INTRODUCTION

Staphylococcus aureus is a major human pathogen, responsible for a large number of nosocomial infections. The pathogenesis of S. aureus has been attributed to its ability to produce a diverse range of secreted proteins (haemolysins, lipases, proteases) and cell-wall-associated proteins (protein A and adhesins) (Novick, 2000; Projan & Novick, 1997).

Protein A (Spa) is a major surface protein of S. aureus strains. The biological properties of Spa have been extensively studied. It has the ability to interact with several host components (immunoglobulins G, A and E, platelets, von Willebrand factor), suggesting a role as a virulence factor in S. aureus infections (Cheung et al., 1997; Hartleib et al., 2000). In several animal models (peritonitis, subcutaneous model, mastitis and arthritis), the isogenic spa mutant causes less severe infections than the wild-type strain (Hartleib et al., 2000; Palmqvist et al., 2002). The binding of protein A to IgG antibodies is involved in evading the immune response, perhaps by preventing opsonization (Projan & Novick, 1997).

The regulation of the production of virulence determinants such as Spa in S. aureus involves several global regulatory loci. The agr (Morfeldt et al., 1988; Peng et al., 1988) and sarA (Cheung et al., 1992) loci are the best characterized of these loci. Several other regulatory loci have also been described: sar homologues (Cheung et al., 2001; Manna & Cheung, 2001, 2003; McNamara et al., 2000; Schmidt et al., 2001; Tegmark et al., 2000), sae (Giraudo et al., 1994), arl (Fournier et al., 2001) and srr (Yarwood et al., 2001). In S. aureus, virulence determinant production is modulated in response to growth conditions (Chan & Foster, 1998a, b; Lindsay & Foster, 1999; Novick, 2000), reflecting the ability of S. aureus to adapt and to survive in many different environmental niches. The mechanism by which the external signals are transduced to modify gene expression is crucial for understanding how S. aureus adapts to its host. This adaptation often involves two-component systems, generally consisting of a sensor (histidine kinase) and a response regulator (Stock et al., 1989). The sensor is an integral membrane protein that becomes phosphorylated in response to environmental signals. The phosphate is then transferred to a conserved residue in the response regulator. The response regulator is often a transcription factor, the affinity of which for DNA is modulated by phosphorylation (Stock et al., 1989). To date, four different two-component systems that control virulence have been identified in S. aureus: Agr, Sae, Srr and Arl. The two-component system ArlS–ArlR is involved in several cell
activities. Production of a multidrug-resistance efflux pump, NorA, was increased in an arlS transposon insertion mutant (Fournier et al., 2000). The arlS mutant formed a biofilm on polystyrene surfaces, probably because of altered activity of secreted peptidoglycan hydrolases (Fournier & Hooper, 2000). The arl locus is also involved in the regulation of several virulence factors, mainly protein A, and some secreted proteins (α-toxin, β-haemolysin, coagulase, lipase). The arl locus probably exerts its effects on virulence factors mostly via the agr and/or sarA regulatory pathway (Fournier et al., 2001).

Bacterial DNA is maintained in a negatively supercoiled state, which favours DNA recombination, replication, transcription and transposition. As the level of DNA supercoiling varies with the cellular energy charge, it can change rapidly in response to nutritional and environmental conditions. Several independent studies have provided evidence that DNA supercoiling plays a role in the transduction of environmental signals to the bacterial nucleoid. In particular, changes in temperature, osmolarity and oxygen availability have been shown to change DNA supercoiling (Dorman, 1991; Hsieh et al., 1991). In Escherichia coli, it has been estimated that roughly 50% of the supercoiling is constrained by proteins bound to DNA. The remaining supercoils are maintained actively at the cost of ATP hydrolysis, via topoisomerase activities (Hatfield & Benham, 2002). Thus, in E. coli, factors responsible for modifying DNA supercoiling fall into three general categories: those affecting DNA topoisomerases, those altering DNA structure, and those influencing cellular energetics (Drlica, 1992).

In this study, we showed that protein A gene expression is regulated by DNA supercoiling in response to environmental conditions such as high osmolarity, and that the Arl system may interact with the mechanism by which modulators of DNA topology (high osmolarity or DNA gyrase inhibitors) control spa expression. We also provide evidence for a relationship between the arlRS deletion and topological changes in plasmid DNA conformation.

**METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. Staphylococci were grown in trypticase soy broth (TSB) at 37°C. Cultures of 150 ml in 500 ml flasks were inoculated at a starting OD$_{600}$ value of 0.025 prior to growth at 37°C. When required, antibiotics were added at the following concentrations: 7 mg chloramphenicol l$^{-1}$; 20 mg erythromycin l$^{-1}$; 10 mg kanamycin l$^{-1}$; 3 mg tetracycline l$^{-1}$.

**DNA transformation.** Plasmid DNA was isolated and used to transform S. aureus by electroporation as previously described (Novick, 1991). Chromosomal DNA was isolated from S. aureus as described by Stahl & Pattee (1983). Transformation with high molecular mass chromosomal DNA was performed as previously described (Stahl & Pattee, 1983).

**Plasmid construction.** A transcriptional spa::lacZ reporter fusion had previously been constructed by cloning PCR fragments into pBF50. pBF50 is an integrative promoterless transcriptional vector (Table 1) (Fournier et al., 2001). It contains the attP site of staphylococcal phage L54a and is capable of integrating specifically into the chromosomal attB site in the geh gene, which encodes lipase (Lee et al., 1991).

**β-Galactosidase activity.** β-Galactosidase activity was determined as previously described (Fournier et al., 2001). Briefly, cells were assayed for β-galactosidase (LacZ) activity using the chemiluminescent reporter assay system (Galacton-Light Plus, Applied Biosystems). Bacterial cells were grown to different OD$_{600}$, harvested, washed and resuspended in lysis buffer supplemented with 50 μg lysostaphin ml$^{-1}$.

**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RN4220</td>
<td>8325-4, nitrosoguanidine-induced restriction mutant used as a primary recipient for plasmids propagated in E. coli</td>
<td>Kreiswirth et al. (1983)</td>
</tr>
<tr>
<td>RN6390BF</td>
<td>Wild-type derivative of 8325-4</td>
<td>Fournier et al. (2001); Peng et al. (1988)</td>
</tr>
<tr>
<td>BF21</td>
<td>RN6390BF arlR::cat</td>
<td>Fournier et al. (2001)</td>
</tr>
<tr>
<td>MT5</td>
<td>8325 gyrB142 hisG15 pig-131</td>
<td>Fournier &amp; Hooper (1998)</td>
</tr>
<tr>
<td>BF41</td>
<td>RN6390BF gyrB142</td>
<td>This study</td>
</tr>
<tr>
<td>BF42</td>
<td>RN6390BF arlR::cat gyrB142</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
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<tr>
<td>pE194</td>
<td>3.7 kb S. aureus plasmid, Em$^r$</td>
<td>Shivakumar et al. (1980)</td>
</tr>
<tr>
<td>pT181</td>
<td>4.4 kb S. aureus plasmid, Tet$^r$</td>
<td>Khan &amp; Novick (1983)</td>
</tr>
<tr>
<td>pUB110</td>
<td>4.5 kb S. aureus plasmid, Km$^r$</td>
<td>Jalanlko et al. (1981)</td>
</tr>
<tr>
<td>pBF50</td>
<td>12 kb shuttle promoterless transcriptional lacZ fusion vector carrying the attP site of phage L54a; Ap$^r$ (E. coli), Tet$^r$ (S. aureus)</td>
<td>Fournier et al. (2001)</td>
</tr>
<tr>
<td>pBFSpa</td>
<td>300 bp fragment containing the spa promoter cloned upstream of the lacZ gene of pBF50</td>
<td>Fournier et al. (2001)</td>
</tr>
</tbody>
</table>
and 15 μg DNase ml⁻¹. The mixture was incubated for 30 min at 37°C, and the β-galactosidase and protein concentrations of the supernatant were then determined. β-Galactosidase activity is expressed in relative light units (RLU). Assays were performed on duplicate samples and the mean values were determined. The results presented here are representative of at least two independent experiments.

**Plasmid supercoiling analysis.** Strains were grown at 37°C in TSB medium with appropriate antibiotics to an OD₆₀₀ of 0.25–2.0. After centrifugation, cell pellets were stored at −20°C. Cells were thawed and plasmid DNA extracted. Plasmid DNA was separated on 1% agarose gels containing 80 or 140 mg ml⁻¹ chloroquine, a DNA intercalator, in TBE buffer (90 mM Tris/borate, 2 mM EDTA). Chloroquine was removed by soaking the gels in distilled water for at least 4 h before staining with ethidium bromide (3 μg ml⁻¹). The gels were scanned and subjected to densitometry using National Institutes of Health Image, a public domain image-processing program. The experiment was repeated three to four times and a representative set of values is shown.

**Protein A analysis.** Cultures were grown to an OD₆₀₀ of 2.0 and centrifuged. Cell-wall-associated proteins were extracted with lysostaphin in a hypertonic medium (30% raffinose), as described previously (Cheung & Fischetti, 1988; Fournier et al., 2001). Briefly, cell-wall-associated proteins (30 μg) were resolved on a 10% SDS-polyacrylamide gel, electrophoresed onto nitrocellulose Hybond-C Pure and probed with rabbit anti-staphylococcal protein A antibody (Sigma) at a 1:15000 dilution. Bound antibody was detected with donkey anti-rabbit immunoglobulin G conjugated to peroxidase (1:20 000) and the ECL Western blotting detection system (Amersham). Quantification of signals from Western blots was performed by densitometric analysis of the autoradiograms using NIH Image. Arbitrary units (AU) correspond to the integrated density measured by the program. The experiment was performed twice and a representative set of values is shown.

**Measurement of transformation efficiency.** pT181 and pE194 were extracted from strain RN4220 and the same amount of DNA was then transformed into different strains by electroporation. Bacteria were grown on TSA plates containing either tetracycline (pT181) or erythromycin (pE194). The competence of each *S. aureus* recipient was assessed by counting the number of transformants. Data are representative of at least four independent determinations.

**RESULTS AND DISCUSSION**

**Repression of spa expression in the presence of high osmolarity**

Several studies have demonstrated that NaCl, sucrose, alkaline pH, glucose, temperature and several ions modify the expression of virulence genes (Chan & Foster, 1998a, b; Regassa & Betley, 1992; Regassa et al., 1992). A transcriptional *spa::lacZ* fusion was previously inserted as a single copy into the chromosome of the wild-type strain RN6390BF (Fournier et al., 2001).

The presence of 1 M NaCl dramatically repressed *spa* expression (Fig. 1a). The expression level of *spa* was about four- to sevenfold lower in the presence of 1 M NaCl than in its absence (Fig. 1a). To determine whether NaCl repression is due to a general osmotic stress effect or specifically to sodium or chloride ions, we studied the effect on *spa* transcription of other osmoles: another

Fig. 1. Effect of high osmolarity on the expression of the *spa::lacZ* fusion (a) and on plasmid DNA supercoiling (b, c).
(a) Expression of the *spa::lacZ* fusion in the wild-type strain (RN6390BF) in the presence of different osmoles: 1 M NaCl (▪), 1 M KCl (□) and 1 M sucrose (■); ○, absence of osmolyte. β-Galactosidase activity is expressed in relative light units (RLU) per g protein. OD₆₀₀; rather than time, was plotted on the x axis because the presence of certain additives slows down growth. (b) Plasmid DNA supercoiling in the absence and presence of 1 M NaCl. Strain RN6390BF (pE194) was grown to an OD₆₀₀ of 2.0. After plasmid extraction, topoisomers were separated in agarose gels containing 80 μg chloroquine ml⁻¹ in Tris/borate buffer (in these conditions, less negatively supercoiled topoisomers migrate more rapidly). (c) Scan analysis of topoisomer distribution in the plasmids separated in the gel shown in (b).
ionic osmolyte similar to NaCl (KCl) and a non-ionic osmolyte (sucrose). Sucrose (1 M) and KCl (1 M) similarly repressed \textit{spa} expression (Fig. 1a). Thus, the repression effect on \textit{spa} is likely specific for the osmotic stress response.

Expression of several virulence genes of \textit{S. aureus}, such as those encoding \textit{a}-toxin, protein A and toxic-shock syndrome toxin-1, have been shown to be dependent on osmolarity (Chan & Foster, 1998a, b; Regassa & Betley, 1992; Regassa \textit{et al.}, 1992). One of the distinguishing characteristics of \textit{S. aureus} is its considerable tolerance of NaCl. \textit{S. aureus} is the most halotolerant, non-halophilic eubacterium known. It is generally accepted that the lowest water activity that allows the growth of this organism is 0.86 (equivalent to 3.5 M), and the reduction of water activity is commonly used as a method of food preservation. This organism is probably the most common cause of food-poisoning outbreaks (Graham & Wilkinson, 1992).

Effect of high osmolarity on DNA supercoiling

High osmolarity alters the degree of plasmid DNA supercoiling (Dorman, 1991). Thus, we examined the effect of high osmolarity on the topoisomer distribution of pE194 (Shivakumar \textit{et al.}, 1980) isolated from RN6390BF. Strains were grown at 37°C in TSB medium with appropriate antibiotics to an OD\textsubscript{600} of 2.0 and plasmid DNA extracted. As expected, plasmid preparations isolated from cells grown in high-osmolarity medium contained more supercoiled topoisomers than those grown in low-osmolarity conditions (Fig. 1b, c).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Effect of novobiocin on plasmid DNA supercoiling. (a) Effect of novobiocin on pE194 supercoiling in the wild-type strain; the concentration of chloroquine in the gel was 80 \(\mu\)g ml\(^{-1}\). The numbers above the lanes indicate the concentration of novobiocin (5, 20 and 40 ng ml\(^{-1}\)) in the culture medium. (b) Scan analysis of topoisomer distribution in the plasmids separated in the gel shown in (a). (c) Effect of novobiocin on pE194 supercoiling in the wild-type strain; the concentration of chloroquine in the gel was 7 \(\mu\)g ml\(^{-1}\).}
\end{figure}

It is also noteworthy that the pathogenicity of \textit{S. aureus} involves the colonization of a high-salt environment, the mammalian skin. Thus, response to NaCl could be an important factor for adaptability and pathogenicity in \textit{S. aureus}.
Protein A gene expression is modified by novobiocin

As the expression of the protein A gene is dramatically repressed by high salt concentrations (1 M NaCl) and as high osmolarity alters the degree of plasmid DNA supercoiling, we looked at the effect of changing the degree of DNA supercoiling on protein A gene expression. We first determined the concentration of novobiocin, a DNA gyrase inhibitor, which relaxes DNA. Strain RN6390BF containing pE194 was grown at 37 °C in TSB medium containing different concentrations of novobiocin and the topoisomer distribution of pE194 was examined. No differences in DNA supercoiling were observed between the peak of distribution of plasmids from bacteria grown without antibiotic and those grown with 5 ng novobiocin ml\(^{-1}\) (Fig. 2a, b). However, DNA relaxed slightly with 20 ng novobiocin ml\(^{-1}\) and even more at 40 ng novobiocin ml\(^{-1}\) (Fig. 2a, b). With a lower concentration of chloroquine (7 μg ml\(^{-1}\)) in the gel, we also observed an increase of relaxed DNA (Fig. 2c). Thus, as expected, 20 and 40 ng novobiocin ml\(^{-1}\) resulted in plasmid relaxation.

We then tested the effect of novobiocin on the expression of the protein A gene in cells harbouring the spa::lacZ fusion. The wild-type strain was grown in TSB medium in the absence and presence of novobiocin. spa expression was similar in the absence and presence of 40 ng novobiocin ml\(^{-1}\) at the beginning of the exponential phase (OD\(_{600}\) 0.1) (Fig. 3a). Subsequently, novobiocin induced spa expression at all growth stages. At the end of the exponential phase (OD\(_{600}\) 2.0), spa expression was 26-fold higher when bacteria were grown with 40 ng novobiocin ml\(^{-1}\) (Fig. 3a). The highest concentration of novobiocin used (40 ng ml\(^{-1}\)) corresponds to one-quarter of the minimal inhibitory concentration (160 ng ml\(^{-1}\)), and did not affect the growth rate (data not shown). Thus, the novobiocin-induced increase in protein A gene expression was not simply due to alterations in the growth rate. We also found that the level of production of the cell-wall-associated protein A was much higher (15-fold) in the presence of novobiocin than in its absence (Fig. 3b).

**Effect of gyrB mutations on DNA supercoiling and protein A gene expression**

DNA supercoiling is regulated by several enzymes. DNA gyrase is the bacterial topoisomerase that introduces negative supercoils into DNA by using the free energy created by ATP hydrolysis. This enzyme consists of two proteins, A and B; the active enzyme is an A\(_2\)B\(_2\) complex. The A protein (GyrA) is responsible for the breakage-reunion of DNA (N-terminal domain) and for the DNA–protein interactions (C-terminal domain). The N-terminal domain of the B protein contains the ATPase activity. Coumarins such as novobiocin or coumermycin inhibit the ATPase reaction (Maxwell, 1993). In S. aureus, a gyrB142 allele corresponding to a double mutation (Ile102Ser and Arg144Ile) confers a high level of resistance to novobiocin (Fournier & Hooper, 1998). These mutations are located in the N-terminal domain responsible for the ATPase activity. The Arg144 residue in the S. aureus protein is at the same position as Arg136 in the E. coli homologue. DNA supercoiling activities of the mutants Arg136-His, -Cys and -Ser are reduced to a greater or lesser extent relative to the wild-type enzyme (Contreras & Maxwell, 1992). Thus, it is assumed that, similarly to what is observed in E. coli, the presence of the gyrB142 allele of S. aureus will induce DNA relaxation by modifying the ATPase activity. We determined the effect of the gyrB142 double mutation (Ile102Ser and Arg144Ile) (Fournier & Hooper, 1998) on DNA supercoiling. We analysed the topoisomerase distribution of plasmid preparations from the wild-type strain and the gyrB142 mutant (Fig. 4a, b). The topoisomerase distribution of pE194 in the wild-type strain differed from that in the gyrB142 mutant, ranging from two to three shifts (Fig. 4a, b). Thus, in S. aureus strains carrying the gyrB142 allele, a relaxation of plasmid DNA was observed. Therefore, we used this mutant to study the relationship between DNA supercoiling and spa expression in S. aureus.
The transcriptional spa::lacZ fusion was introduced into S. aureus BF41, a derivative of strain RN6390BF carrying the gyrB142 allele on the chromosome (Table 1). The introduction of the gyrB142 allele into the wild-type strain resulted in a significant increase (sevenfold) in protein A gene expression at the beginning of the exponential phase (OD₆₀₀ 0·1) (Fig. 4c). This sevenfold increase was observed until an OD₆₀₀ of 0·5. At the end of the exponential phase (OD₆₀₀ 2·0), no significant difference in spa expression was observed between the wild-type strain and its gyrB142 mutant (Fig. 4c). These results indicate that the gyrB142 allele increases protein A gene expression mainly during the exponential phase. As this allele also induces DNA relaxation, these results reinforce the hypothesis that there is a relationship between DNA relaxation and an increase in protein A production. Although we measured DNA supercoiling using plasmid DNA, the fact that novobiocin also affected expression from the supercoiling-sensitive chromosomal spa promoter strongly suggests a similar change in chromosomal DNA supercoiling. Thus, this result strongly suggests that protein A gene expression is regulated by DNA supercoiling in the wild-type background.

Both the presence of novobiocin and the gyrB142 allele induce DNA relaxation. However, spa expression is differently modified by these two DNA supercoiling modulators. In the presence of novobiocin, expression of spa was mainly increased at the end of the exponential phase (Fig. 3a), whereas in the presence of the gyrB142 allele, spa expression was mainly increased at the beginning of the exponential phase (Fig. 4c). Novobiocin has to be added to the culture medium before it can enter the cell. Conversely, the gyrB142 allele is already efficient in the cell at the beginning of the growth. This also suggests that the modification of DNA supercoiling is dependent on the growth phase, as spa expression was not modified at the end of the exponential phase with the gyrB142 allele (Fig. 4c).

**Combined effect of high osmolarity and novobiocin on spa expression and DNA supercoiling**

To observe the combined effect of high osmolarity and novobiocin on protein A gene expression, the wild-type strain harbouring the spa::lacZ fusion was grown in TSB medium containing 1 M NaCl in the presence and absence of novobiocin. In parallel, we also determined the DNA topological state of pE194. The concentration of novobiocin used was lower (10 ng ml⁻¹) in the presence of NaCl than in its absence (40 ng ml⁻¹) because cells cannot grow with a higher concentration.

Addition of 10 ng novobiocin ml⁻¹ in the presence of 1 M NaCl increased protein A gene expression by up to fourfold at the end of the exponential phase (OD₆₀₀ 2·0) (Fig. 5a). Thus, we determined the effect of novobiocin on NaCl-induced DNA supercoiling (Fig. 5b, c). Increasing concentrations of novobiocin relaxed plasmid DNA, even in the
presence of high concentrations of NaCl. This result correlates well with the induction of \textit{spa} expression when novobiocin was added to the medium. Furthermore, \textit{spa} expression of bacteria grown in 1 M NaCl and 10 ng novobiocin ml\textsuperscript{-1} was lower than that of bacteria grown in the medium alone (Fig. 5a). The peak of distribution of plasmids from bacteria grown with NaCl and novobiocin was more negatively supercoiled than that from bacteria grown in the medium alone (Fig. 5b, c). Thus, there is a good correlation between DNA supercoiling and \textit{spa} expression.

We found that the expression of \textit{spa} was repressed by high osmolarity and induced by increasing concentrations of novobiocin. Furthermore, the presence of the \textit{gyrB142} allele also increased protein A gene expression. Thus, protein A gene expression appears to be at least regulated by DNA supercoiling. Previous studies on \textit{S. aureus} (Chan \& Foster, 1998a; Lindsay \& Foster, 1999) have suggested that the expression of the genes encoding the \textit{z}-toxin (\textit{hla}), the toxic shock syndrome toxin-1 (\textit{tst}) and protein A (\textit{spa}) is not regulated by DNA supercoiling because the expression of these genes is not affected by 0.5 ng novobiocin ml\textsuperscript{-1}. This novobiocin concentration is much lower than that used here. We found that 1-2 ng novobiocin ml\textsuperscript{-1} did not modify protein A gene expression or DNA supercoiling. Thus, we speculate that the discrepancy observed between the two studies is due to the novobiocin concentrations used. However, another gene (\textit{eta}) is strongly induced when \textit{S. aureus} is grown in the presence of subinhibitory concentrations of novobiocin (Sheehan \textit{et al.}, 1992). These results suggest that the regulation of virulence genes such as \textit{spa} and \textit{eta} is dependent on DNA supercoiling.

DNA superhelicity is known to exert a wide variety of effects on prokaryotic gene expression. Increasing or decreasing DNA supercoiling modifies the transcription initiation reaction of certain promoters and has consequences for

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**Fig. 5.** Effect of combined high osmolarity and novobiocin on \textit{spa} expression (a) and DNA supercoiling (b, c). (a) Expression of the \textit{spa}::\textit{lacZ} fusion in different conditions: ○, absence of NaCl; ●, 1 M NaCl; □, 1 M NaCl and 10 ng novobiocin ml\textsuperscript{-1}. Bacteria were grown as in Fig. 1. \(\beta\)-Galactosidase activity was measured as in Fig. 1. (b) Plasmid DNA supercoiling in the same conditions. Chloroquine gel analysis was performed as in Fig. 1. (c) Scan analysis of topoisomer distribution in the plasmids separated in the gel shown in (b).
other topological perturbations of DNA (e.g. looping or bending) that affect transcription (Dorman, 1991). The relative abundance for 88 proteins has been measured in E. coli strains carrying non-lethal mutations in genes encoding topoisomerase I or gyrase (which raise and lower the level of supercoiling, respectively). The abundances of many proteins are maximal at wild-type supercoiling levels, whereas others exhibit maximal abundance at relaxed levels of DNA supercoiling and a smaller number show maximal abundance at elevated levels of DNA supercoiling (Steck et al., 1993). RNA polymerase-mediated initiation of transcription requires the transient melting of the DNA duplex at the transcription initiation start site. The untwisting stresses imposed by negative supercoiling can facilitate this energetically costly process, which can, in turn, increase the rate of the transcription initiation reaction (Sheridan et al., 2001). However, similarly to gyrA expression in E. coli, spa expression in S. aureus is repressed by elevated levels of DNA supercoiling. It has been suggested that the DNA supercoiling-mediated repression of transcription from the gyrA promoter is facilitated by the superhelical realignment of specific promoter recognition elements into an unfavourable arrangement, either through DNA duplex twisting or through bending (Sheridan et al., 2001; Wang & Syvanen, 1992). The protein A gene is expressed during the exponential phase and it is transcriptionally down-regulated as cells enter the post-exponential phase (Fig. 1a) (Projan & Novick, 1997). Two regulators, SarS and SarA, have been shown to directly bind to the spa promoter (Chien et al., 1999; Tegmark et al., 2000). SarS is an activator, whereas SarA is a repressor of spa expression. During the exponential phase of growth, there is optimal expression of SarS, which binds to the SarS binding site and displaces the SarA repressor complex from the spa promoter. When cells enter the post-exponential phase, SarS levels fall. Therefore, SarA occupies binding sites and spa transcription is inhibited (Gao & Stewart, 2004). Furthermore, binding of Sar proteins can alter the shape of DNA, inducing bending or overwinding of DNA (Schumacher et al., 2001). Since spa transcriptional regulation involves several regulatory sites, it is possible that negative superhelical tension similarly affects the spa promoter of S. aureus and the gyrA promoter of E. coli.

Effect of the Arl system on the regulation of spa expression by DNA supercoiling

The Arl system regulates protein A gene expression through the sarA locus (Fournier et al., 2001). Thus, we studied the combined effect of the arl mutation and the modulation of DNA supercoiling by high osmolarity, novobiocin and the gyrB142 allele on spa expression. We analysed protein A gene expression in the arlRS mutant in the presence and absence of novobiocin. At the beginning of the exponential phase (OD_{600} 0.1), spa expression in the presence of 40 ng novobiocin ml^{-1} was less than twofold lower than that in the absence of novobiocin. The presence of novobiocin increased protein A gene expression by 10-fold in the arlRS mutant at the end of the exponential phase (OD_{600} 2.0) (Fig. 6a). The presence of the gyrB142 mutation in the arlRS mutant slightly increased (2.5-fold) protein A gene expression at the beginning of the exponential phase (OD_{600} 0.1) (Fig. 6b). At higher OD_{600}, the gyrB142 mutation in the arl mutant altered spa expression by less than twofold. Thus, spa expression is not induced by the gyrB mutation in the arl mutant background, whereas it is induced in the wild-type background. We also analysed spa expression in the arlRS mutant in the presence of high osmolarity. In the arlRS null mutant, the expression of spa was less than twofold lower in the presence of 1 M NaCl (or 1 M KCl and 1 M sucrose) than in its absence at all growth stages (Fig. 6c). Thus, spa expression was dramatically repressed by high osmolarity in the wild-type strain but not in the arl mutant.

spa expression in the presence of novobiocin was increased by 10-fold in the arl mutant and by 26-fold in the wild-type strain. Similarly, the gyrB142 mutation, which increased spa expression in the wild-type strain (sevenfold), did not modify spa expression to the same extent in the presence of the arl mutation (2.5-fold). Furthermore, in the presence of 1 M NaCl, spa expression was decreased in the wild-type strain (four- to sevenfold), whereas it was unchanged in the arl mutant (less than twofold). Thus, absence of arlRS decreases the efficiency of DNA supercoiling modulators by a factor of two- to threefold, suggesting that active arl genes are necessary for the full action of DNA gyrase inhibitors and high osmolarity on spa expression. It seems that the Arl system interacts with the mechanism by which modulators of DNA topology (high osmolarity or inhibitors of DNA gyrase) control spa expression. Increased DNA supercoiling due to salt shock is largely prevented by a concentration of coumermycin that had no detectable effect on supercoiling in the absence of salt, suggesting that gyrase is the source of the increased supercoiling associated with salt shock (Hsieh et al., 1991). When E. coli was shifted from a medium lacking salt to one containing 0.5 M NaCl, both the [ATP]/[ADP] ratio and negative supercoiling of plasmid DNA increased within a few minutes (Hsieh et al., 1991). Since in vitro the [ATP]/[ADP] ratio influences the level of supercoiling generated by gyrase (Westerhoff et al., 1988), the physiological response of supercoiling to salt shock could be explained by the sensitivity of gyrase to changes in the intracellular [ATP]/[ADP] ratio (Hsieh et al., 1991; Jensen et al., 1995). Thus, DNA gyrase activity is essential for the transmission of the osmotic signals that change DNA supercoiling levels in bacteria (Bhriain et al., 1989). Furthermore, novobiocin and the gyrB142 allele inhibit the ATPase activity of the DNA gyrase. These observations suggest that DNA gyrase is probably an important enzyme in the modulation of spa expression. The Arl system may act in different ways. It is possible that the Arl system acts indirectly by modulating the expression of several genes in response to modification of DNA supercoiling. Alternatively, the arl mutation itself may affect DNA supercoiling.
The arl mutation affects DNA supercoiling

To determine whether the Arl system can modulate DNA supercoiling, strains RN6390BF and BF21 (Table 1) containing pE194 were grown in parallel at 37°C in TSB medium. The topoisomer distribution of DNA from each strain was determined (Fig. 7a). Significant and reproducible differences in DNA supercoiling were detected in the arlRS mutant. The distribution peak was shifted approximately one to three topoisomers in the arlRS mutant relative to the wild-type strain (Fig. 7a). The plasmid isolated from the arlRS mutant migrated more slowly than that from the wild-type strain on the gel containing 80 μg chloroquine ml⁻¹. This indicates that more highly supercoiled topoisomers were present in plasmid DNA isolated from the arlRS null mutant than in that from strain RN6390BF. To confirm that the Arl system plays a role in DNA supercoiling, we used pUB110 as a DNA supercoiling reporter. pUB110 from the arlRS mutant contained more highly supercoiled topoisomers (two to six shifts) than that from the wild-type strain (Fig. 7b). Thus, deletion of the arlRS locus results in changes in the level of DNA supercoiling.

Effect of the arlRS mutation on genetic competence

To analyse further the role of the arl null mutation in DNA supercoiling, we measured the transformation efficiency. Indeed, the development of genetic competence is related to intracellular DNA supercoiling (Ashiuchi et al., 2002; Chandler & Smith, 1996). We previously showed that the gyrB142 allele, corresponding to a double mutation (Ile102Ser and Arg144Ile), decreases plasmid DNA supercoiling (Fig. 4a, b). Thus, we analysed the genetic competence of strains with different mutations or grown in different conditions by measuring the transformation efficiency with pE194 and pT181. Plasmid DNA isolated from strain RN4220 was introduced into these strains by electroporation. The transformation efficiencies with pE194 and pT181 were 11- and 21-fold higher, respectively, in the arlRS null mutant than in the wild-type strain (Table 2). In the wild-type background, the gyrB142 allele decreased the transformation efficiency by 25-fold for pE194 and by threefold for pT181. Cells were grown with 40 ng novobiocin ml⁻¹, which has similar effects to the gyrB142 allele, and then transformed with pT181 by electroporation. The transformation efficiency was ninefold lower in the presence of novobiocin, confirming the results obtained with the gyrB142 allele. Finally, the combined effects of the arlRS deletion plus either the gyrB142 allele or 40 ng novobiocin ml⁻¹ were similar to those obtained with the arlRS mutation alone (Table 2), suggesting that the arlRS mutation inhibits the effects of
Table 2. Effect of mutations and growth conditions on the transformation efficiency of two plasmids

Transformation competence is expressed as number of transformants per c.f.u. ND, Not determined.

<table>
<thead>
<tr>
<th>S. aureus recipient</th>
<th>Genotype</th>
<th>Competence and ratio using:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pE194 (×10⁹)</td>
<td>Ratio</td>
<td>pT181 (×10⁸)</td>
</tr>
<tr>
<td>RN6390BF</td>
<td>Wild-type</td>
<td>50</td>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td>+ Novobiocin (40 ng ml⁻¹)</td>
<td></td>
<td>ND</td>
<td>4</td>
<td>0-1</td>
</tr>
<tr>
<td>BF21</td>
<td>arlRS</td>
<td>550</td>
<td>11</td>
<td>750</td>
</tr>
<tr>
<td>+ Novobiocin (40 ng ml⁻¹)</td>
<td></td>
<td>ND</td>
<td>1000</td>
<td>25</td>
</tr>
<tr>
<td>BF41</td>
<td>gyrB142</td>
<td>2</td>
<td>0-04</td>
<td>9</td>
</tr>
<tr>
<td>BF42</td>
<td>arlRS gyrB142</td>
<td>620</td>
<td>12</td>
<td>1530</td>
</tr>
</tbody>
</table>

Fig. 7. Effect of the arlRS mutation on plasmid DNA topology in different strains. Wild-type (WT; RN6390BF) and the arlRS mutant (BF21) strains carrying different plasmids: pE194 (a), and pUB110 (b). Strains were grown to an OD₆₀₀ of 2-0. After plasmid extraction, topoisomers were separated in agarose gels containing 80 µg ml⁻¹ (a) or 140 µg ml⁻¹ (b) chloroquine. Scan analyses of topoisomer distribution in the plasmids separated in the gel are presented on the right.
Fig. 8. Plasmid DNA supercoiling at different growth stages. pUB110 DNA supercoiling was measured at different growth stages (OD600 0.25, 1.0 and 4.0). (a) Wild-type strain RN6390BF. (b) arlRS mutant strain BF21. Chloroquine gel analysis was performed as in Fig. 1. Scan analyses of topoisomer distribution in plasmids separated in gels are presented on the right.
novobiocin and the gyrB142 allele. Interestingly, we previously showed that the presence of the arlRS deletion decreases the effect of DNA supercoiling modulators such as high osmolarity, novobiocin and gyrB142 allele on spa expression (Fig. 6). Thus, we confirmed that the arlRS deletion also decreases the effect of DNA supercoiling modulators on genetic competence.

As stated above, DNA topology influences genetic competence (Chandler & Smith, 1996). S. aureus is competent at very early stages of exponential growth (Novick, 1991). The transformation efficiency of the arl mutant was dramatically increased relative to the wild-type strain. However, the presence of the gyrB142 allele or treatment by novobiocin decreased genetic competence. These two results are consistent with each other and suggest that the genetic competence of S. aureus is decreased by the relaxation of DNA. This also strongly suggests the role of the Arl system in the modulation of DNA supercoiling.

DNA topology at different growth stages

To determine DNA topology at different growth stages, we analysed pUB110 DNA supercoiling at OD600 of 0·25, 1·0 and 4·0. No difference or only slight relaxation of DNA was observed between an OD600 of 0·25 and 1·0. However, at the end of the exponential phase (OD600 4·0), a relaxation of DNA supercoiling was observed in the wild-type strain (Fig. 8a). In E. coli, plasmid DNA relaxes as cells enter the stationary phase (Conter, 2003). Similarly, we observed DNA relaxation when cells entered stationary phase, even though the expression of topoisomerase genes is not modified at this growth stage (data not shown), suggesting that other factors such as cellular energy (ATP) are also involved in the regulation of DNA supercoiling in S. aureus. In the presence of the arlRS mutation, we observed no DNA relaxation at an OD600 of 4·0 (Fig. 8b), confirming that the Arl system is involved in the regulation of DNA supercoiling.

In conclusion, we showed that the protein A gene is regulated by several DNA supercoiling modulators, such as high osmolarity, novobiocin and gyrB mutation, indicating that spa expression is controlled by DNA supercoiling. Absence of arlRS decreased the effect of DNA supercoiling modulators on spa expression, suggesting that the Arl system is involved in the control of spa expression by DNA supercoiling. We also provide evidence for a relationship between the arlRS deletion and topological changes in plasmid DNA conformations.

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