INSTITUT PASTEUR

Microbes and Infection 13 (2011) 1073–1080

www.elsevier.com/locate/micinf

Original article

# Salicylic acid enhances *Staphylococcus aureus* extracellular adhesin protein expression

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Received 28 December 2010; accepted 9 June 2011
Available online 21 June 2011

**Abstract**

One of the virulence factors required by *Staphylococcus aureus* at the early stages of infection is Eap, a secreted adhesin that binds many host proteins and is upregulated by the two-component regulatory system *sae*RS. The *S. aureus* Newman strain harbors a mutation in *sae*S that is thought to be responsible for the high level of Eap expression in this strain. This study was designed to ascertain whether salicylic acid (SAL) affects the expression of Eap and the internalization of *S. aureus* into epithelial cells. The strain Newman treated with SAL exhibited increased levels of *eap* transcription and protein expression. Furthermore, SAL treatment increased the *eap* promoter activity. SAL treatment enhanced Eap expression in the Newman and in other *S. aureus* strains that do not carry the mutation in *sae*S. Internalization of *S. aureus eap* and *sae* mutants into the MAC-T epithelial cells was significantly decreased compared with the wild-type counterparts. In conclusion, we demonstrated that a low concentration of SAL increased *S. aureus* Eap expression possibly due to enhancement of *sae*. SAL may create the conditions for *S. aureus* persistence in the host, not only by decreasing the capsular polysaccharide expression as shown before, but also by enhancing Eap expression.


*Keywords:* *Staphylococcus aureus*; Salicylic acid; Extracellular adhesin protein

## 1. Introduction

*Staphylococcus aureus* is a leading cause of both nosocomial and community-acquired infections (e.g., superficial and deep-seated abscesses, endocarditis, pneumonia, septicemia, and osteomyelitis) [1]. The capacity of this species to cause a wide variety of diseases partially depends on its ability to express many virulence factors acting in concert. To initiate infection, *S. aureus* has to adhere to host cells and extracellular matrix substrates through a number of adhesins [2]. The adhesins anchored to the *S. aureus* cell wall were defined as "Microbial Surface Components Recognizing Adhesive Matrix Molecules" [2]. The extracellular adhesin protein (Eap) is instead a secreted adhesin that belongs in the group of proteins called "Secreted Expanded Repertoire Adhesive Molecules", which play an important role at the early stages of infection [3]. The *eap* gene is conserved among *S. aureus* clinical isolates. The role of Eap in adherence of *S. aureus* to eukaryotic cells has been convincingly demonstrated [4–7]. Once secreted, Eap attaches to the *S. aureus* surface and acts as a surface adhesin to bind a significant number of host molecules such as fibrinogen, collagen and fibronectin, among other proteins [4]. This adhesin is particularly relevant to the *S. aureus* Newman strain, which expresses a truncated version of the fibronectin-binding protein (FnBP), one of the major *S. aureus* adhesins [8]. Expression of Eap has been shown to depend upon *sae*RS (henceforth named *sae* in this paper), an

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doi:10.1016/j.micinf.2011.06.003

important two-component regulatory system in *S. aureus* that controls expression of many secreted proteins [9]. The *S. aureus* Newman strain shows a high expression of the *sae* operon and Eap due to an amino acid substitution within the first membrane-spanning domain of the sensor histidine kinase SaeS [10–12]. Many environmental factors, such as glucose concentration and pH, among others, were found to affect expression of *sae* [13].

Salicylic acid (SAL), the major metabolite of aspirin produced *in vivo*, interferes with expression of *S. aureus* virulence factors and global regulators [27,33]. A recent study from our laboratory demonstrated that exposure of encapsulated *S. aureus* strains to a low concentration of SAL (50 μg/ml) reduced capsular polysaccharide (CP) production [16]. The concentration of SAL used matched the antiplatelet concentration achievable in human plasma after ingestion of aspirin within therapeutic range. Furthermore, we have shown that low capsule expression caused by SAL was accompanied by enhanced expression of *sae*, the cognate regulator of Eap [16]. Here we hypothesize that SAL may affect the expression of the secreted adhesin Eap. The present study was designed to assess the effect of SAL on Eap expression in *S. aureus*, which may modify the capacity of *S. aureus* to invade mammary epithelial cells.

## 2. Materials and methods

### 2.1. Bacterial isolates and growth conditions

Bacterial strains and plasmids used in this study are described in Table 1. Bacteria were stored in tryptic soy broth medium with 20% glycerol at −20 °C until use. *S. aureus* was routinely cultured at 37 °C at 200 rpm for 18 h in casamino acids-yeast extract-glycerophosphate broth without glucose (CYGPw) [17]. Since glucose affects the *sae* expression [9] the experiments were performed in media without glucose. SAL was added, when required, to the culture medium to a concentration of 50 μg/ml (serum concentration normally attained after ingestion of aspirin). The SAL concentration used in the experiments did not affect the growth of the *S. aureus* strains used in this study (data not shown). For cell invasion experiments, bacterial cells were collected by centrifugation, washed with sterile saline solution and suspended in invasion medium (see below) to a density of *ca.* $10^7$ CFU/ml.

Table 1
Strains and plasmids utilized in the present study.

| *S. aureus* strains/plasmids | Description | Source |
|---|---|---|
| Newman | Clinical isolate (ATCC 25904); CP5 producer | [46] |
| AH12 | Strain Newman *eap*::Ery$^r$ | [28] |
| NewHG | Strain Newman with repaired *sae*S | [11] |
| Wood46 | Human isolate | ATCC10832 |
| HU-27 | Human isolate | Dr. Sordelli's lab |
| Brazilian clone | Human isolate | Dr. Centrón's lab |
| ALC4483 | Newman *sae* single-deletion mutant | [47] |
| ALC4241 | Strain Newman with pALC4231 | Dr Cheung's lab |
| ALC3257 | Strain Newman with pALC1484 | [48] |
| pALC1484 | A derivative of pSK236 containing the promoterless $gfp_{uvr}$ gene preceded by an *S. aureus* ribosome binding site | [49] |
| pALC4231 | A derivative of pALC1484 with the *eap* promoter driving the $gfp_{uvr}$ gene | Dr Cheung's lab |

### 2.2. Internalization assays

The established bovine mammary epithelial cell line MAC-T [18] was generously provided by Nexia Biotechnologies (Quebec, Canada). MAC-T cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), insulin (5 μg/ml), hydrocortisone (5 μg/ml), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) (Sigma). For each experiment, MAC-T cells were seeded at $1.5 \times 10^5$ cells/well in 24-well tissue culture plates and grown for 24 h at 37 °C with 5% $CO_2$ to reach confluence. Internalization assays were performed as described previously [19]. Briefly, confluent MAC-T monolayers (approximately $1 \times 10^6$ cells/well) were inoculated with *S. aureus* suspended in fresh growth medium without antibiotics (invasion medium) to a multiplicity of infection of 40. Plates were subjected to centrifugation at $1000 \times g$ for 20 min to deposit bacteria on the monolayer surface and to synchronize the bacterial internalization into cells. After incubation for 1 h at 37 °C under 5% $CO_2$, wells were washed with PBS and then 1 ml of invasion medium supplemented with 2.5 μg of lysostaphin (Sigma) was added to each well to kill extracellular bacteria. Incubation of co-cultures with lysostaphin proceeded for an additional 2 h at 37 °C under 5% $CO_2$. Supernatants were collected and plated on tryptic soy agar to confirm 100% bacterial killing by lysostaphin. The monolayer was washed four times with sterile PBS, treated for 5 min at 37 °C with 100 μl of 0.25% trypsin–0.1% EDTA (Gibco BRL) and lysed by the addition of 900 μl of 0.025% Triton X-100 (USB) in sterile distilled water to release intracellular staphylococci. The number of released CFU was determined by quantitative plating on TSA agar. MAC-T cell viability was evaluated by trypan blue exclusion.

### 2.3. Transcriptional fusion studies

After overnight culture, *S. aureus* strains harboring the recombinant plasmids (see Table 1) were diluted 1:100 and grown with or without 50 μg/ml SAL at 37 °C with shaking in CYGPw broth. Aliquots were transferred every hour for 9 h to microtiter plates and assessed for cell density ($OD_{650nm}$) and fluorescence in a FL600 fluorescence spectrophotometer (BioTek Instruments). Promoter activities were plotted as the mean fluorescence/$OD_{650nm}$ ratio to minimize variations due to changes in cell density from experiment to experiment, using the average values from triplicate readings.

### 2.4. Real-time (qRT) PCR

Bacterial RNA was extracted using Trizol (Gibco BRL) and silica beads 0.1 mm in diameter (Biospec) and was subjected

to DNAse treatment using the TURBO DNA*free*TM kit (Ambion) according to the manufacturer's instructions. cDNA synthesis was performed with the Transcriptor First Strand cDNA Synthesis kit (Roche) using random hexamer primers. 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: *eap*-f 5′ TAG AGG TAT CGG GGA ACG TG-3′; *eap*-r 5′ TTG GTG TTG ATG TGC CAT TT-3′; *clf*A-f 5′ ATG TGA CAG TTG GTA TTG ACT CTG G 3′; *clf*A-r 5′ TAG GCA CTG AAA AAC CAT AAT TCA GT 3′ [20]; *fnb*A-f 5′ ACA AGT TGA AGT GGC ACA GCC 3′; *fnb*A-r 5′ CCG CTA CAT CTG CTG ATC TTG TC 3′ [20]; *emp*-f 5′ CAG AAT CGC CTA GAT ATA CAC ATC CA 3′; *emp*-r 5′ GCA TGC CCT GGT GTA ACA AAA TT 3′; *sdr*C-f 5′ AAA AGG CAT GAT ACC AAA TCG A 3′; *sdr*C-r 5′ AAT TCT CCA TTC GTA TGT TCT G 3′; *gyrB*-f 5′-GGT GCT GGG CAA ATA CAA GT-3′; and *gyrB*-r 5′-TGG GAT ACC ACG TCC GTT AT-3′. Cycling conditions were: 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 55 °C for 10 s and 72 °C for 15 s, and 1 cycle of 40 °C for 30 s. The *gyr*B 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 represents the difference in threshold cycle ($C_T$) between the target and control (*gyr*B) genes treated with SAL minus the difference in $C_T$ between the untreated target and control genes [21].

### 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

Protein extracts were prepared from SDS cell surface extracts as described by Hussain et al. [22], except that the use of dialysis to remove SDS was omitted. Briefly, to prepare cell surface proteins, staphylococci were grown in 40 ml cultures in CYGPw at 37 °C until $OD_{600nm} = 1.3$ and then centrifuged at $6000 \times g$ for 15 min. The pellet was resuspended in extraction buffer (125 mM Tris–HCl pH 7.0, 2% SDS), heated at 95 °C for 3 min, and then centrifuged at $10{,}000 \times g$ for 3 min. An equal volume of 2× Laemmli sample buffer [23] was added to the protein extracts prior to boiling them for 3 min and separating them by SDS–10% PAGE. SDS–PAGEs were stained with the Coomassie colloidal technique [24,25].

### 2.6. In-gel tryptic digestion and MS/MS analysis

The protein bands were excised with a scalpel and destained with a freshly prepared 50 mM $HCO_3NH_4$, 25 mM $HCO_3NH_4$ solution containing 50% acetonitrile and then 100% acetonitrile. After washing with water the sample was reduced, alkylated and digested with trypsin [26]. Tryptic peptides were extracted from the gel pieces with 50% acetonitrile/0.5% TFA, concentrated nearly to dryness in a SpeedVac vacuum centrifuge and diluted to a total volume of 5 µl with 50% acetonitrile/0.5% TFA. Samples were mixed 1:1 with 3 mg/ml hydroxicinnamic acid in 50% acetonitrile/0.5% TFA. Samples were analyzed by a MALDI–TOF spectrometer, Ultraflex II (Bruker), in the mass spectrometry facility (CEQUIBIEM, Buenos Aires, Argentina). Calibrated mass spectra were searched against a monthly updated copy of the NCBI nr protein database using bacteria as a taxonomy filter in the PNACL Proteomics Facility version of the Mascot search tool (Matrix Science). The criteria for protein identification were based on the manufacturer's definitions. The protein score is $-10 \times \log p$, where $p$ is the probability that the observed match is a random event. A protein score higher than 81 was considered significant ($p < 0.05$).

### 2.7. Nucleotide sequencing

A 300-base fragment of *sae*S was amplified from strains Newman, Wood46, HU27 and one representative strain of the Brazilian clone using the following primer pairs: *sae*S-f 5′-ATG CTA ATA CCG TGA ATG TCC A-3′ and *sae*S-r 5′-TGG CCG TTA AAC CAC ATT AAA-3′. PCR products of the expected sizes were purified from 1% agarose gels with a Wizard® SV Gel and PCR Clean-Up System (Promega) and were sequenced using a Beckman Coulter, CEQ2000XL sequencer by Cromátida, Argentina. Multiple DNA sequence alignments were performed with CodonCode Aligner (version 3.7.1), available at http://www.codoncode.com/aligner/download.htm.

### 2.8. Statistical analysis

Non-parametric data were analyzed with the Mann–Whitney test using the GraphPad software (PRISM, version 4.0). $p$ Values lower than 0.05 were considered significant.

## 3. Results

### 3.1. SAL affects Eap expression by S. aureus

Previous reports have shown that SAL alters expression of several virulence factors [15,27]. Recently, we demonstrated that a low concentration of SAL increased *sae* expression by the *S. aureus* strain Newman [16]. The effect of SAL on the *S. aureus* strain Newman surface protein profiles was assessed by SDS–PAGE. The results revealed that some bands were slightly increased but unexpectedly one major band of *ca.* 60 kDa was substantially enhanced by SAL treatment of *S. aureus* Newman (Fig. 1, lanes 1 and 2). This band may correspond to Eap since it is the major band of the appropriate size (~60–70 kDa) found in surface protein profiles [6]. To confirm this, we analyzed the 60 kDa band by MS/MS (MALDI–TOF) and the protein was identified as Eap (Score = 296, $p < 0.05$, see Materials and methods). These results demonstrate that SAL enhances Eap expression at the protein level in *S. aureus* strain Newman.

### 3.2. SAL affects Eap expression in S. aureus other than the Newman strain

The Newman strain bears a single amino acid variation within the first N-terminal transmembrane loop of SaeS compared with the SaeS of other strains [12]. It has been recently

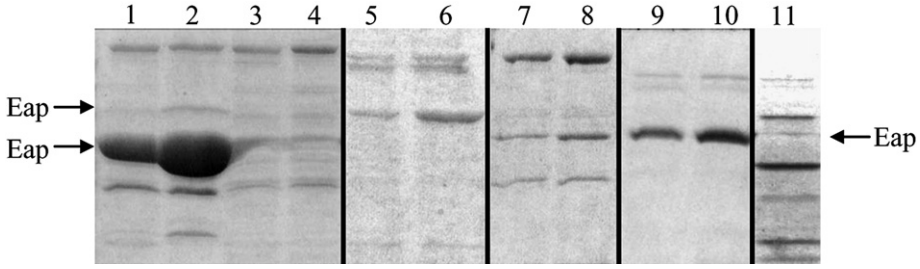


Fig. 1. Expression of Eap in different *S. aureus* strains after SAL treatment. Surface proteins were subject to SDS–PAGE. Bacteria were grown in CYGPw and proteins were extracted at an $OD_{600nm} = 1.3$. Equivalent volumes of cell extracts were loaded into each lane. Lane 1, Newman; lane 2, Newman + SAL; lane 3, NewHG; lane 4, NewHG + SAL; lane 5, Wood46; lane 6, Wood46 + SAL; lane 7, HU27; lane 8, HU27 + SAL; lane 9, Brazilian clone; lane 10, Brazilian clone + SAL; lane 11, AH12. Eap polypeptides were confirmed in each fraction by MS/MS (MALDI–TOF).

shown that this variation in SaeS may partially be responsible for the increased Eap expression seen in the Newman strain [11]. To assess whether or not the increased level of Eap expression after SAL treatment was due to the mutation described in the *saeS* from strain Newman, we performed SDS–PAGE of surface proteins from strain NewHG, a repaired *sae*S Newman derivative. The results showed that under the experimental conditions utilized, the NewHG strain expressed Eap at negligible levels which impeded the determination of any further increase in Eap expression (Fig. 1, lanes 3 and 4). Therefore, the effect of SAL was tested on other *S. aureus* strains including Wood46, the clinical isolate HU27, and a strain representative of the methicillin-resistant *S. aureus* Brazilian clone that did not harbor the mutation in *sae*S, as was confirmed by nucleotide sequence analysis (data not shown). Interestly, all these strains showed a higher Eap expression after SAL treatment (Fig. 1). Taken together, these results suggest that the effect of SAL on *S. aureus* does not depend upon the single amino acid variation within *sae*S found in the Newman strain.

### 3.3. Expression of the eap transcript is enhanced by SAL treatment

SAL treatment of *S. aureus* was previously shown to have an effect on expression of virulence factors at the transcriptional level [14,27,33]. To investigate the SAL-mediated effect on *eap* expression, we measured the activity of the *eap* promoter after SAL exposure through a $gfp_{uvr}$ reporter gene fusion analysis. As shown in Fig. 2A, treatment with SAL enhanced the *eap* promoter activity in the Newman strain. Further analysis by qRT-PCR (Fig. 2B) showed that the levels of *eap* transcripts from strains Newman and Wood46 after SAL treatment were also increased. In our previous report [16] we demonstrated that SAL treatment enhanced expression of the global regulator *sae* which controls expression of many secreted and anchored surface proteins. In addition, there is no expression of *eap* transcripts in the Newman *sae* mutant [Newman *vs.* Newman *sae* both without SAL: $2^{(-\Delta\Delta CT)} = 0.0005 \pm 0.00026$].

The results obtained in this study, in conjunction with those previously described, led us to suggest that *S. aureus* enhanced expression of *eap* after SAL treatment may be attributable to the increased expression of *sae*, its cognate regulator.

The effect of SAL on expression of other adhesins was ascertained by qRT-PCR. In strain Newman the level of *fnb*A transcripts was significant enhanced after treatment with SAL (Fig. 3). Besides a slight increment of the level of *emp* and *sdr*C transcripts was observed. However, the *clf*A expression was not modified by SAL. Furthermore, SAL exposure caused a significant increased level of adhesins (*fnb*A, *emp* and *sdr*C) transcripts in strain Wood46 (Fig. 3).

### 3.4. SAL induces enhanced cellular internalization of S. aureus

We have previously shown that pretreatment of bacteria with SAL enhanced the capacity of *S. aureus* to invade epithelial cells. This finding was attributed to a negative effect of SAL on CP5 expression [16]. In the present study we investigated whether the increased expression of Eap generated by SAL contributes to the incremented capacity of *S. aureus* to invade MAC-T epithelial cells. Unexpectedly, the AH12 mutant grown in the presence of SAL exhibited a similar capacity of internalization compared with that obtained with the Newman strain treated with SAL (Fig. 4). However, in agreement with previous reports [5,28], we have found that internalization of the *eap* mutant (AH12) was significantly decreased when compared with that of the wild-type strain. *S. aureus* strain AH12 organisms treated with SAL showed an increased ability of internalization when compared with untreated organisms (Fig. 4). Such increased ability cannot be attributed to Eap increased expression since the AH12 does not produce Eap (Fig. 1). Furthermore, the *sae* mutant of the Newman strain was internalized at lower levels than its wild-type counterpart (Fig. 4). SDS–PAGE analysis of surface proteins showed that the levels of Eap expression in the *sae* mutant were undetectable (data not shown). Exposure of the *S. aureus sae* mutant to SAL led to a mild increase in *S. aureus* internalization but at a level still much lower than that of the untreated parental control (Fig. 4). This result was expected since *sae* regulates many other factors in addition to Eap, e.g.: *sae* downregulates CP expression thus contributing to enhanced *S. aureus* internalization [16]. Taken together our results showed that the capacity of internalization of the *S. aureus* Newman strain into MAC-T cells would be independent of the increased Eap expression caused by SAL.

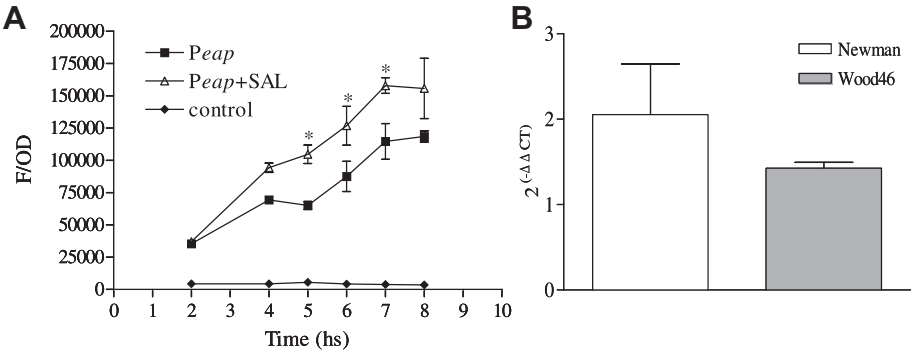


Fig. 2. The effect of SAL on the *eap* transcription. (A) Expression of *gfp* driven by the *eap* promoter (P*eap*) of strain Newman was measured during the growth cycle. Fluorescence values were expressed as GFP fluorescence related to $OD_{650nm}$ (F/OD) to minimize variations in fluorescence due to varying cell densities. Control: *gfp* driven by no promoter. Data represent the arithmetic mean ± SD of 3–4 independent experiments. Mann–Whitney test, $*p = 0.0286$ ($p < 0.05$). (B) Real Time PCR of the *eap* transcript from strains Newman and Wood46 treated or not treated with SAL. Changes in gene expression are shown as normalized mean fold change $[2^{(-\Delta\Delta CT)}] \pm$ SEM (differences of *eap* gene expression with 50 μg/ml of SAL to an $OD_{650nm} = 1.4$ compared to *eap* gene expression without treatment). Data were normalized to *gyr*B expression. $2^{(-\Delta\Delta CT)} > 1$ represents significant increased expression by SAL and $2^{(-\Delta\Delta CT)} < 1$ indicates significant decreased expression by SAL. The data represent the mean of duplicate measurements from 3 independent experiments. $2^{(-\Delta\Delta CT)}$: Newman = 2.05 ± 0.59; Wood46 = 1.42 ± 0.07.

## 4. Discussion

The surface protein profiles of several *S. aureus* strains treated or not treated with SAL were evaluated in the present study. We have found that SAL treatment increased the expression of Eap by *S. aureus*. This enhancement in Eap expression caused by SAL was also demonstrated at the transcriptional level by qRT-PCR. The expression of Eap is controlled in *S. aureus* by the combined action of multiple regulatory systems and different environmental stimuli [9,29,30]. Several studies have demonstrated that the global regulator *sae* controls Eap production at the transcriptional level [9,12,31]. In the *S. aureus* strain Newman an increased Eap production was reported [9]. This distinctive feature of the Newman strain was attributed to a point mutation in *sae*S that provokes a single amino acid change (Leu 18 to Pro 18) in the first transmembrane loop [11,32]. Interestingly, Schafer et al. [32] observed the upregulation of *sae* and *eap* upon exposure of the Newman strain to a biocide (Perform®) and SDS. In agreement with this result, we have found high levels of *eap* transcript and Eap after treatment with SAL, even above the high baseline levels displayed by the Newman strain. In contrast to SDS effect, enhanced Eap expression by SAL was also observed in other *S. aureus* strains with wild-type SaeS. This observation agrees with the previous demonstration that *sae* can be activated by SAL in the *S. aureus* Newman strain [16]. Recently, Mainiero et al. [11] have showed that certain *sae* target genes (*coa*, *fnbA*, *eap*, *sib*, *efb*, *fib*, *sae*) are highly expressed in the Newman strain due to the requirement of a high level of SaeR phosphorylation. In addition, we demonstrated in a previous study that treatment with SAL diminished CP expression by the Newman strain [16], although the *cap* gene belongs to the group of the *sae* target genes that are independent of the SaeS mutation [11]. Our results suggest that the effect of SAL on Eap expression would be independent of the SaeS mutation (Leu 18 to Pro 18) under the experimental conditions utilized.

Previous studies revealed that SAL downregulates *mgrA* transcription in *S. aureus* [16,33]. Other authors showed that treatment of *S. aureus* with SAL activated the transcriptional factor sigma B [15,27]. On the other hand, previous reports found non influence of $\sigma^B$ on *sae* expression in the *S. aureus* strains Newman, COL and GP268 [34,35]. Geiger et al. [36] demonstrated that neither *agr* nor *sig*B had influence on *sae* promoter activities in the strain Newman. In addition, the existence of an as yet unidentified regulator of *sae* target genes (for example *eap*, *hla*, *emp*), which may function together with SaeR, has been hypothesized [35,37,38]. Whether SAL may also affect this putative regulator deserves to be investigated deeply.

Recently, we demonstrated that the ability of *S. aureus* to be internalized into epithelial cells was increased by a low concentration of SAL. This finding, which was attributed to a diminished CP expression [16], allowed us to hypothesize that an increased expression of certain adhesins, such as Eap, may contribute to this process. Contrary to what was expected, we observed similar levels of internalization of Newman and its *eap* mutant (AH12) both exposed to SAL. However, the mutant AH12 exhibited a significantly lower level of invasion of MAC-T epithelial cells compared with the parental strain. These results concur with those of Haggar et al. [5] who showed a diminished adhesion of strain AH12 organisms to fibroblasts and keratynocytes compared with the parental *S. aureus* Newman strain. Eap plays an important role in the establishment of disease because it enhances the adhesion of *S. aureus* to eukaryotic cells [7]. Both FnBPA and FnBPB are truncated in the Newman strain leading to deficient adherence *in vitro* [8]. However, the loss of functional FnBPs would be partially compensated by the increased level of Eap present in this strain [39]. Treatment of the AH12 mutant with SAL provoked a significant increased ability of cellular invasion compared with the untreated strain. This fact may be attributed to a decreased expression of CP [16] and the consequent unmasking of other adhesins in the AH12 strain grown in the presence of SAL. From our results it can be inferred that other

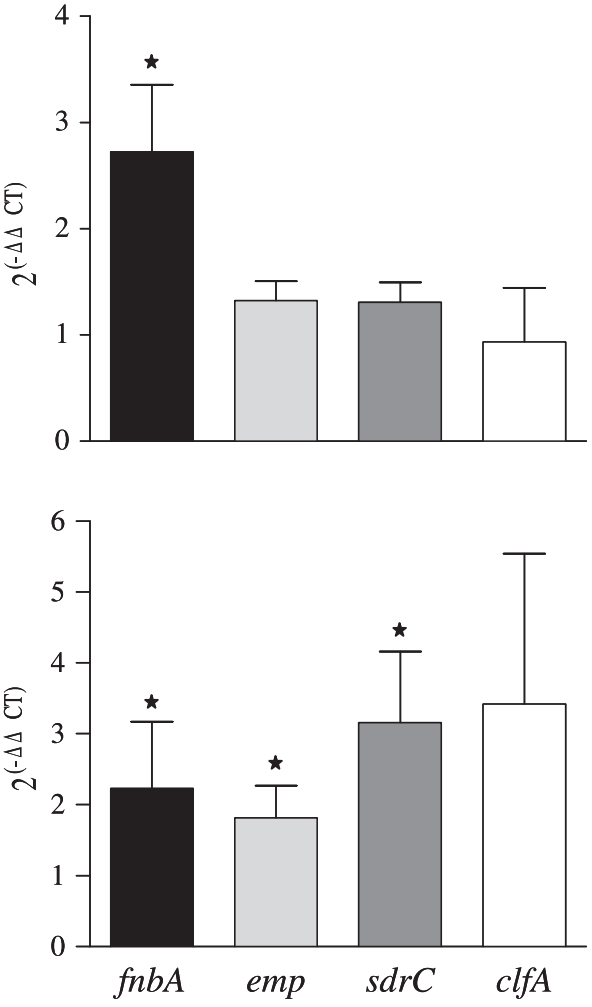


Fig. 3. The effect of SAL on the *fnb*A, *emp*, *sdr*C and *clf*A transcription. Real Time PCR of *fnb*A, *emp*, *sdr*C and *clf*A transcripts from strains Newman (upper panel) and Wood46 (lower panel) treated or not treated with SAL. Changes in gene expression are shown as normalized mean fold change $[2^{(-\Delta\Delta CT)}] \pm$ SEM. Data were normalized to *gyr*B expression. $2^{(-\Delta\Delta CT)} > 1$ represents significant increased expression by SAL and $2^{-(\Delta\Delta CT)} < 1$ indicates significant decreased expression by SAL. The data represent the mean of duplicate measurements from 3 independent experiments. $2^{(-\Delta\Delta CT)}$ Newman: *fnb*A $= 2.72 \pm 0.63$; *emp* $= 1.32 \pm 0.18$; *sdr*C $= 1.31 \pm 0.19$; *clf*A $= 0.93 \pm 0.51$; Wood46: *fnb*A $= 2.23 \pm 0.94$; *emp* $= 1.81 \pm 0.46$; *sdr*C $= 3.16 \pm 1.00$; *clf*A $= 3.42 \pm 2.12$.

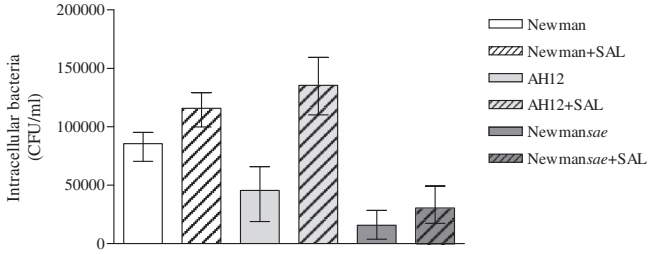


Fig. 4. SAL enhances the invasion ability of the *S. aureus* strain Newman. Confluent MAC-T cells were tested for staphylococcal invasion using strains Newman, Newman *eap* (AH12) and Newman *sae* pretreated or not with 50 μg/ml of SAL. Each bar represents the median and range value of intracellular CFU/ml ($n = 9$–12). Newman + SAL: $1.1 \times 10^5$ CFU/ml *vs.* AH12 + SAL: $1.4 \times 10^5$ CFU/ml, $p > 0.05$; AH12: $4.6 \times 10^4$ CFU/ml *vs.* AH12 + SAL, $p < 0.0001$; Newman: $8.5 \times 10^4$ CFU/ml *vs.* AH12, $p = 0.0002$; Newman *vs.* Newman *sae*: $1.6 \times 10^4$, $^*p = 0.0006$; Newman *sae vs.* Newman *sae* + SAL: $3 \times 10^4$ UFC/ml, $p = 0.0107$; Newman *vs.* Newman + SAL, $p = 0.0061$; Mann–Whitney test ($p < 0.05$ was considered significant).

adhesins different from Eap (see Fig. 3) may also contribute to internalization of strain Newman organisms into the MAC-T epithelial cells. In fact, we observed that other adhesins such as *emp* and *sdr*C are enhanced after SAL treatment in strain Newman. In this regard, a recent study showed a novel staphylococcal internalization mechanism that involves the major autolysin Atl [40].

Steinhuber et al. [12] demonstrated that a Δ*sae* mutant of strain ISP479C had a decreased rate of invasion into human endothelial cells compared with the parental wild type, while the Newman strain organisms and its Δ*sae* derivative exhibited significantly reduced invasion rates. In the present study, it was demonstrated that the invasion rate of the *S. aureus* Newman *sae* mutant into MAC-T cells was decreased compared with the parental strain. Moreover, the treatment with SAL caused a moderate increase in the Newman *sae* invasion rate, which did not reach the levels found in the Newman strain. Therefore, these results demonstrate that the *sae* deficiency affects virulence factors involved in internalization of *S. aureus* strain Newman into MAC-T cells. Certainly, the effect of SAL on other regulator systems (e.g. *mgrA*) [16] of the Newman *sae* mutant may not contribute in a large extent to the epithelial cellular invasion.

Taken together our findings allow us to speculate that SAL concomitantly provokes a reduction of CP [16] and an enhancement of Eap which also increase the capacity of internalization of *S. aureus* strain Newman. Joost et al. [6] showed that Eap contributes to the development of chronic infection. In addition, we also showed in an animal model of staphylococcal disease that loss of CP expression may lead to persistence of *S. aureus* [41,42]. Accordingly, we have also found higher incidence of non encapsulated *S. aureus* in patients with chronic osteomyelitis compared with those with acute osteomyelitis [43]. SAL likely attenuates *S. aureus* virulence in endovascular infections by targeting global staphylococcal regulatory pathways [27,44]. In a recent study, Eisen et al. [45] reported that administration of aspirin to patients developing *S. aureus* infective endocarditis was associated with a reduced occurrence of acute valve replacement surgery. In spite of this undisputed empirical finding, our data on SAL treatment of *S. aureus* cells and its ensuing effect on enhanced Eap expression and internalization into MAC-T cells calls for a revision of the risk-benefit analysis of prophylactic or therapeutic aspirin treatment in patients with *S. aureus* infection. SAL, the major aspirin metabolite, may create the conditions for *S. aureus* persistence in the infected host, not only by reducing CP expression as we have showed before [16] but also by enhancing Eap expression.

## Acknowledgments

We are grateful to Lorena Medina and Constanza Giai for their expert technical assistance. We thank Dr. Muzaffar Hussain (Institute of Medical Microbiology, University Hospital of Müenster, Müenster, Germany) for kindly providing *S. aureus*

strain AH12. We thank Dr. Christiane Wolz (Institute of Medical Microbiology, University Hospital, Tübingen, Germany) for kindly providing *S. aureus* NewHG strain. We thank Dr. Daniela Centrón (Department of Microbiology, Medicine College, University of Buenos Aires, Buenos Aires, Argentina) for kindly providing *S. aureus* the Brazilian clone strain utilized in this study. This work was supported in part by grants from ANPCyT (PICT 06/00991), CONICET (PIP 5933) and Universidad de Buenos Aires (UBACyT M-070, M-406, 90200061), Buenos Aires, Argentina.

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