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Transcriptional Profiling of XdrA, a New Regulator of *spa* Transcription in *Staphylococcus aureus*▽

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Transcription of *spa*, encoding the virulence factor protein A in *Staphylococcus aureus*, is tightly controlled by a complex regulatory network, ensuring its temporal expression over growth and at appropriate stages of the infection process. Transcriptomic profiling of XdrA, a DNA-binding protein that is conserved in all *S. aureus* genomes and shares similarity with the XRE family of helix-turn-helix, antitoxin-like proteins, revealed it to be a previously unidentified activator of *spa* transcription. To assess how XdrA fits into the complex web of *spa* regulation, a series of regulatory mutants were constructed; consisting of single, double, triple, and quadruple mutants lacking XdrA and/or the three key regulators previously shown to influence *spa* transcription directly (SarS, SarA, and RNAIII). A series of *lacZ* reporter gene fusions containing nested deletions of the *spa* promoter identified regions influenced by XdrA and the other three regulators. XdrA had almost as strong an activating effect on *spa* as SarS and acted on the same *spa* operator regions as SarS, or closely overlapping regions. All data from microarrays, Northern and Western blot analyses, and reporter gene fusion experiments indicated that XdrA is a major activator of *spa* expression that appears to act directly on the *spa* promoter and not through previously characterized regulators.

Global gene regulation is essential for the pathogenic success of *Staphylococcus aureus*. The tightly regulated expression of virulence factors enables it to adapt to hostile environments and to cause a wide range of infections. The temporal modulation of virulence factors occurs throughout infection progression *in vivo* and has also been widely documented *in vitro* (1, 11). Virulence gene expression is coordinated by a highly complex, interconnected regulatory network that responds to both endogenous and external stimuli (8). This network consists of well-characterized loci, such as the *agr* two-component quorum sensor system (1, 54); the various members of the SarA regulatory protein family, including SarS, SarT, SarU, SarV, SarX, SarZ, Rot, MgrA, and TcaR (17); the main stress response alternative sigma factor, SigB (7); and two-component sensor-transducer systems *saeRS* (44), *arlRS* (42), and *srrAB* (61, 76). Other loci with different attributed primary functions, or whose main functions are unknown, e.g., *ccpA* (70, 71), *spoVG* (69), *codY* (60), *msrR* (33), and *msa* (65), have also been shown to contribute to virulence and/or to modulate virulence factor expression, creating links between virulence and other cellular traits such as metabolism and antibiotic resistance phenotypes.

Protein A, encoded by *spa*, is one of the most widely studied and characterized staphylococcal cell wall-anchored surface components (21, 51). It is best characterized for its ability to bind the Fc region of IgG, attenuating antibody-mediated opsonization (24, 59). Protein A has also been shown to bind to von Willebrand factor, to activate tumor necrosis factor (29, 31), and to induce inflammation by triggering B-cell proliferation (4). The contribution of protein A to virulence has been debated, but studies have shown that protein A-deficient *S. aureus* strains are phagocytosed more efficiently *in vitro* and show decreased virulence in murine models of septic arthritis and pneumonia (58).

Numerous regulatory elements modulate *spa* gene expression either directly or indirectly (30). The SarS DNA-binding transcriptional regulator, encoded upstream of *spa*, is the main activator and has been shown to bind directly to the *spa* promoter (56). Activation is counteracted by SarA (20, 72) and by the effector molecule of the *agr* system, RNAIII (20, 55), both of which bind to the *spa* promoter and repress transcription. RNAIII-dependent *spa* repression occurs not only at the transcriptional level but also at the posttranscriptional level via an antisense mechanism (34). Most other loci shown to influence *spa* transcription belong to intricate regulatory cascades that eventually control *spa* indirectly, through modulation of SarS, SarA, or RNAIII. Some regulators, such as MgrA and Rot, have been proposed to act both directly and indirectly, although their exact roles in direct regulation have not been well characterized (56). Comparative levels of *spa* transcription during growth, and the influence of different regulators on expression levels, differ enormously among different strain backgrounds (12, 36). Most *spa* regulation studies have been performed on strains derived from NCTC8325, which have mutations reducing SigB activity (41) and inactivating TcaR (52), both of which indirectly affect *spa* transcription. SarT and SarU, which are part of the cascade leading to *spa* regulation, make up part of the variable *S. aureus* core genome and are also absent from some *S. aureus* clonal complexes (12, 43).

We recently identified a new DNA-binding transcriptional regulator encoded by open reading frame (ORF) SA1665 in the *S. aureus* N315 genome (23). We have named this protein XdrA (XRE-like DNA-binding regulator, A) because it shares similarity with XRE (xenobiotic response element) family he-

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lix-turn-helix, antitoxin-like proteins and is not homologous to Sar family proteins. Deletion of *xdrA* increased β-lactam resistance in all clinical methicillin-resistant *S. aureus* (MRSA) isolates tested (23). Although the essential prerequisite for methicillin resistance is the expression of PBP2a, encoded by the *mecA* gene, more than 40 additional chromosomal loci, including both regulatory and structural genes, are known to modulate resistance phenotypes (5, 6). Some of these factors directly or indirectly influence processes impacting resistance, such as cell wall biosynthesis and autolysis, but the functions of several others and/or their roles in resistance are unknown. XdrA was found to bind to the *mecA* promoter/operator region but not to alter *mecA* transcription or PBP2a production (23), leaving the mechanism by which *xdrA* deletion increased resistance levels unclear.

Here we further characterized this protein by performing microarray analysis to identify the XdrA transcriptome in the clinical isolate CHE482, a rapidly growing MRSA strain which belongs to clonal complex 45 (CC45) and which is highly prevalent among MRSA strains circulating in Zurich, Switzerland (62). The gene with the greatest fold difference in transcription levels was *spa*. Here we confirmed the influence of *xdrA* deletion on *spa* transcription in different strain backgrounds and analyzed the interplay of XdrA with other key *spa* regulators. Our results indicated that XdrA acts as a direct activator of *spa* transcription.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. Bacteria were grown on sheep blood or Luria-Bertani (LB) (Difco Laboratories, Detroit, MI) agar plates, and liquid cultures were grown in LB medium with shaking at 180 rpm at a broth/flask volume ratio of 1:4. Media were supplemented with the following antibiotics when appropriate: 50 μg/ml kanamycin, 10 μg/ml erythromycin, 10 μg/ml tetracycline, or 100 μg/ml ampicillin. Cell growth was measured by absorbance (optical density at 600 nm [OD_{600}]).

Microarrays. Overnight cultures of *S. aureus* were diluted 100-fold into fresh prewarmed LB medium and were grown to an OD of 0.5. Cells were then mixed (1:2) with RNAprotect bacterial reagent (Qiagen), incubated at room temperature for 5 min, harvested by centrifugation at 4°C, and snap-frozen. RNA isolation was performed as described by Cheung et al. (16), and the RNeasy kit, with on-column DNase I digestion (Qiagen), was used for RNA purification. RNA integrity was checked on an Agilent BioAnalyser, model 2100. cDNA was synthesized using SuperScript II reverse transcriptase and random hexamer primers (Invitrogen) and was labeled with either Cy3- or Cy5-dCTP (GE Healthcare). Microarray analysis was performed using the Bacterial Microarray Group at St. George's University of London (BμG@S) SAv1.1.0 microarray, and hybridization was carried out as described by Witney et al. (75). The array design is available in BμG@Sbase (http://bugs.sgul.ac.uk/A-BUGS-17) and ArrayExpress under accession no. A-BUGS-17. Slides were scanned using an Affymetrix 428 scanner, and data were extracted using BlueFuse, version 3.0 (BlueGnome, Cambridge, United Kingdom). Three biological replicates of both wild-type and mutant strains were hybridized against each other in dye-swap experiments, and data from all six arrays were normalized and analyzed using GeneSpring, version 7.2 (Silicon Genetics). The list of differentially expressed genes (see Table 3) includes those with a 3-fold or greater change in the expression level and P values of ≤ 0.05 by the t test, with the Benjamini and Hochberg false-discovery-rate correction applied.

Detection of the *sarT-sarU* genomic region. Primers sarT-U.F and sarT-U.R (Table 2) were used to detect the core variable RD5 genomic region by PCR. These primers were also used to amplify a single digoxigenin (DIG)-labeled probe for the simultaneous detection of *sarT* and *sarU* transcripts by Northern blot analysis.

Deletion mutants. The original *sarA* and *agr* mutations in strains LR12 and RN6911, respectively, both carried a tetracycline resistance marker. In order to create a complete set of regulatory mutants, both loci were also deleted by allelic replacement, using the erythromycin resistance gene (*ermB*) from pEC1. To create the new *agr* operon and *sarA* deletion mutants, regions from both upstream and downstream of these loci were amplified from *S. aureus* COL and cloned into pEC1 on either side of the *ermB* gene. To delete the *agr* operon, a 1.256-kb region upstream of RNAIII was amplified using primers agr.upF and agr.UpR, and a 1.455-bp fragment downstream of *agrA* was amplified using primers agr.downF and agr.downR. To delete the *sarA* gene, a 1.103-kb fragment upstream of *sarA* was amplified using primers sarA.upF and sarA.upR, and a 1.204-kb region downstream of *sarA* was amplified using primers sarA.downF and sarA.downR. The primers are listed in Table 2.

pEC1 constructs isolated from *E. coli* DH5α were then digested with HindIII and EcoRI, and the fragments containing the upstream and downstream regions flanking *ermB* were subcloned into the *S. aureus* suicide plasmid pAD21 to create pAD21Δ*agr* and pAD21Δ*sarA*. These plasmids were electroporated into *S. aureus* strain RN4220, and erythromycin-resistant transformants were screened for loss of kanamycin resistance.

All regulatory mutations (interruption of *sarS* with pAD21, replacement of *sarA* by insertion of either *ermB* or *tetL*, or replacement of the *agr* operon by insertion of either *ermB* or *tetM*) were transduced into either CHE482 or CHE482Δ*xdrA* using phage 80α, in order to create the full set of single, double, triple, and quadruple regulatory mutants. The genotypes of all mutants were confirmed by Southern blotting (data not shown).

Northern blotting. Overnight cultures were diluted to an OD of 0.05 in fresh prewarmed LB medium. To monitor the temporal patterns of gene transcription, cultures were sampled at five different growth stages. Total RNA was extracted as described by Cheung et al. (16). RNA samples (8 μg) were separated in a 1.5% agarose–20 mM guanidine thiocyanate gel. DIG-labeled probes were amplified using the PCR DIG probe synthesis kit (Roche). Table 2 lists the primer pairs used for the amplification of DIG-labeled probes. All Northern blot experiments were repeated at least twice using at least two independently isolated RNA samples.

Western blotting. Cell envelope proteins were prepared as previously described (68). Bacteria were harvested at an OD of 4.0 and were resuspended in SMM buffer (0.5 M sucrose, 0.02 M maleate, 0.02 M $MgCl_2$ [pH 6.5]). Lysostaphin (100 mg/liter) was added, and cells were lysed at 37°C. Protoplasts were centrifuged, and the supernatant containing cell envelope proteins was precipitated with 10% trichloroacetic acid. Proteins were resuspended in phosphate-buffered saline (PBS). Proteins (500 ng per sample) were separated in 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Zug, Switzerland). Protein A was detected using a goat anti-mouse IgG antibody conjugated to horseradish peroxidase (dilution, 1:5,000). Protein detection was performed using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Lausanne, Switzerland).

Hemolysis. Hemolytic activities were compared on sheep blood agar plates. Strains were grown overnight in LB medium in a 96-well microtiter dish at 37°C. Cultures were then stamped onto a sheep blood agar plate, which was incubated first at 37°C for 24 h and then at 4°C for 48 h before being scanned.

Primer extension. RNA was extracted from CHE482 cultures that were grown to an OD of 1.0, as described above. Primer extension reactions were performed with 20 μg of total RNA and 3 pmol of the 5′-biotin-labeled primers spa.PEbio1 and spa.PEbio3 (Table 2), using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Sequencing reactions were performed using the Thermo Sequenase cycle sequencing kit (U.S. Biochemicals). The Biotin Chromogenic Detection kit (Fermentas) was used for detection.

***lacZ* reporter gene fusions.** Nine different-length fragments of the *spa* promoter region were amplified, all using the reverse primer spa.lacZR and one of the forward primers spa.lacZF1 to -F9. Fragments were ligated upstream of the promoterless *lacZ* gene in the *E. coli-S. aureus* shuttle vector pBUS-*lacZ*, and ligations were transformed into *E. coli* DH5α. The resulting fusion constructs, pspa_P-*lacZ1* to pspa_P-*lacZ9* (p1 to p9), were then transformed into *S. aureus* RN4220 before being transduced into CHE482 and its corresponding *xdrA*, *sarS*, *sarA*, and *agr* regulatory mutants.

β-Galactosidase activity. β-Galactosidase activity was compared qualitatively by patching strains containing *spa* promoter-*lacZ* fusions onto LB medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal; 40 μg/ml) (Fermentas) and incubating the plates overnight at 37°C. β-Galactosidase activity was also measured quantitatively by performing *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma) cleavage assays according to the standard protocol (28).

Microarray data accession numbers. Fully annotated microarray data from this study have been deposited in BμG@Sbase (http://bugs.sgul.ac.uk/E-BUGS-105) and ArrayExpress under accession no. E-BUGS-105.

TABLE 1. Strains and plasmids

Strain or plasmid[a]	Relevant genotype[b]	Reference or source
Strains		
S. aureus		
RN4220	Restriction-negative derivative of NCTC8325-4	40
CHE482 (1)	Clinical MRSA isolate; CC45, ST45, SCC*mec* type N1; *blaZ* SarT$^-$ SarU$^-$	23
CHE482Δ*xdrA* (2)	CHE482 containing a markerless deletion of *xdrA*	23
ZH44	Clinical MRSA isolate; CC8, ST8, SCC*mec* type II; kanamycin resistant	23
ZH44Δ*xdrA*	ZH44 containing a markerless deletion of *xdrA*	This study
COLn	Early clinical MRSA isolate COL cured of plasmid pT181; CC8, ST250, SCC*mec* type I	39
COLnΔ*xdrA*	COLn containing a markerless deletion of *xdrA*	This study
Newman	Clinical methicillin-susceptible *S. aureus* strain (ATCC 25904); CC8, ST8	2, 22
NewmanΔ*xdrA*	Newman containing a markerless deletion of *xdrA*	This study
RN6911	RN4220 Δ*agr*::*tetM*	55
LR12	RN4220 Δ*sarA*::*tetL*	This study
NM520	CHE482 Δ*sarA*::*ermB*	This study
NM521	CHE482 Δ*agr*::*ermB*	This study
sarA strain (3)	CHE482 Δ*sarA*::*tetL*	This study
sarS strain (4)	CHE482 *sarS*::p*sarS*	This study
agr strain (5)	CHE482 Δ*agr*::*tetM*	This study
xdrA sarA strain (6)	CHE482 Δ*xdrA* Δ*sarA*::*tetL*	This study
xdrA sarS strain (7)	CHE482 Δ*xdrA sarS*::p*sarS*	This study
sarS sarA strain (8)	CHE482 *sarS*::p*sarS* Δ*sarA*::*tetL*	This study
xdrA agr strain (9)	CHE482 Δ*xdrA* Δ*agr*::*tetM*	This study
sarS agr strain (10)	CHE482 *sarS*::p*sarS* Δ*agr*::*tetM*	This study
sarA agr strain (11)	CHE482 Δ*sarA*::*ermB* Δ*agr*::*tetM*	This study
xdrA sarS sarA strain (12)	CHE482 Δ*xdrA sarS*::p*sarS* Δ*sarA*::*tetL*	This study
xdrA sarS agr strain (13)	CHE482 Δ*xdrA sarS*::p*sarS* Δ*agr*::*tetM*	This study
xdrA sarA agr strain (14)	CHE482 Δ*xdrA* Δ*sarA*::*ermB* Δ*agr*::*tetM*	This study
sarS sarA agr strain (15)	CHE482 *sarS*::p*sarS* Δ*sarA*::*ermB* Δ*agr*::*tetM*	This study
xdrA sarS sarA agr strain (16)	CHE482 Δ*xdrA sarS*::p*sarS* Δ*sarA*::*ermB* Δ*agr*::*tetM*	This study
E. coli DH5α	F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ$^-$	Invitrogen
Plasmids		
pBUS1	*S. aureus-E. coli* shuttle vector; *tetL*	63
pAW17	*S. aureus-E. coli* shuttle vector; *aac-aph*	63
pAZ106	Suicide vector containing promoterless *lacZ* reporter gene; *ermB*	14
pBUS-*lacZ*	pBUS1 containing a 4.5-kb EcoRI/Asp718 fragment from pAZ106, carrying the promoterless *lacZ* reporter gene; *tetL*	This study
pEC1	*E. coli* plasmid; *ori* ColE1 *bla ermB*	9
pAD21	*S. aureus* suicide vector derived from pAW17 by removal of pAMα1-*ori*	52
pAD21Δ*agr*	pAD21 with a 3.3-kb fragment comprising *ermB* flanked by the upstream and downstream sequences of the *agr* operon; *aac-aph*	This study
pAD21Δ*sarA*	pAD21 with a 3.3-kb fragment comprising *ermB* flanked by the upstream and downstream sequences of *sarA*; *aac-aph*	This study
p*sarS*	pAD21 with a 390-bp insert containing an internal portion of the COL *sarS* gene; *aac-aph*	52
pME26	pAW17 containing the *xdrA* gene; *aac-aph*	23
pME27	pBUS1 containing the *xdrA* gene; *tetL*	23

[a] Numbers in parentheses are alternate strain designations.
[b] CC, clonal complex; ST, sequence type; SCC*mec*, staphylococcal cassette chromosome *mec*.

RESULTS

Differential gene expression resulting from *xdrA* deletion. Transcriptional profiling had previously shown that *xdrA* was strongly expressed during early growth stages, with transcription beginning to decrease between ODs of 1.0 and 2.0 (23). Therefore, microarrays to compare the whole-genome transcriptional profiles of wild-type *S. aureus* CHE482 versus CHE482Δ*xdrA* were performed on RNA harvested at an OD of 0.5, at maximal *xdrA* expression. Statistical analysis of the results indicated that potentially 24 ORFs were upregulated and 26 downregulated by a factor of 3-fold or more in the *xdrA* deletion mutant (Table 3).

Confirmation of microarray results by Northern blot analysis. Northern blotting was used to confirm the transcriptional profiles of regulated ORFs. Four upregulated and four downregulated ORFs with the highest differential expression were selected (Table 3, asterisks). Transcription was compared in two different strain backgrounds: CHE482 (CHE482, CHE482Δ*xdrA*, and the *trans*-complemented mutant CHE482Δ*xdrA* pME26) and ZH44 (ZH44, ZH44Δ*xdrA*, and the *trans*-complemented mutant ZH44Δ*xdrA* pME27). The selected ORFs and their genomic contexts, along with the corresponding Northern blots, are shown in Fig. 1.

All upregulated ORFs tested, including *fadX* (encoding a

TABLE 2. Primers used in this study

Primer function and name	Nucleotide sequence (5′–3′)[a]	Source or reference
Construction of plasmids for allelic replacement		
agr.upF	ATTTA GAATTC ATATGAATGCTGAAGTAGAT	This study
agr.upR	ATTAA GGATCC GTTATCTTCGTATAGTACTA	This study
agr.downF	ATTTA CTGCAG TTCAATTGTAAATCTTGTTG	This study
agr.downR	AATAT AAGCTT TTGATACATCTAGTATAGAA	This study
sarA.upF	ATTTA GAATTC GAGACTTTATTCATATGCTT	This study
sarA.upR	ATTTA GGATCC CTTAAGAATGAGTTGACTAT	This study
sarA.downF	ATTTA CTGCAG TGATAGATGATACATTCTAT	This study
sarA.downR	ATTAT AAGCTT TTATCTTCACGTACAAGATT	This study
Primer extension		
spa.PEbio1	BIO-TGTTACGCCACCAGATATAA	This study
spa.PEbio3	BIO-ACCTAGTTTACGAATTGAATA	This study
Amplification of DIG-labeled probes		
SA1665F	TTCGTATAGAGGCTGGTTAG	23
SA1665R	AATTGGTTGGTTATCTGGAT	23
spaF	TGTAGGTATTGCATCTGTAA	52
spaR	AAGTTAGGCATATTCAAGAT	52
sarSF	TTGATGAGCGTAATACTTAC	52
sarSR	GAGCTAATAATTGTTCAGCA	52
RNAIII.F	TATAACTAGATCACAGAGATG	This study
RNAIII.R	TTAGTACTATACGAAGATAAC	This study
sarA.F3	TATTGACATACATCAGCGAA	This study
sarA.R1	GTTTGCTTCAGTGATTCGTT	This study
fadXF	TAGCGAATGATATTCATAGT	This study
fadXR	TAGTTGATAGTCGTCAACTA	This study
SAR0995F	ATCATTGAACGCTCTTGCAA	This study
SAR0995R	ATAGCGTGTCAATGCTTCAA	This study
SAR0996F	CTAATGTTAAACATACAACT	This study
SAR0996R	TATTCTGCCGTATATTGATT	This study
SAR1741F	GTATGAATTAATGCCGATTA	This study
SAR1741R	CGTCACCATAACCAATATAT	This study
SAR2413F	CAACGCTTACAAGGCTATAA	This study
SAR2413R	GCTACACAGTTCACTCTAAT	This study
SAR2589F	GGTTATGCATTAATGCAAGT	This study
SAR2589R	TAACGTGGAATACTTCTGTT	This study
mtlAF	CCAATGTTACTTGGTGCAAT	This study
mtlAR	TAAGTTGCAACACCAGTCAT	This study
sarT-U.F	GATTGGTCTATACCGATATA	This study
sarT-U.R	GTTGTATTGGAGTAACTGAA	This study
Amplification of *spa* promoter fragments for *lacZ* reporter gene constructs		
spa.lacZF1	AATTT GGATCC GAATCAATTATTAGCAGATAA	This study
spa.lacZF2	AATTT GGATCC TCAGCACATAATGAACAACTT	This study
spa.lacZF3	AATTT GGATCC TGTATTAAACCGCTTTCATTA	This study
spa.lacZF4	AATTT GGATCC ACTTCCTGAATAAATCTTTCA	This study
spa.lacZF5	AATTT GGATCC AAATATCTCTATATTTTATC	This study
spa.lacZF6	AATTT GGATCC TATTAATCGAAATAGCGTGA	This study
spa.lacZF7	AATTT GGATCC TTATAAGTTGTAAAACTTAC	This study
spa.lacZF8	AATTT GGATCC AATATAGATTTTAGTATTGC	This study
spa.lacZF9	AATTT GGATCC GTATTGCAATACATAATTCG	This study
spa.lacZR	AATTT GAATTC ACCTAGTTTACGAATTGAATA	This study

[a] Restriction sites are underlined; BIO denotes 5′ biotinylation.

putative acetyl coenzyme A [acetyl-CoA] transferase), SAR0995 (encoding a conserved hypothetical protein), SAR1741 (encoding a type III leader peptidase family protein), and SAR2413 (encoding a putative short chain dehydrogenase), gave very weak signals in the parent strains but clear upregulation upon XdrA inactivation, with at least partial restoration to wild-type levels in the *trans*-complemented mutants, confirming the microarray data. The microarrays indicated that the largest transcriptional alteration was the downregulation of *spa* in CHE482Δ*xdrA*, by a factor of 32.2 fold. Northern blot analysis confirmed strong attenuation of *spa* transcription in both strain backgrounds, with wild-type levels of transcription restored by *trans*-complementation. Other downregulated ORFs that were confirmed included SAR0996, encoding a putative regulatory protein, which is transcribed divergently from the up-

TABLE 3. ORFs differentially regulated by *xdrA* deletion in *S. aureus* CHE482

Gene ID[a]	Name	Gene product	Fold change in expression[e]
Upregulated genes			
SAR0153	*capC*	Capsular polysaccharide synthesis enzyme	4.2
SAR0154	*capD*	Capsular polysaccharide synthesis enzyme	3.4
SAR0179		Putative transporter protein	3.8
SAR0227*	*fadX*	Putative acetyl-CoA transferase	9
SAR0228		Putative glutamine amidotransferase class I	3
SAR0420		Putative membrane protein	3.5
SAR0995*		Putative regulatory protein	13.4
SAR1740		DNA repair protein (partial)	13.5
SAR1741*		Type III leader peptidase family protein	14.7
SAR1812	*acuA*	Acetoin utilization protein	5.5
SAR1813		Histone deacetylase family protein	4.1
SAR2396		DeoR family regulatory protein	4.1
SAR2413*		Putative short chain dehydrogenase	8.7
SAR2587		Hypothetical protein	4.3
SAR2610		Putative L-serine dehydratase, alpha chain	4.7
SAR2611		Putative L-serine dehydratase, beta chain	4.9
SAR2612		Putative membrane protein	5.3
SAR2643	*crtM*	Squalene desaturase (pseudogene)	4
SAR2645		Putative glycosyl transferase	5
SAR2646		Putative phytoene dehydrogenase related protein	4.3
SAR2647		Putative membrane protein	3.6
SAR2762		Hypothetical protein	3.7
MW0377[b]		Conserved hypothetical protein	12
MW0378[b]		Conserved hypothetical protein	14.3
Downregulated genes			
SAR0114*	*spa*	Immunoglobulin G binding protein A precursor	32.5
SAR0178		Putative D-isomer specific 2-hydroxyacid dehydrogenase	3.2
SAR0435		Exotoxin	7.8
SAR0696		Putative exported protein	3.1
SAR0787	*sstA*	FecCD transport family protein	3.1
SAR0788	*sstB*	FecCD transport family protein	3.0
SAR0789	*sstC*	ABC transporter ATP-binding protein	3.2
SAR0790	*sstD*	Lipoprotein	3.3
SAR0806		Putative S30EA family ribosomal protein	5.3
SAR0866		Hypothetical protein	3.2
SAR0996*		Conserved hypothetical protein	7.7
SAR1041		Conserved hypothetical protein	3.4
SAR1042	*purQ*	Putative phosphoribosylformylglycinamidine synthase I	4.3
SAR1043	*purL*	Putative phosphoribosylformylglycinamidine synthase II	3.2
SAR1450	*tdcB*	Putative threonine dehydratase	3.5
SAR1451	*ald2*	Alanine dehydrogenase 2	4.8
SAR1849		Proline dehydrogenase	3.2
SAR1938 (SA1665; *xdrA*)		Putative DNA-binding protein	71.4
SAR2244*	*mtlA*	PTS system, mannitol-specific IIBC component	15.2
SAR2245		Putative transcriptional antiterminator	5.5
SAR2247	*mtlD*	Putative mannitol-1-phosphate 5-dehydrogenase	3.5
SAR2589*		Putative transporter protein	6.4
SAR2593		Putative transcriptional regulator	3.5
SAR2605	*ddh*	D-Specific D-2-hydroxyacid dehydrogenase	3.3
SACOL0478[c]		Exotoxin 3	8.6
SA0393[d]	*set15*	Exotoxin 15	8.1

[a] Gene identifications (IDs) refer to *S. aureus* strain MRSA252 unless otherwise indicated. Genes selected for Northern blot analysis are marked with asterisks.
[b] Gene probes on microarrays were obtained from *S. aureus* MW2.
[c] Gene probes on microarrays were obtained from *S. aureus* COL.
[d] Gene probes on microarrays were obtained from *S. aureus* N315.
[e] $P < 0.05$.

regulated ORF SAR0995, and SAR2589, encoding a protein of unknown function with similarity to small-molecule transporters. SAR2589 was strongly expressed in both strain backgrounds, and transcription decreased by significant amounts in both *xdrA* mutants, increasing again upon complementation with *xdrA* in *trans*. Decreased transcription of SAR0996 by XdrA inactivation, and complementation in *trans*, was also confirmed in both strains. The inverse regulation of SAR0996 and the divergently transcribed SAR0995 suggested that there may be promoter exclusion controlled by the presence/absence of XdrA, although this requires further investigation. Northern blotting could not,

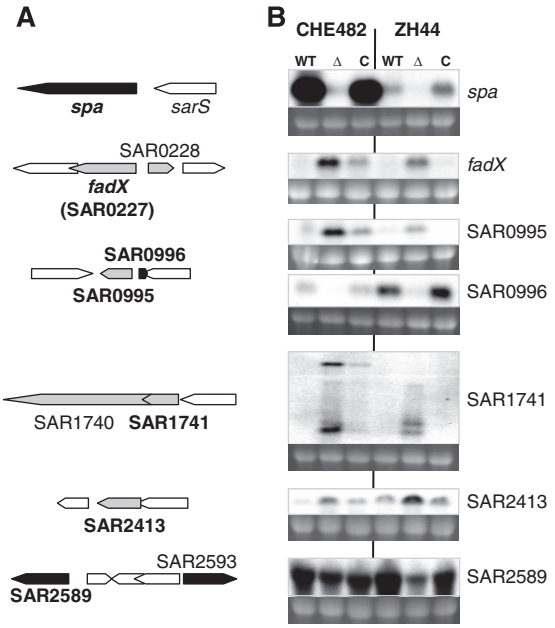


FIG. 1. Northern blotting of ORFs up- or downregulated from microarrays of CHE482 against CHE482Δ*xdrA*. (A) Locus maps of selected ORFs either upregulated (gray) or downregulated (black) in CHE482Δ*xdrA*. The designations of ORFs used as probes for Northern blotting are shown in boldface. (B) Northern blots of RNA extracted from strains CHE482 and ZH44: WT, wild type; Δ, Δ*xdrA* deletion mutant; C, mutant complemented with *xdrA* in *trans*. ORFs/genes used as probes are given on the right. Ethidium bromide-stained 16S rRNA bands are shown beneath the transcripts as an indication of RNA loading.

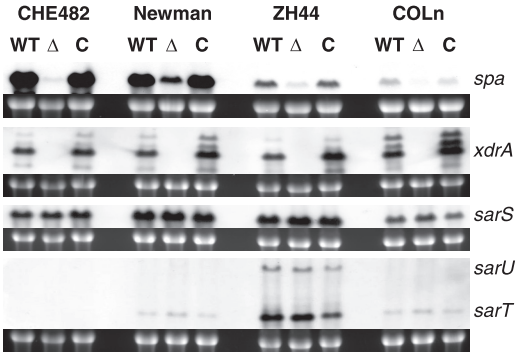


FIG. 2. Northern blot comparison of *spa* transcription in different strain backgrounds. RNA was extracted from strains CHE482, Newman, ZH44, and COLn. WT, wild type; Δ, Δ*xdrA* deletion mutant; C, mutant complemented with *xdrA* in *trans*. Levels of *spa* transcription differed greatly among the four different strain backgrounds; however, *spa* levels were significantly decreased in all *xdrA* deletion mutants and were restored to wild-type levels by *trans*-complementation. Northern blotting was also performed to compare the levels of *spa* with levels of *xdrA*, *sarS*, *sarU*, and *sarT* present in each of the strains. Probes used for hybridization are given on the right. Ethidium bromide-stained 16S rRNA bands are shown beneath the transcripts as an indication of RNA loading.

however, confirm the 15-fold downregulation of *mtlA*, which forms part of a mannitol-specific phosphotransferase (PTS) system (data not shown); the reasons for this discrepancy also need further exploration.

Effect of *xdrA* deletion on the transcription of *spa*, *sarS*, *sarU*, and *sarT*. The effect of *xdrA* deletion on the transcription of *spa* was monitored and compared in different strain backgrounds, including strain CHE482, which belongs to CC45, and three strains belonging to CC8: strain Newman, the clinical isolate ZH44, and the highly methicillin resistant strain COLn. Although levels of *spa* expression differed greatly between these strains, transcription was always significantly decreased by *xdrA* deletion and fully restored by *trans*-complementation (Fig. 2). Levels of *spa* transcription were also compared to levels of *xdrA*, *sarS*, *sarT*, and *sarU* within these different strain backgrounds. These Northern blot analyses showed that levels of *xdrA* were very consistent in all wild-type strains tested. A PCR screen revealed that the core variable region containing *sarT* and *sarU* was absent from CHE482 but present in the other three strains. Northern blot analyses confirmed that *sarT* and *sarU* were not present in CHE482 and showed variability in the levels of *sarS*, *sarT*, and *sarU* among the different strain backgrounds; however, no specific relationships between the levels of these regulators and the overall levels of *spa* transcription could be ascertained (Fig. 2).

Transcriptional profiling of *spa* and selected regulators in CHE482 throughout growth. The regulatory network controlling *spa* transcription is extensive (25, 26, 30, 34, 56, 61, 72). Currently at least 15 different regulatory elements have been shown to influence levels of *spa* transcription; some of these are shown in Fig. 3. So far, SarS, SarA, and RNAIII have been found to be the main regulators shown to act directly on the *spa* promoter; most other regulators influence transcription indirectly, through modulation of one or more of these regulators. The microarray results indicated that XdrA did not influence *spa* indirectly via one of its known regulators, because the transcriptome data set for XdrA did not overlap significantly with those previously published for any other regulator in the SAMMD database (53). Like that of many MSCRAMM proteins, protein A expression is growth stage dependent. RNA of CHE482 was sampled throughout growth, at ODs of 0.25, 0.5, 1, 2, and 4, and was used in Northern blot analyses to compare the temporal transcription of *spa* to those of its direct regulators *sarS*, *sarA*, and RNAIII, and to that of *xdrA* (Fig. 4). *spa* was transcribed weakly during early growth, with transcription increasing during exponential growth and decreasing only slightly by the final sampling point at an OD of 4.0. The major *spa* transcriptional activator SarS was transcribed at a consistently strong level throughout all growth stages, decreasing slightly at an OD of 4.0. SarA is proposed to displace SarS during the post-exponential growth phase, consequently decreasing *spa* transcript activation (26). Northern blotting of *sarA* showed transcription over all growth stages measured; however, *sarA* transcription profiles are difficult to interpret and correlate with SarA activity because of growth-phase dependent modulation in transcript initiation from the three different *sarA* promoters (15). RNAIII transcription was only barely detectable at an OD of 0.5 but then increased steadily throughout exponential growth and into post-exponential growth. This correlates with the published mechanism of *spa* repression by RNAIII, whereby RNAIII accumulation during post-exponential growth represses *spa* transcription. Although the microarray results and Northern blot analyses all

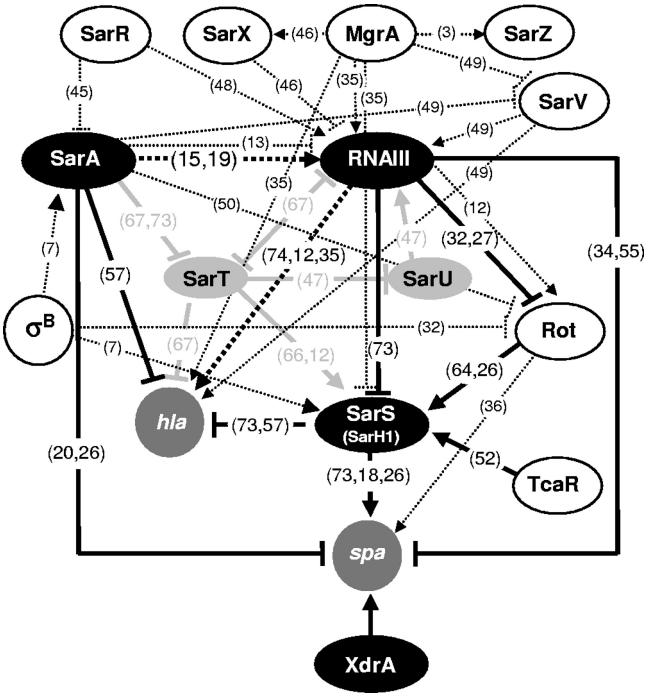


FIG. 3. Regulatory network controlling *spa* and *hla* expression. Regulatory loci directly or indirectly influencing *spa* transcription, either positively (arrows) or negatively (blocked arrows), are indicated. Regulators analyzed here are represented by filled ovals. SarT and SarU, shown as light shaded ovals, form part of the *S. aureus* core variable region RD5, which is absent from strain CHE482. Other regulatory connections reported in the literature are shown by broken lines. These regulatory connections are documented in references 3, 7, 12, 13, 15, 18–20, 26, 27, 32, 34–36, 45–50, 52, 55, 57, 64, 66, 67, 73, and 74. Reference numbers are given in parentheses on the figure.

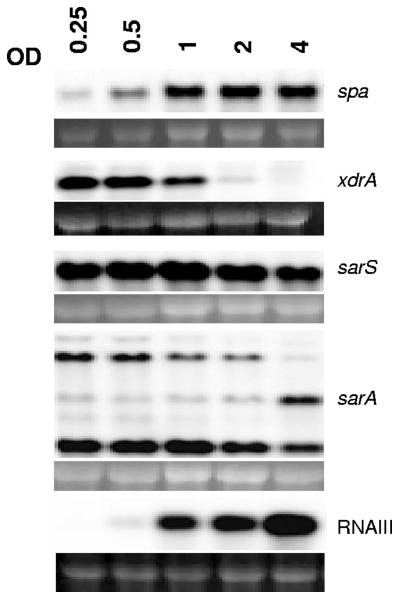


FIG. 4. Transcriptional profiles of *spa* and the regulators *xdrA*, *sarS*, *sarA*, and RNAIII over exponential growth and into early stationary phase. Northern blotting was performed on RNA extracted from strain CHE482 at the OD values indicated. Ethidium bromide-stained 16S rRNA bands are shown beneath the transcripts as an indication of RNA loading.

indicated that XdrA had a positive regulatory effect on *spa* transcription, Fig. 4 shows an inverse relationship between *spa* and *xdrA* transcription, with *xdrA* transcripts steadily decreasing over growth, as *spa* transcripts increase.

Impact of regulator mutations on *spa* transcription. A series of mutants were constructed to monitor *spa* transcription in the presence and absence of the four regulators: SarA, SarS, RNAIII, and XdrA. Fifteen mutants were constructed, including single mutants of all regulators, all combinations of double and triple mutants, and a quadruple mutant devoid of all four regulators (Table 1). Because of differences in the growth rates of some mutants, RNA was sampled at several time points over growth at standardized ODs, in order to produce comprehensive transcriptional profiles for each of the strains (Fig. 5A). Northern blot profiling of all mutants required the loading of samples onto four different gels. Wild-type CHE482 samples were loaded onto each gel as a control, to enable transcription comparisons between different gels (Fig. 5B). Profiling of the single regulatory mutants showed that *xdrA* deletion led to a massive decrease in *spa* transcription over all growth stages tested, and an even larger decrease, with no distinct *spa* transcripts detected, was observed in *sarS* mutants. Deletion of *sarA* or the *agr* operon interfered with the temporal expression of *spa*. In the *sarA* mutant, transcription was much stronger than in the wild type at the first two sampling stages, although it was very similar to that in the wild type over the last three samples, indicating that SarA represses *spa* transcription during early-exponential growth. In the *agr* mutant, transcription levels also appeared higher in both the first and the last samples, indicating derepression of *spa* during both early and late growth stages.

Transcription patterns became much more complex in the double, triple and quadruple mutants. Analysis of the double mutants showed some expected results, in that when both activators were absent (SarS and XdrA), there was no detectable transcription, whereas when both repressors (SarA and RNAIII) were deleted, there was derepression in both the earliest and the latest samples. Deletion of both repressors did not appear to have an additive effect: transcript levels in the double mutant did not appear significantly higher than those in either of the single mutants. Complicated patterns emerged, however, when combinations of activators and repressors were inactivated together. For instance, analysis of single mutants indicated that SarS was essential for initiating *spa* transcription; however, when one or more of the repressors were also deleted, transcription could once again be detected. Transcription in the *xdrA* mutant also increased significantly when *sarA*, *agr*, or both were deleted. Transcription in both *sarS* and *xdrA* mutants was higher throughout growth when *sarA* was deleted than when *agr* was missing, suggesting that SarA is a stronger repressor of *spa* than RNAIII, and was much greater when both repressors were absent. Transcription was also higher when repressors were inactivated in the *xdrA* mutant background than in the *sarS* mutant background, suggesting again that SarS is the stronger *spa* activator. In double and triple mutants, the effects of *sarA* and *agr* mutation on the growth phase-dependent expression of *spa* were even more exagger-

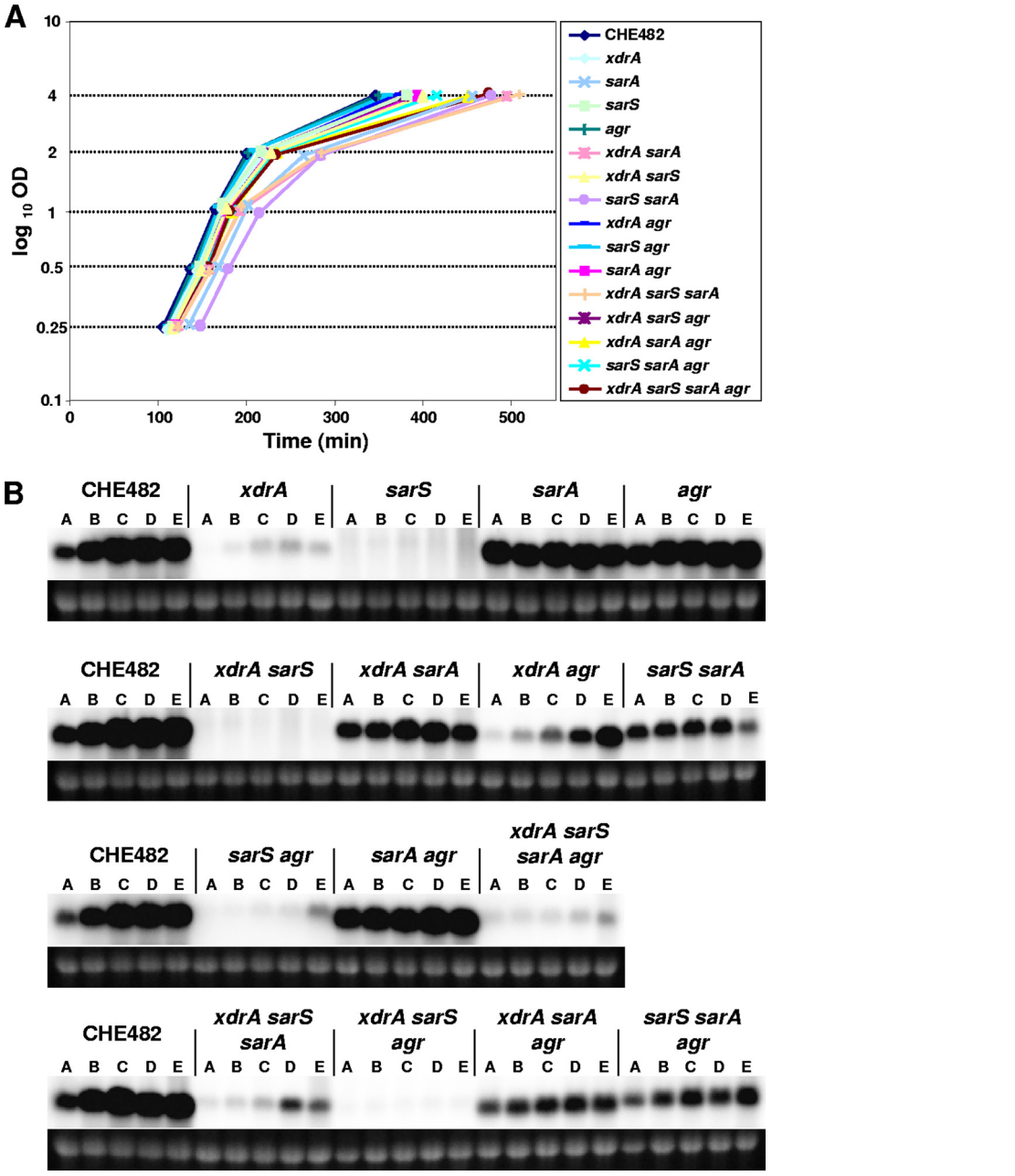


FIG. 5. Northern blot analysis of *spa* transcription in CHE482 regulatory mutants. (A) Growth curves of regulatory mutants, showing the five OD sampling points for RNA extraction. (B) Profiles of *spa* transcription over growth in wild-type CHE482 and strains containing single, double, triple, or quadruple mutations in the regulatory loci *xdrA*, *sarS*, *sarA*, and *agr*. RNA was extracted from cultures harvested at OD values of 0.25, 0.5, 1, 2, and 4 (lanes A to E, respectively). Ethidium bromide-stained 16S rRNA bands are shown beneath the transcripts as an indication of RNA loading.

ated, with *sarA* mutation enhancing *spa* transcription during early growth and *agr* mutation increasing expression levels in the later samples. Residual levels of transcription were low but clearly detectable in the quadruple mutant.

Western blot analysis of protein A production. Cell wall-associated proteins were isolated from the wild type and all 15 regulatory mutants at an OD of 4.0 (Fig. 6). Western blot analysis showed that the levels of protein A produced closely mirrored the levels of *spa* transcription in each of the strains

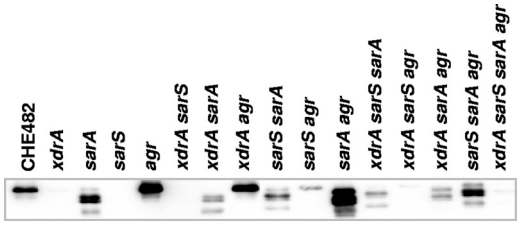


FIG. 6. Protein A expression in *spa* regulatory mutants. Cell envelope proteins were harvested at an OD of 4.0 and were probed with a goat anti-mouse IgG antibody conjugated to horseradish peroxidase.

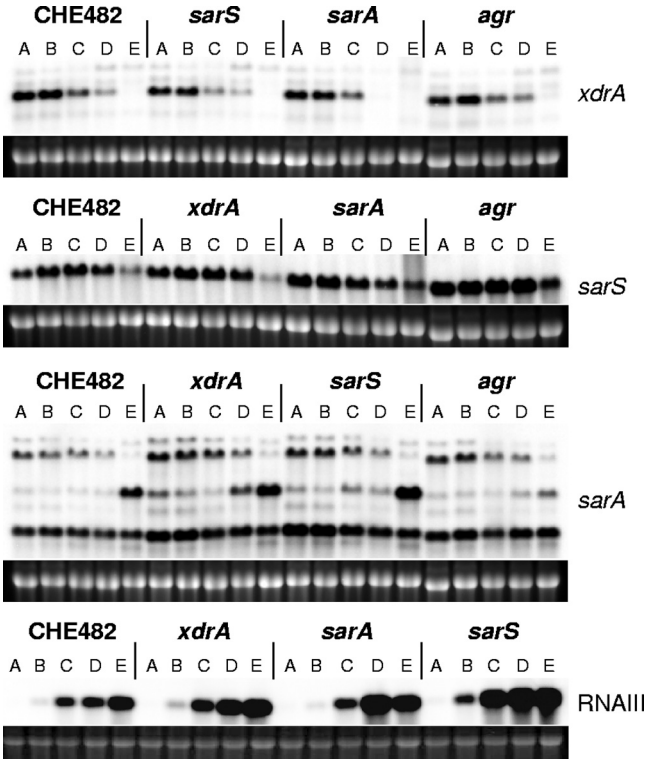


FIG. 7. Transcriptional profiles of *xdrA*, *sarS*, *sarA*, and RNAIII in wild-type CHE482 and in the single *xdrA*, *sarS*, *sarA*, and *agr* regulatory mutants. RNA was extracted from cultures harvested at OD values of 0.25, 0.5, 1, 2, and 4 (lanes A to E, respectively). Ethidium bromide-stained 16S rRNA bands are shown beneath the transcripts as an indication of RNA loading.

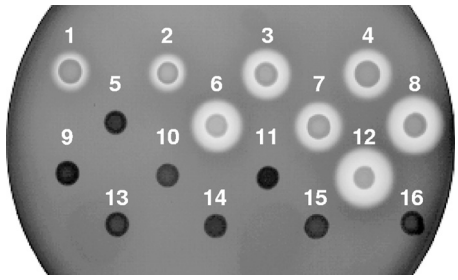


FIG. 8. Hemolytic activities of CHE482 regulatory mutants. Hemolysis zones surrounding colonies of regulatory mutants were compared on sheep blood agar. Numbers correspond to alternate strain designations in Table 1. Deletion of *xdrA* had no visible effect on autolysis. Disruption of *sarS* or deletion of *sarA* increased hemolysis, with an additive effect seen when both were missing (strains 8 and 12). Deletion of the *agr* operon had an overriding effect, abolishing hemolysis in all mutants.

tested. The *xdrA* deletion greatly decreased amounts of cell wall-associated protein A in all combinations of regulatory mutants tested, albeit with the weakest effect in the *agr* mutant. Proteolytic cleavage of protein A was observed in all *sarA* mutants, suggesting an upregulation of proteases, as is generally observed in *sarA* mutants (37, 38).

Effect of *xdrA* deletion on transcription of *sarA*, *sarS*, and RNAIII. The microarray results indicated that XdrA did not influence any previously characterized *S. aureus* regulatory loci. Northern blotting confirmed that deletion of *xdrA* did not significantly influence the expression of *sarS*, *sarA*, or RNAIII, nor did mutation of any of these three regulators influence *xdrA* transcription (Fig. 7). Because of differences in growth kinetics between mutants, sampling points were standardized at defined ODs. It seems that *agr* deletion enhanced *sarS* transcription, as reported previously by Tegmark et al. (73), and that *sarS* inactivation, conversely, resulted in a slight derepression of RNAIII. *sarS* upregulation in the *agr* mutant may also contribute to the higher levels of protein A in *agr* and *agr xdrA* mutants than in the wild type (Fig. 6).

Hemolysis. Hemolysis of CHE482 and the 15 regulatory mutants on sheep blood agar plates (Fig. 8) confirmed previously published findings, in that deletion of *agr* severely decreased hemolysis while deletion of *sarS* or *sarA* increased hemolysis (57, 74). Deletion of *xdrA* had no effect on hemolysis in CHE482, further indicating that XdrA influences *spa* directly and not through known regulators that also control *hla* expression.

***spa* promoter analysis.** Primer extension was performed to confirm the transcriptional start site (TSS) for *spa*. The results from two different primers both showed transcription initiating at an adenine nucleotide (nt), 25 nt upstream of the *spa* TTG translational start codon. This TSS was 7 nt upstream of the previously published *spa* TSS (26) but corresponded to a faint band described in those investigators' primer extension experiments (Fig. 9). DNase I footprinting experiments and electrophoretic mobility shift assays (EMSA) were performed on several biotin-labeled DNA fragments, covering different overlapping regions of the *spa* promoter. Unfortunately, the data from these experiments gave inconclusive results, which did not allow us to identify a localized XdrA binding site within the *spa* promoter (data not shown).

Therefore, a series of nine different *lacZ* fusion plasmids were constructed, containing successively smaller fragments of the *spa* promoter (Fig. 10A). These reporter gene plasmids, pspa_p-*lacZ1* to pspa_p-*lacZ9* (p1 to p9), were then transformed into RN4220, from which they were transduced into CHE482 and the single *xdrA*, *sarS*, *sarA*, and *agr* regulatory mutants. The phenotypes of all fusion-containing strains were then compared by growth on LB agar containing Xgal (Fig. 10B) and by ONPG cleavage assays (Fig. 10C).

The results from fusion p1, containing the full-length *spa* promoter, closely mirrored those from Northern blotting; the wild type and the *agr* and *sarA* mutants all appeared dark blue on LB-Xgal plates, and the *xdrA* and *sarS* mutants appeared white (Fig. 10B). The results from the remaining fusion constructs, representing a series of nested *spa* promoter deletions, identified the regions of the promoter influenced by inactivation of the four regulatory loci. Presumably, increased β-galactosidase activity represented either increased activator binding, reduced repressor binding, or a combination of both.

All strains containing fusion p9 produced very low levels of β-galactosidase, ranging from 3.6 to 16.9 Miller units (MU). These values, however, were higher than the negligible background levels of β-galactosidase detected from cultures of CHE482 containing the empty fusion vector pBUS-lacZ (<0.4 MU). The p9 construct contained a promoter fragment of 63

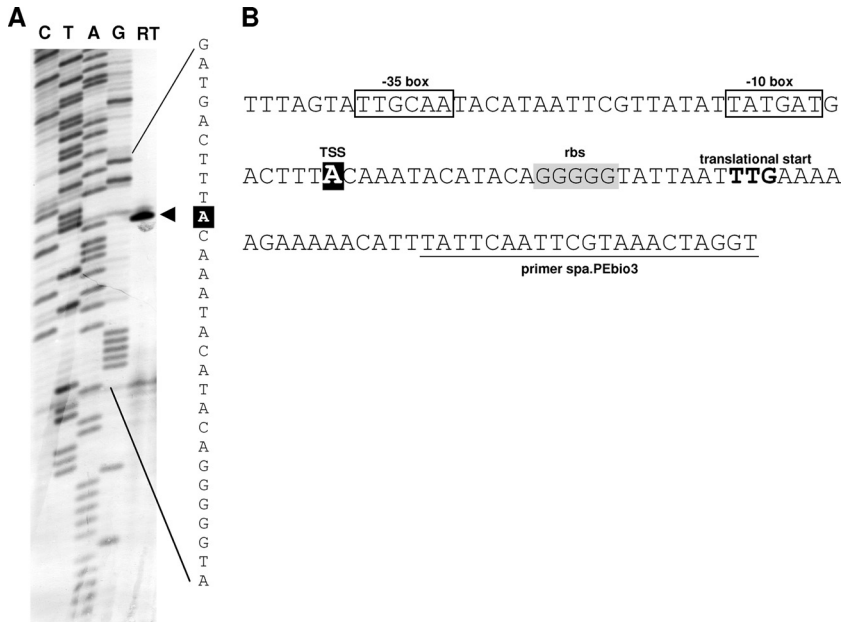


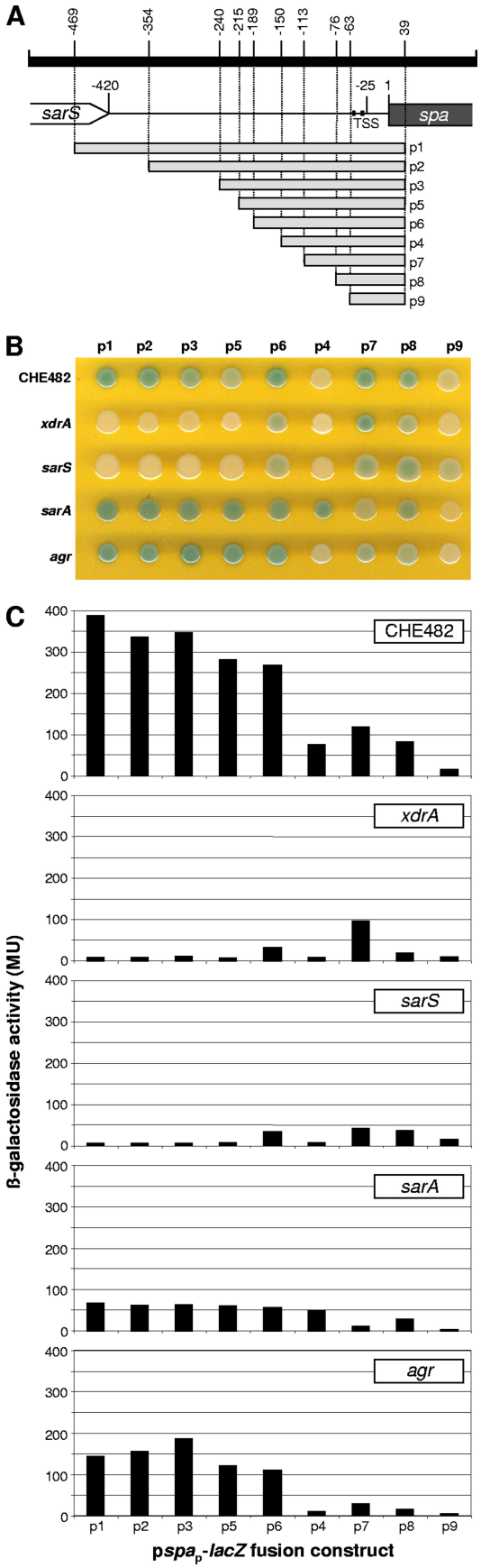
FIG. 9. Primer extension determination of the *spa* TSS. (A) Lanes C, T, A, and G show the dideoxy-terminator sequencing ladder obtained using complementary dideoxy terminators; lane RT contains the reverse transcription product obtained using primer spa.PEbio3. The TSS is indicated by an arrowhead, and the corresponding nucleotide is highlighted in white on a black background. (B) Sequence of the *spa* promoter region. The TSS is shown in white on a black background; predicted −10 and −35 regions are boxed; the predicted ribosome binding site (rbs) is highlighted in gray; and the translational start site (TTG) of *spa* is shown in boldface. The sequence of primer spa.PEbio3 is underlined.

nt, extending just 3 nt upstream of the predicted −35 box. Consistently low level expression of this fusion in all strains indicated that this truncated promoter contained no *cis*-acting regulatory elements, and the low levels of basal *spa* transcription corresponded well with transcript levels seen in Northern blots when all four regulators were absent (Fig. 5B).

The expression of the fusion constructs in CHE482 showed how different promoter lengths influenced *spa* expression in the wild type. On LB-Xgal plates, expression levels appeared to be consistently high in plasmids p1 to p6, through truncation of the promoter from 469 to 189 nt, indicating that this region did not play a significant role in regulation. Expression then decreased significantly in fusion p4, once the region between nt −150 and −189 had been deleted, suggesting that this region is important for activator binding. Expression then increased again once the promoter had been truncated to 113 nt in fusion p7, decreasing again to a basal level in fusion p9. A similar pattern of expression was reflected in the ONPG hydrolysis results, with expression decreasing sharply in fusion p4 and increasing again in p7 and p8, although only to levels approximately one-half to one-third of those seen in the full-length promoter (Fig. 10C).

xdrA and *sarS* mutants gave very similar reporter gene expression profiles, indicating that they act in similar ways on the same or closely overlapping regions of the promoter. As in Northern blot analyses, there was minimal *spa* expression from full-length promoters in both *sarS* and *xdrA* mutants. When the promoters became truncated to 189 nt, expression increased significantly in both mutants. Transcription in these two backgrounds also increased in Northern blots when one or both of the repressors were missing, thereby indicating that when the promoter is truncated to 189 nt, one or more of the repressors can no longer bind. Hence, the promoter region between 189 to 215 nt appears important for repressor binding. The strongest levels of transcription in both *sarS* and *xdrA* mutants were seen when the promoter was truncated to between 76 and 150 nt, once again suggesting limited repressor binding when promoters were truncated to this length. The levels of ONPG hydrolysis from fusions in *xdrA* and *sarS* mutants agreed well with the Northern blot analysis results, with expression levels from p1 to p5 being lower in the *sarS* mutant than in the *xdrA* mutant (Fig. 10C).

The patterns of reporter gene expression in the *agr* and *sarA* mutants indicated that these two repressors acted on similar promoter regions. In both mutants, expression was consistently high in fusions p1 to p6. Expression of p4 decreased in the *agr* mutant, as it did in the other four strains; however, it remained high in the *sarA* mutant, indicating that the region between −113 and −150 was not as essential for transcript activation when *sarA* was missing. This could correspond with the higher levels of expression in Northern blots for the *sarA xdrA sarS* triple mutant than for the *agr xdrA sarS* triple mutant (Fig. 5B). When strains containing fusions p1 to p6 were grown on LB-Xgal plates, levels of β-galactosidase in *sarA* and *agr* mutants appeared as strong as in the wild type, if not stronger, which would be consistent with the levels of *spa* transcription observed in Northern blot analyses (Fig. 10B). However, ONPG cleavage assays, while showing expression profiles consistent with those seen on LB-Xgal plates, showed lower levels of β-galactosidase activity in 8-h cultures of the *sarA* and *agr* mutants than in the wild type (Fig. 10C). These relatively low levels compared to those in the wild type were also observed in ONPG assays performed on 16-h and 24-h cultures (data not shown). The reasons for the low levels of β-galactosidase ac-



tivity in these cultures, especially for the *sarA* mutant, are unknown but are probably linked to changes in the growth phenotype and global regulation of strains lacking SarA.

DISCUSSION

The regulon of XdrA was found to comprise potentially more than 50 ORFs in *S. aureus* CHE482. None, however, had previously been shown to influence β-lactam resistance levels. Therefore, more in-depth characterization of the XdrA regulon, to identify which member(s) contributes to enhanced β-lactam resistance in the absence of *xdrA*, is ongoing.

The gene with the largest fold change in transcription was *spa*, suggesting that the *spa* promoter is one of the major targets of XdrA. Decreased *spa* transcription and successful *trans*-complementation to wild-type levels was confirmed in four different strains that differed greatly in their levels of *spa* transcription and belonged to two unrelated clonal complexes; suggesting that the function of XdrA as a positive regulator of *spa* is widely conserved in *S. aureus*.

Strain CHE482 was chosen for extended analysis of *spa* regulation because of its high level of *spa* transcription and the subsequent dramatic decrease upon *xdrA* deletion. Regulation was also likely to be somewhat simplified in CHE482, since it lacked the RD5 core variable genomic region containing the regulators SarT and SarU, which play a central role in feedback regulatory circuits between *sarA*, *sarS*, and RNAIII (12, 66, 67). Northern blot analyses showed that the regulatory loci SarS, SarA, RNAIII, and XdrA had little transcriptional influence on one another in strain CHE482.

To assess how XdrA fits into the complex web of *spa* regulation, *spa* transcription levels throughout growth were compared in a series of regulatory mutants. The temporal expression patterns of *spa* have been shown to be highly strain variable, especially during post-exponential growth phases, with transcription decreasing significantly in some backgrounds while remaining much more stable in others (10). In CHE482, *spa* transcription increased steadily throughout exponential growth and decreased only slightly thereafter. The transcriptional profiles of single *sarS*, *sarA*, and *agr* mutants correlated well with previously published findings (73); no discernible *spa* transcripts were present in the *sarS* mutant, in contrast with increased transcription in the *sarA* and *agr* mutants. Transcription was markedly decreased in the *xdrA* mutant at all sampling points.

Levels of *spa* transcription increased again in both *sarS* and *xdrA* mutant backgrounds when either *sarA* or *agr*, or both, was also deleted. Mutants containing different combinations of activators and repressors gave rise to different levels and temporal patterns of *spa* transcription, showing the relative contri-

FIG. 10. Identification of regulatory regions within the *spa* promoter of CHE482. (A) Map showing regions of the *spa* promoter that were fused to *lacZ* to create fusion constructs p1 to p9. Fusion constructs were introduced into wild-type CHE482 and the single regulatory mutants lacking *xdrA*, *sarS*, *sarA*, or the *agr* operon. (B) Phenotypes of fusion-containing strains grown on LB agar containing Xgal. (C) β-Galactosidase activity detected in 8-h cultures of fusion-containing strains.

butions of each of the regulatory loci at the different growth stages tested. Overall, *spa* transcript levels were lowest when SarS was inactivated, followed by the levels observed when *xdrA* was deleted; levels were increased, especially at later sampling points, when *agr* was absent and were highest when *sarA* was deleted. The effect of *agr* deletion on *spa* transcription in CHE482 was much less pronounced than that reported for NCTC8325-derived strains, substantiating previous reports that the effect of *agr* on the temporal expression of *spa* is strongly strain dependent (12).

High levels of *spa* transcription in *sarS sarA* double mutants previously led Gao and Stewart (26) to hypothesize that the role of SarS was not to activate the transcription of *spa* but to occlude repressor binding. Our results, from double, triple, and quadruple regulatory mutants, partially agree, in that they show that neither SarS nor XdrA is essential for the initiation of *spa* transcription and that relatively high levels of transcription are reached in corresponding *sarA* or *agr* double or triple mutants. However, our results also showed that transcription levels in double, triple, and quadruple mutants lacking one or more of the activators (SarS or XdrA) are never as high as in the wild type. The low levels of transcription in the *xdrA sarS sarA* and *xdrA sarS agr* triple mutants and in the quadruple mutant, which contains neither repressor, suggest that SarS and XdrA do stimulate transcription and that their function is not only to obstruct repressor binding. Otherwise, wild-type transcription should be restored by deleting both repressors. The same logic argues that XdrA can activate *spa* transcription in the absence of the other three regulators, since transcription in the *sarS sarA agr* triple mutant is significantly higher than in the quadruple mutant, where *xdrA* is also deleted.

Levels of β-galactosidase activity from reporter gene fusions, containing nested deletions of the *spa* promoter, identified potential *cis*-acting regulatory regions. Consistently low levels of p9 expression in all strains indicated that this minimal promoter, extending only 3 nt upstream of the predicted −35 promoter element, initiated a basal level of expression but lacked any *cis*-acting regulatory regions required for induction. The relative expression levels of other fusion plasmids in the wild-type strain CHE482 and in mutants lacking *xdrA*, *sarS*, *sarA*, or *agr* indicated that regions between p5 and p6 (nt −215 to −189) and p4 and p7 (nt −150 to −113) were involved in repressor binding; the second region corresponds to a previously identified SarA-responsive promoter element, which would lie between nt −121 and −115 (26). The fusions also indicated that the region between p6 and p4 (nt −189 to −150) was required for activator binding. Expression from fusions p7 and p8 was higher than that from p9 in all strains, indicating that this region enhanced transcription, although various levels of p7 and p8 expression in the five strains made its exact role in regulation difficult to interpret. Fusion results from CHE482, showing lower overall expression in fusions p4, p7, and p8, correlated with previous observations that this operator region, extending from immediately upstream of the −35 box to approximately nt −137, was required for SarS binding and hence for full activation of *spa* transcription (26, 73).

For the scope of this study, however, the most important results are those showing that *xdrA* deletion and *sarS* inactivation had very similar effects on *spa* promoter fragments, indicating that they act on the same or closely overlapping regulatory sequences. This potential interaction of XdrA and SarS at the same *cis* elements is likely to account for the apparent redundancy between these two activators when one or more of the repressors are absent. Such a redundancy would, once again, account for the lower levels of *spa* transcription in the triple and quadruple mutants when both XdrA and SarS are missing. No such redundancy can be seen, however, when both repressors SarA and RNAIII are present, since no *spa* transcription was detected in the single *sarS* mutant, and only very low levels were detected in the single *xdrA* mutant.

Therefore, the results presented here indicate that XdrA is a major activator of *spa* that acts on the same *cis*-regulatory elements as SarS, or closely overlapping elements, within the *spa* promoter. All the current evidence suggests that XdrA regulates *spa* directly and does not join the interconnected regulatory network linking other well-characterized regulators of *spa*.

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