mice were bred and maintained in the vivarium of the Department of Microbiology, School of Medicine, University of Buenos Aires in accordance with the guidelines set forth by the U.S. National Institutes of Health. For 50% lethal dose (LD_{50}) studies, 6-week-old, male CF-1 mice were injected intraperitoneally (ip) with 0.5 ml of a suspension containing the bacterial strain and 2% (w/v) brewer's yeast in BHI broth [25]. Three groups, each comprising 10 mice, from three separate tests received serial log dilutions of bacteria. The estimation of the LD_{50} was made after 7 days using a software for probit analysis [26].

2.6. Bacterial interference assays in mice

Different types of bacterial interference assays were performed in vivo. In one of them, groups of 10−20 mice were inoculated by the intranasal (ina) route with 10 μl of a suspension containing approximately 10^7 CFU of the NK41 mutant or heat-killed Newman (Newman-HK) strain during three consecutive (−3, −2, and −1) days. At day 0 animals were challenged by the ina route with 10^7 CFU of *S. aureus* $Newman_{gfp+}$ or MRSA HU-71_{gfp+} (representing the Cordobes clone) or MRSA $Hde288_{gfp+}$ strains (representing the Pediatric clone). To assess simultaneous nasal infection, groups of mice were inoculated by ina route with a 50:50 mixture composed of the clinical isolate *of S. aureus* $Hde288_{gfp+}$ and the NK41 mutant, at a total inoculation dose of 1×10^7 CFU/10 μl. For both types of assays, the bacterial interference phenomenon was evaluated by quantitative differential cultures of the nasal tissues of mice that were CO_2-euthanized 24 h after bacterial challenge. The area around the nasal region was wiped with 70% ethanol, and the nose was excised and homogenized in 400 μl TSB using a tissue grinder. Dilutions of the tissue homogenate were plated onto TSA plates. To determine the number of challenge strain CFU per nose counts were made under UV light as described above. On the other hand, NK41 mutant organisms were plated onto TSA supplemented with Ka to check preservation of the mutation. In the heat-killed bacterial experiments, the Newman strain suspensions were incubated in a water bath at 72 °C during 60 min in order to kill all organisms, as verified by control plating. Previously, the bacterial were diluted in PSS to achieve the desirable concentration.

To ascertain the pALC1743 loss frequency, a group of 11 mice was ina inoculated with 10 μl of *S. aureus* $Hde288_{gfp+}$ suspension (3.6×10^7 CFU/nose). A day after, the nose were excised, homogenized in 400 μl of TSB and plated. After 24 h of incubation at 37 °C, the fluorescent *gfp*+ (with pALC1743) (median value: 6080 CFU/nose) and the white (without pALC1743) colonies (median value: 32 CFU/nose) were recorded and checked as Hde288 strain (oxacillin resistant and manitol growth positive). The resulting estimation of pALC1743 in vivo loss frequency was lower than 9×10^{-7}.

2.7. Real-time (qRT) PCR

Nasal RNA of mice was extracted using Trizol (Gibco) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the LightCycler FastStart DNA Master SYBR Green I (Roche) equipment and kits. cDNA was subjected to real-time using the following primer pairs: *il-1ß*-f 5′- TTG ACA GTG ATG AGA ATG ACC-3′; *il-1ß*-r 5′-CAA AGA TGA AGG AAA AGA AGG-3′; *tnf-α* f **5′-**CCA CCA CGC TCT TCT GTC TA-3′; *tnf-α* r 5′-GAA ACC ATT TGG GAA CTT CT-3′; *cox-2* f **5′-**GCT GTA CAA GCA GTG GCA AAG-3′; *cox-2* r 5′-GCG TTT GCG GTA CTC ATT GAG A-3′; *gapdh*-f 5′-GAA GGT GGT GAA GCA GGC AT-3′; and *gapdh*-r 5′-TCG AAG GTG GAA GAG TGG GA-3′. Cycling conditions for *il*-1β were: 95 °C for 10 m followed by 40 cycles of 95 °C for 15 s, 53 °C for 30 s and 60 °C for 45 s. Cycling conditions for *tnf*-α, *cox*-2 and *gapdh* were: 95 °C for 2 m followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 m. The *gapdh* gene was used to normalize data. The number of copies of each sample transcript was determined with the aid of the LightCycler software. The $2^{-(\Delta\Delta CT)}$ value represent the difference in threshold cycle (*CT*) between the target and control (*gapdh*) genes of group of mice inoculated with NK41, Newman-HK or Newman minus the difference in *CT* between control mice group (inoculated with PSS) target and control genes [27].

2.8. Cell culture of human nasal epithelial cell (HNEC) and A549 cells

HNEC were obtained and cultured as previously described [28]. The A549 human airway epithelial cell line (ATCC CCL-185) was grown in F-12 Kaighn's medium (Gibco) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin. A549 cells were cultured at 37 °C under 5% CO_2 and grown to 80% confluence. Prior to each experiment, cells were seeded at 1.5×10^5 cells/well in 24 well-tissue culture plates and grown for 24 h under the same conditions. The cell viability was determined by the trypan blue exclusion method.

2.9. Internalization assays

Confluent A549 cell and HNEC monolayers (approximately 2.5×10^5 cells/well) were washed with sterile PBS and then inoculated with bacteria suspended in fresh growth medium without antibiotics (invasion medium) to produce a multiplicity of infection of 40. After incubation for 1 h at 37 °C under 5% CO_2, the wells were washed with PBS and then 1 ml of invasion medium supplemented with 25 μg/ml per well of lysostaphin was added to each well to eliminate extracellular bacteria. Incubation of co-cultures with lysostaphin proceeded for an additional 2 h at 37 °C under 5% CO_2. The monolayer was washed four times with sterile PBS, detached with 0.25% trypsin-0.1% EDTA and lysed by the addition of 0.025% (v/v) Triton X-100. The CFU number was determined by quantitative plating on TSA. Cell viability was evaluated by trypan blue exclusion.

2.10. Interleukin measurements

Prior to stimulation, A549 cells were incubated for 24 h in F-12 Kaighn's medium without FBS. Confluent A549 monolayers were inoculated with the *S. aureus* Newman strain or the NK41 mutant as described in Internalization assays above. After lysostaphin treatment the cells were incubated with 1% FBS or without antibiotics. Then, supernatants were harvested at 6 and 24 h after cell infection and ELISA tests (R&D) for IL-8 (detection range >3.5 pg/ml) and IL-6 (detection range >0.70 pg/ml) were performed in duplicate according to the manufacturer's instructions. The optical density (OD) of the samples was measured at a wavelength of 450 nm. In parallel, the number of viable bacteria intracellular were assessed by quantitative plating on TSA after detaching and lysing the infected monolayers.

2.11. Bacterial interference assays in cell culture

Two types of bacterial interference assays were performed on human airway epithelial cells. In one of them, the A549 cell monolayers were first infected with the NK41 mutant and 24 h later co-infected with the Newman_{gfp+} or HU-71_{gfp+} or Hde288_{gfp+} virulent strains as described in the internalization assays. In the other protocol, A549 cell monolayers were infected with a suspension of the NK41 mutant along with a suspension of the virulent strain (50:50 proportion). The control cells were infected with the same absolute amount of challenging bacteria present in the mixture. In both cases, the experiments were then continued as described in the Invasion Assays section.

2.12. Statistical analysis

In order to obtain a statistical assessment of virulence for mice of the *S. aureus* Newman and NK41 mutant strains, the 7 day survival ratios from three separate tests were pooled for estimation of the LD_{50} by a computarized program for probit analysis (PASW Statistics 18). Multiple comparisons of interleukin levels and mutant characterization on cell cultures were performed by the Kruskal–Wallis test. Nonparametrical data was analyzed with the Mann–Whitney test using the GraphPad software (version 4.0; PRISM). *P* values lower than 0.05 were considered statistically significant.

3. Results

3.1. Characterization of the aroA::Ka^R mutant

The *aro*A gene of *S. aureus* was mutagenized by allelic replacement (see Materials and methods). More than 800 white, Em^S and Ka^R colonies were screened to assess the loss of the pMAD-1 vector. Only one (named NK41) displayed homologous recombination between the chromosomal *aroA* gene and

the mutated copy of *aro*A::Ka^R besides the loss of the plasmid. Nucleotide sequence analysis revealed that the NK41 mutant possessed the Ka^R gene inserted in position 1334 of the *aro*A gene. As expected, the NK41 mutant did not grow on DMM agar plates without addition of the three aromatic amino acids as well as PABA and DHB. The auxotrophic phenotype was due to the mutated gene was no longer able to express the enzyme 5-enolpyruvylshikimate 3-phosphate synthase involved in the synthesis of shikimic acid and chorismic acid [29]. The wild type phenotype was restored in the NK41 mutant by complementation assays. The NK41 mutant was very stable and exhibited a reversion frequency lower than 1×10^{-12}. Furthermore, probit analysis was performed to compare the LD_{50} of the auxotrophic NK41 mutant and its parental strain. The LD_{50} of the Newman strain (9×10^6 CFU with 95% confidence intervals [3.7×10^6–3.1×10^7 CFU]) was statistically significant ($p < 0.05$) different from the LD_{50} of the NK41 mutant (9.5×10^7 CFU with 95% confidence intervals [3.4×10^7–3.2×10^8 CFU]). This statistical increase in the LD_{50} of the NK41 mutant confirmed its attenuation in mice.

3.2. Bacterial interference studies in mice

Initially, we conducted in vivo experiments in order to provoke colonization of the mice noses with the attenuated NK41 mutant at levels similar to those of the wild type Newman strain. To obtain this, it was necessary to administer during 3 consecutive days 10^7 CFU of the NK41 mutant by the ina route. The results demonstrated that the level of NK41 recovered 1 day after the last inoculation was similar to that observed with a single administration of the same dose of the parental Newman strain (Fig. 1A). This scheme was followed in one type of bacterial interference assays. Twenty-four hour after the last administration, groups of mice previously colonized with the NK41 mutant and the control groups (without any previous application) were challenged by the ina route with a similar dose of the Newman$_{gfp+}$ strain or clinical isolates of *S. aureus* (HU-71$_{gfp+}$ and Hde288$_{gfp+}$). We chose the clinical isolates HU-71 and Hde288 because they represent, respectively, highly prevalent MRSA lineages in the argentinean hospital environment and community [30]. The *S. aureus* Hde288 strain represents the Pediatric clone and HU-71 strain the Cordobes clone, as determined by PFGE after digestion of the genome with the enzyme *Sma*I [31]. Twenty-four hours after the challenge with the virulent strains, the animals were sacrificed and the noses were removed. Aliquots of the homogenates were seeded onto TSA plates for CFU counts as described in Materials and methods. Fig. 2 shows that the previous nasal colonization with NK41 attenuated mutant significantly reduced the number of CFU of *S. aureus* clinical isolates HU-71$_{gfp+}$ (Fig. 2B) and Hde288$_{gfp+}$ (Fig. 2C), as well as the parental Newman$_{gfp+}$ strain (Fig. 2A). Similar experiments were designed to ascertain whether also Newman-HK (60 min at 72 °C) strain was capable to interfere with nasal colonization by a pathogenic strain of *S. aureus*. No significant difference in the number of Hde288$_{gfp+}$ recovered from noses between Newman-HK colonized and control mice was observed (Fig. 1B).

Furthermore, the activation of immune innate response in nasal cavity from NK41, Newman-HK and Newman ina inoculated mice according to the scheme described above, was ascertained by qRT-PCR experiments of IL-1β, TNF-α and Cox-2 transcripts formed 24 h after the last administration (time coincident with the challenge). Our results showed that as the mutant as parental strain (it lives or heat-killed) not induced the expression of the pro-inflammatory cytokines and Cox-2 transcripts (Table 2).

In a second type of bacterial interference assays, the attenuated NK41 mutant was administered simultaneously (50:50 mixture at total dose of 1×10^7 CFU/nose) with the *S. aureus* Hde288$_{gfp+}$ clinical isolate by ina route. Mice were

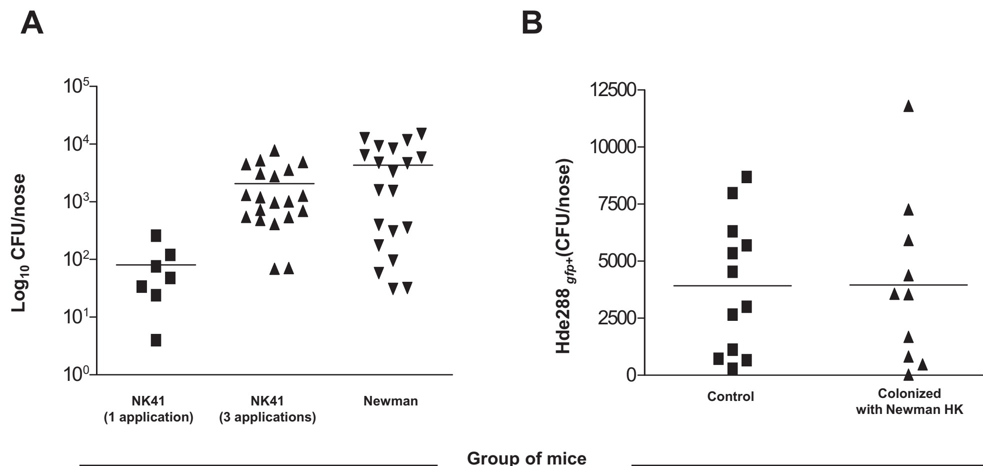

Fig. 1. **(A)** Colonization of the mouse nares by *S. aureus* Newman and the NK41 mutant. Groups of 10–20 mice were inoculated with a suspension of 10^7 CFU by the ina route. Each point represents the CFU/nose from each mouse at 24 h post-inoculation (day 0). At day −1, mice were inoculated with 1 application of *S. aureus* Newman strain (▼), and the NK41 mutant (■). Colonization of NK41 mutant was also determined after three daily consecutive applications (days −3; −2; −1) (▲). **(B)** Mouse nasal inoculation with heat-killed Newman and interference with *S. aureus* Hde288$_{gfp+}$ isolate. Groups of 10–13 mice were inoculated with a suspension of 10^7 CFU of Newman-HK (▲) by the ina route during three consecutive (−3; −2 and −1) days. At 0 day, mice were ina challenged with the same doses of *S. aureus* Hde288$_{gfp+}$. Twenty-four hour after challenge mice were sacrificed and their noses removed. The mice control group were inoculated with PSS and Hde288$_{gfp+}$ challenged (■). The horizontal lines represent the median value.

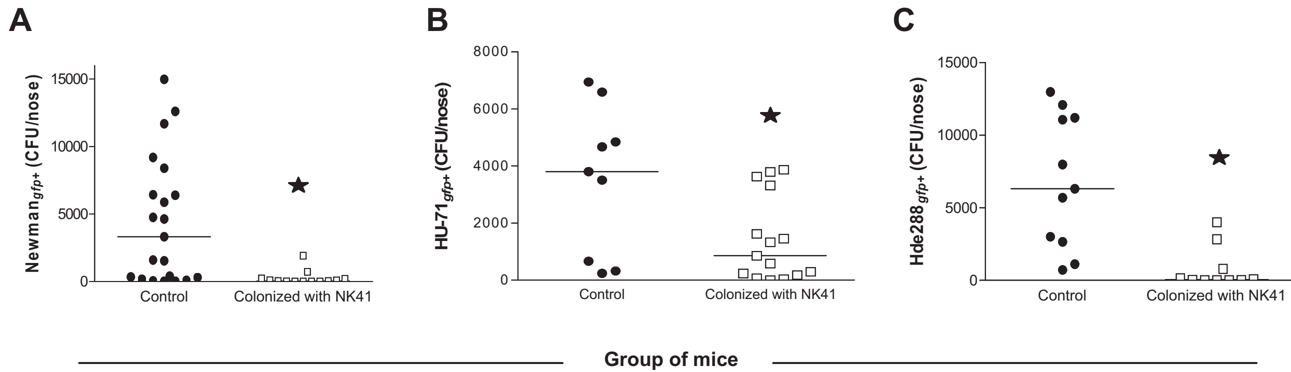

Fig. 2. Previous mouse nasal colonization with the NK41 mutant interferes with the *S. aureus* Newman strain and clinical isolates. Groups of 10–15 mice were inoculated with a suspension of 10^7 CFU by the ina route during three consecutive (−3; −2 and −1) days. At 0 day, mice were ina challenged with the same doses of *S. aureus* Newman strain **(A)**, HU-71 **(B)** and Hde288 **(C)**. Twenty-four hours after challenge mice were sacrificed and their noses removed. The horizontal lines represent the median value. In all cases, mice previously colonized with NK41 (□) showed a significant decrease in the CFU number of the challenging strains compared with a control group without previous colonization (●), (*) $p < 0.01$, Mann–Whitney test.

sacrificed at 24 h after inoculation, and the number of CFU in nostrils were determined. The CFU number recovered of strain Hde288$_{gfp+}$ (median: 2.2×10^2 CFU/nose) was significantly lower than that of the mutant NK41 found (median: 1.4×10^3 CFU/nose) ($p = 0.04$, Mann–Whitney test) when both were inoculated in a suspension mixture. Taken together, these results show that the NK41 mutant interferes with the colonization and establishment of virulent clinical isolates of *S. aureus* under the evaluated conditions.

3.3. Physio-immunological evaluation of NK41 in respiratory epithelial cells

As it was expected, the internalization levels of the NK41 mutant (Median: 2.1×10^4 CFU/ml and 2.8×10^2 CFU/ml) did not differ from those of the parental Newman strain (Median: 2.2×10^4 CFU/ml and 3.5×10^2 CFU/ml) in the A549 cell line and HNECs, respectively. The viability of either A549 cells or HNEC infected with the parental strain Newman within a period of 24 h after infection was 64%, whereas cells infected with the NK41 mutant exhibited a level of viability similar to the one found in cells without *S. aureus* infection (89% and 87%, respectively) ($p = 0.0005$, Mann–Whitney test compares with A549 cells and HNECs).

In order to determine the reaction of the respiratory epithelium against the NK41 mutant and its parental strain, the levels of pro-inflammatory cytokines synthesized were measured. The levels of IL-6 and IL-8 induced by the NK41 mutant in respiratory epithelial cells were significantly lower compared to the levels triggered by the parental Newman strain organisms (Fig. 3A and Fig. 3B). The NK41 mutant (median: 1.1×10^4 CFU/ml) remained viable within epithelial cells at 24 h post infection. The impact that the NK41 mutant generated in respiratory epithelial cells was different to that exerted by the parental strain.

Table 2
Cytokines and Cox-2 transcripts level from nares of inoculated mice.

Bacteria ina inoculated	*il1-ß*[a]	*tnf-α*[a]	*cox-2*[a]
NK41	1.15 ± 1.12	0.85 ± 0.52	0.66 ± 0.34
Newman	1.18 ± 0.97	0.82 ± 0.66	0.61 ± 0.36
Newman-HK	1.13 ± 1.09	0.83 ± 1.03	0.62 ± 0.26

[a] *il1-ß*, *tnf-α* and *cox-2* transcript levels from mice inoculated with NK41, Newman or Newman-HK by ina route for three consecutive days. Changes in gene expression are shown as mean fold change [$2^{-(\Delta\Delta CT)}$] ± SD. Data were normalized to *gapdh* expression. Control mice group was inoculated with PSS. The data represent the mean of duplicate measurements from 3 independent experiments with 5 mice for groups.

3.4. Bacterial interference studies in cell culture

To assess the bacterial interference phenomenon by the NK41 mutant two types of protocols of mixed infection were conducted in epithelial cells. First, monolayers of A549 cells infected with the NK41 mutant during 1 day, were co-infected with the parental Newman$_{gfp+}$ strain or clinical isolates Hde288$_{gfp+}$ and HU-71$_{gfp+}$ The results showed that the cells previously infected with the NK41 mutant were significantly less invaded by the virulent wild type strains compared with those of the control group (Fig. 4). During the assays, the viability of the A549 cells infected with the NK41 mutant was similar to the uninfected cells. This result indicates that the effect of bacterial interference caused by the NK41 mutant in the murine model can also be achieved in human respiratory epithelial cells. In the mixed infection model using the simultaneous time assay, the A549 cell monolayers were infected with a suspension of the NK41 mutant along with the virulent strains Newman$_{gfp+}$, Hde288$_{gfp+}$ or HU-71$_{gfp+}$ in a 50:50 proportion. The attenuated NK41 mutant significantly hindered the internalization of virulent strains in human respiratory epithelial cells (Fig. 5). The results of this experiment showed that the NK41 mutant was able to interfere with the cellular invasion in concurrent infections with either the parental Newman strain or the clinical isolates HU-71 and Hde288.

4. Discussion

Nasal carriage is a major risk factor for subsequent infections caused by *S. aureus*. The source of 80% of bloodstream

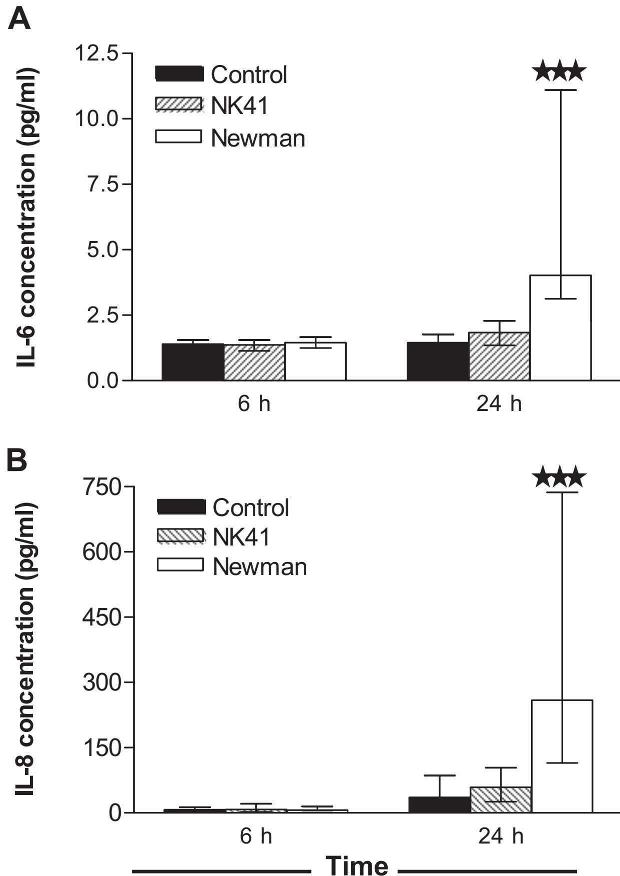

Fig. 3. Induction of pro-inflammatory interleukins by the NK41 mutant and the Newman strain in respiratory epithelial cells. Bars represent medians and ranges of IL-6 (A) and IL-8 (B) concentration measured by ELISA (pg/ml) of three independent experiments performed in duplicated ($n = 6$). A549 cells infected with *S. aureus* Newman strain exhibited at 24 h post infection levels of IL-6 and IL-8 significantly higher than those measured in uninfected cells and cells infected with the mutant NK41, (***) $p < 0,0001$; Kruskal–Wallis test.

infections by *S. aureus* is endogenous [32]. It is hypothesized that the incidence of *S. aureus* infections may be reduced by elimination of nasal carriage. In this study, we report the construction of an *aro*A mutant of the Newman strain (named NK41) that was able to interfere with community and hospital clinical isolates of *S. aureus*. The attenuated *aro*A mutant may potentially be used for reduction of nasal colonization by more virulent strains. We have chosen to mutate the *aro*A gene because the enzyme coded by this gene (5-enolpyruvylshikimate 3-phosphate synthase) is involved in the initial steps towards aromatic amino acid synthesis. In a previous work, we have demonstrated that a blockage of this biosynthetic pathway makes the bacterium auxotrophic for aromatic amino acids, PABA (a precursor of folic acid), and DHB (a quinone precursor) [18]. The allelic replacement method enables the construction of well-defined mutants and has been successfully utilized in the study of several pathogenic bacteria. In this investigation, the *aro*A gene of *S. aureus* Newman strain was interrupted in the position 1334 by insertion of the Ka^R gene. The NK41 mutant exhibited the expected auxotrophic phenotype and only proliferated in minimal medium supplemented with Trp, Phe, and Tyr as well as

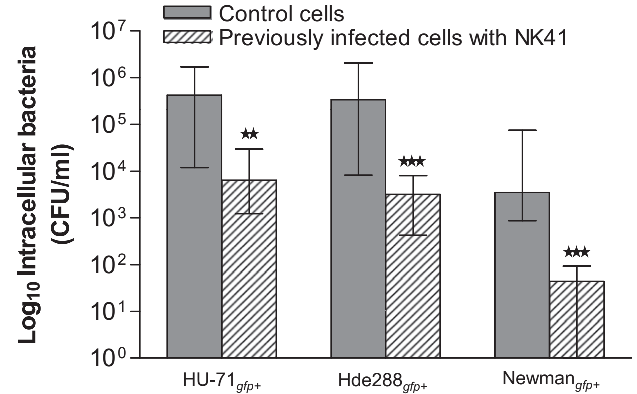

Fig. 4. Previous infection of epithelial respiratory cells with the NK41 mutant interferes with the internalization of *S. aureus* Newman strain and clinical isolates. Bars represent medians and ranges of three independent experiments performed in triplicate ($n = 9$). Respiratory epithelial cells were infected with the NK41 mutant 24 h previous challenge with *S. aureus* Newman, HU-71 or Hde288 strains. The number of virulent intracellular bacteria recovered from the cells previously infected with the NK41 mutant showed a significant decrease compared with those without previous NK41 infection (***) $p < 0.0001$, (**) $p < 0.001$Mann–Whitney test.

PABA and DHB. Moreover, the reversion frequency of the NK41 mutant was less than 1×10^{-12} which demonstrates the stability of the auxotrophic phenotype. Restoration of the wild type phenotype was assessed by complementation. Through the significant increase in the LD_{50}, the NK41 mutant showed to be attenuated compared with the parental strain. Requirement of PABA, that is not synthesized by mammals, has been singled out as the likely reason for reduced virulence of aromatic-dependent mutants [33]. Moreover, the human nasal niche is a relatively nutrient-deficient environment [34]. Therefore, we can speculate that the growth of the NK41 mutant will be severely restricted in vivo making an *aro*A mutant of *S. aureus* an attractive tool for controlled trials in humans. However, we cannot ignore that the *aro*A auxotrophy of the NK41 mutant may limit the long term nasal colonization.

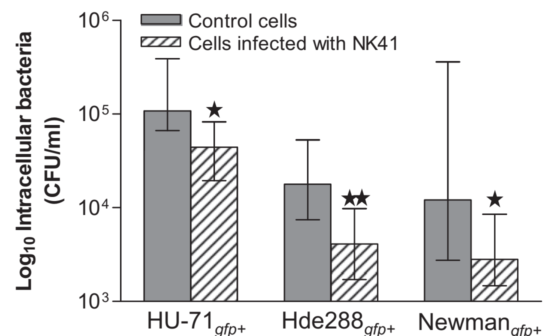

Fig. 5. Simultaneous infection with the NK41 mutant of epithelial respiratory cells interferes with the internalization of *S. aureus* Newman strain and clinical isolates. Bars represent medians and ranges of three independent experiments performed in triplicate ($n = 9$). Respiratory epithelial cells were infected with a 50:50 mixture composed of *S. aureus* Newman or HU-71 or Hde288 strains and the NK41 mutant. The number of virulent intracellular bacteria recovered from cells infected simultaneously with the NK41mutant exhibited a significant decrease compared with the control groups (*) $p < 0.01$, (**) $p < 0.001$Mann–Whitney test.

Recently, nasal colonization studies in healthy human volunteers were performed with *S. aureus* strain 8325-4 [34]. Indeed, the 8325-4 strain had been used in many studies for a long period and might be less virulent, even though it is not a genetically modified strain. Unlike the Newman strain, *S. aureus* 8325-4 does not express CP5 [35]. The *S. aureus* capsule is an important virulence factor involved in bacterial establishment in the nostrils [36]. It is important to mention that *S. aureus* has several adhesins involved in the adherence to host components, which have redundant and pleitropic effects. In spite of the fact that the molecular basis of *S. aureus* nasal colonization remains incompletely understood, it has been suggested that wall teichoic acid is essential for nasal colonization and could mediate the interaction with HNEC and A549 cells [37]. Moreover, Corrigan et al. recently demonstrated that the ability of *S. aureus* Newman to adhere to nasal epithelial cells is clearly multifactorial and involves SdrD, SdrC, as well as ClfB and IsdA [38]. In the absence of functional FnPBs, Eap plays an important role in the internalization of *S. aureus* strain Newman [39]. Therefore, although we do not know the exact nature of the *S. aureus* Newman adhesins interactions and their relative contribution to nasal colonization, we may speculate that ClfB [38], Eap, teichoic acid, as well as other adhesin/s can partially compensate for the loss of Fn binding, thus mediating cellular adherence.

In the past, bacterial interference was achieved by inoculation of the *S. aureus* 502A strain and this procedure was suggested as a method to eradicate staphylococcal nasal carriage in infants and in patients with recurrent furunculosis [16,40–42]. This treatment, however, was occasionally complicated by serious infections [43] and even fatal ones caused by *S. aureus* 502A [44]. Furthermore, the genetic defect or the defined features of the genetic background that confers minimal pathogenic properties to the 502A strain remains unknown. The observed effect on the eradication of colonization with the 502A strain provides an extremely important precedent in the use of active colonization with a live-attenuated strain of *S. aureus*.

Kiser et al. [36] suggested that the nasal normal flora of mice can inhibit the experimental colonization by *S. aureus* through competition of the binding sites and the availability of nutrients and/or secreted products. The authors developed a murine nasal colonization model in which the animals were previously treated with streptomycin to reduce the normal flora from the nasal cavity. This procedure enabled the Newman strain of *S. aureus* to colonize the nasal cavity for 7 days after-infection. In contrast, in this study a murine model of nasal colonization was obtained without any eradication of normal nasal microbiota. However, three intranasal applications of the NK41 mutant by the ina route were required to obtain a number of CFU similar to that of one single administration of the parental strain. Interestingly, previous or simultaneous ina inoculation with the NK41 mutant reduced the colonization and establishment not only of the wild type Newman strain but also the clinical isolates representative of the Pediatric and Cordobes MRSA clones. The MRSA used in the assays were isolated from the community and the hospital environment, respectively. Indeed, these clinical isolates can potentially cause infections in individuals at risk (e.g. patients undergoing cardiac surgery) since it has been shown that the nostril is the gateway causing bacteremia in hospitalized patients [32].

Recently, Margolis et al. [45] established that *S. aureus* resident in noses of neonatal rats prevents the nasal colonization by other *S. aureus*. Moreover, negative association between *S. aureus*, *Staphylococcus epidermidis*, and actinobacterial groups (e.g., *Propionibacterium* spp., *Corynebacterium* spp.) suggests microbial competition during colonization of the human nostrils [46,47]. Whether or not NK41 interferes with colonization of other bacterial species of the human nasal microbiota deserves to be studied more deeply. It would be important to assess whether or not the bacterial interference effect that the NK41 mutant exerted over other *S. aureus* strains is also wielded on bacterial strains of the human nasal microbiota such as *S. epidermidis*, *Corynebacterium* spp. or *Streptococcus pneumoniae*, among other prevalent pathogens.

Interestingly, the octapeptide AIP produced by the NK41 mutant belong to group I of *agr* (AIP_I) while the clinical isolates Hde288 and HU-71 produce autoinducers belonging to group II of *agr* (AIP_{II}). It can then be speculated that, in addition to the interference phenomenon by competition, the NK41 mutant would also inhibit the activation of the *agr* system from those *S. aureus* organisms within an *agr* group different from AIP_I, thus affecting the expression of a variety of genes involved in tissue colonization. Whether or not the activity of the *agr* system is relevant to nasal carriage of the clinical strains of *S. aureus* is not completely understood [48] and deserves to be studied more exhaustively.

The innate immune response plays a major role in the elimination of colonizing pathogens and represents a barrier to be overcome by bacteria in order to inhabit the nasal vestibule. Using our murine nasal model we observated that the pro-inflammatory cytokines and Cox-2 transcript levels were similar among NK41 mutant and Newman or Newman-HK. Moreover, the transcript levels obtained were as lower as those ascertained in the mice PSS inoculated (control group). These data suggest that the low number of CFU of wild type strains recoverd from NK41-colonized mice was not caused by the action of the innate immune response. On the other hand, the ability of the NK41 mutant to invade the airway epithelial cells was similar to that observed by the parental Newman strain. However, the effects induced by the NK41 mutant differed markedly from those of the wild type strain. After 24 h of infection, the NK41 mutant did not affect the epithelial cell viability nor induced the synthesis of pro-inflammatory cytokines IL-6 and IL-8. These results suggest that the NK41 mutant possess diminished virulence. Interestingly, the NK41 mutant reduced the cellular internalization of clinical isolates Hde288 and HU-71 of *S. aureus* as well as the Newman strain organisms. This effect was observed not only in consecutive mixed infections of epithelial cells but also in simultaneous mixed infections. Clements et al. [49] evidenced *S. aureus* within human ciliated nasal epithelial cells and

proposed it as an intracellular reservoir to recurrent *S. aureus* rhinosinusitis. Besides, it remains unclear whether the squamous cells are of major importance for continued colonization or less differentiated cells play a more critical role [50].

In summary, we constructed a stable and attenuated mutant of *S. aureus* Newman strain by insertion of the Ka^R gene in the *aro*A gene using homologous recombination. Using a murine model of nasal colonization we demonstrated that a *S. aureus aroA* mutant was able to interfere with the colonization not only of its parental strain but also of prevalent *S. aureus* clinical strains in hospitals. The same effect was observed in a human respiratory epithelial cell infection model. The present study demonstrates the usefulness of the *aroA* phenotype attenuated *S. aureus* to be used for interference. Construction of unmarked *aro*A deletion mutants of *S. aureus* for therapeutic potential reduction of the pathogenic staphylococci load in the upper respiratory is currently under way in our laboratory. Further studies are required, however, to assess the use of the live-attenuated strain in individuals at risk, due to the complex and multifactorial nature of the nasal colonization by this pathogen.

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