

Staphylococcus aureus ArcR Controls Expression of the Arginine Deiminase Operon^{∇†}

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We identified a single open reading frame that is strongly similar to ArcR, a member of the Crp/Fnr family of bacterial transcriptional regulators, in all sequenced *Staphylococcus aureus* genomes. The *arcR* gene encoding ArcR forms an operon with the arginine deiminase (ADI) pathway genes *arcABDC* that enable the utilization of arginine as a source of energy for growth under anaerobic conditions. In this report, we show that under anaerobic conditions, *S. aureus* growth is subject to glucose catabolic repression and is enhanced by arginine. Likewise, glucose and arginine have reciprocal effects on the transcription of the *arcABDCR* genes. Furthermore, we show using a mutant deleted for *arcR* that the transcription of the *arc* operon under anaerobic conditions depends strictly on a functional ArcR. These findings are supported by proteome analyses, which showed that under anaerobic conditions the expression of the ADI catabolic proteins depends on ArcR. Bioinformatic analysis of *S. aureus* ArcR predicts an N-terminal nucleotide binding domain and a C-terminal helix-turn-helix DNA binding motif. ArcR binds to a conserved Crp-like sequence motif, TGTGA-N₆-TCACA, present in the *arc* promoter region and thereby activates the expression of the ADI pathway genes. Crp-like sequence motifs were also found in the regulatory regions of some 30 other *S. aureus* genes mostly encoding anaerobic enzymatic systems, virulence factors, and regulatory systems. ArcR was tested and found to bind to the regulatory regions of four such genes, *adh1*, *lctE*, *srrAB*, and *lukM*. In one case, for *lctE*, encoding L-lactate dehydrogenase, ArcR was able to bind only in the presence of cyclic AMP. These observations suggest that ArcR is likely to play an important role in the expression of numerous genes required for anaerobic growth.

Staphylococcus aureus is an important human pathogen and one of the major causes of community- and hospital-acquired infections worldwide. It colonizes primarily the squamous epithelium of the anterior nares (11), causing a variety of diseases ranging from simple skin infections to life-threatening diseases such as endocarditis, toxic shock syndrome, and chronic infections that require the successful adaptation of the pathogen to the human host (33). In the absence of oxygen, *S. aureus* can grow by fermentation of glucose or by using an alternative terminal electron acceptor such as nitrate (39, 41). When neither glucose nor nitrate is available, arginine can serve as the sole energy source for growth. The main bacterial arginine catabolic pathway is the arginine deiminase (ADI) pathway, encoded by the *arc* operon (13). The pathway comprises three reactions catalyzed by ADI (EC 3.5.3.6), ornithine carbamoyl transferase (EC 2.1.3.3), and carbamate kinase (EC 2.7.2.2), resulting in the conversion of arginine into ornithine, ammonia, and CO₂, with the concomitant production of 1 mol of ATP per mol of arginine consumed. The *arc* operon may also contain genes encoding ArgR and Crp/Fnr regulatory proteins

as well as transport proteins (2). ADI operons have been described for a wide variety of bacteria, including mycoplasma, halobacteria, *Pseudomonas* spp., *Bacillus* spp., and lactic acid and dental bacteria. In *Streptococcus rattus*, the ADI pathway functions as a key defense mechanism against acidification (12). Ammonia generation by ADI catabolism of arginine is believed to be crucial for pH homeostasis and a major factor in promoting dental health (7). In those bacteria that lack a respiratory chain, the ADI system is subject to carbon catabolite repression, while arginine serves to induce its expression (16, 17, 24).

An ADI gene cluster is present in the genomes of all nine *S. aureus* strains that have been fully sequenced. We noted that they all possess an open reading frame (ORF) encoding a 234-amino-acid protein related to the Crp/Fnr family of regulatory proteins. The *S. aureus* ORF is significantly related to the *Bacillus licheniformis* and *Lactobacillus sakei* ArcR proteins, which control the expression of the ADI catabolic pathway (34, 35, 52). Accordingly, the *S. aureus* ORF was named ArcR. The *S. aureus arcR* gene, encoding ArcR, is, unlike other previously reported ADI gene clusters, located immediately downstream of the *arcABDC* genes. Inspection of the region upstream of *arcABDC* revealed a conserved palindromic sequence motif, TGTGA-N₆-TCACA, that is the recognition site for Crp and some Fnr-related regulatory proteins (4). Bioinformatic analysis identified 15 more identical copies of this motif in the regulatory region of genes involved in anaerobic metabolism, including genes coding for lactate dehydrogenase, alcohol dehydrogenase, and pyruvate formate-lyase as well as

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristic(s)	Source or reference
Bacterial strains		
<i>E. coli</i> XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^q lacZΔM15 Tn10(Tc^r)</i>]	Laboratory stock
<i>S. aureus</i> RN4220 (NCTC 8325)	Restriction-minus derivative of 8325-4, primary recipient for propagation of <i>E. coli</i> plasmids, contains an 11-bp deletion in the <i>rsbU</i> gene	32
<i>S. aureus</i> SH1000	8325-4 carrying an intact <i>rsbU</i> gene	S. Foster (28)
<i>S. aureus</i> SH1000 Δ <i>arcR</i>	SH1000 in which the <i>arcR</i> gene was deleted and replaced by the Ωkm-2 kanamycin cassette	This study
Plasmids		
pUC18	Cloning vector; <i>lacZ</i> Ap ^r	MBI/Fermentas
PMUTIN-4	pUC18-based suicidal vector for gram-positive bacteria; P _{spac} - <i>lacZ</i> P _{penP} - <i>lacI</i> Ap ^r Em ^r	51
pAUL-A	Temperature-sensitive shuttle vector; <i>lacZ</i> Em ^r	47
pBR322::Ωkm-2	pBR322 carrying the Ωkm-2 cassette; Ap ^r Km ^r	43
pGEM-T Easy vector	An EcoRV-linearized vector with 3'-added T bases at both ends	Promega
pET28a(+)	A T7-based protein expression vector carrying an N-terminal His tag/thrombin tag	Novagen

the ADI pathway genes. These findings prompted us to analyze the role of ArcR in regulating the expression of the *arc* operon and of other genes that function in anaerobic growth. We show here that *S. aureus* ArcR positively controls the transcription of the *arcABDC* genes via binding to the upstream regulatory region and that a similar mechanism likely operates with some other anaerobic genes.

MATERIALS AND METHODS

Strains, media, and culture conditions. Bacterial strains and plasmids used in this study are described in Table 1. *S. aureus* strains were grown at 37°C in tryptone soy broth (TSB) (Difco) and brain heart infusion broth (Difco) supplemented with erythromycin (12 μg ml⁻¹) and kanamycin (200 μg ml⁻¹) where appropriate. Phage transductions were carried out with φ11 as described previously (40). *Escherichia coli* was grown in Luria-Bertani medium with the addition of ampicillin (100 μg ml⁻¹) or kanamycin (50 μg ml⁻¹) as needed. *S. aureus* aerobic liquid cultures were grown at 37°C in an air orbital shaker at 250 rpm. For limiting oxygen conditions, the standard anaerobic growth conditions used for growth of cultures were agitation at 100 rpm in an orbital shaker at 37°C in TSB medium supplemented with cysteine (5.7 mM) to scavenge traces of oxygen and 0.001% resazurin as a redox indicator. Wheaton serum bottles (100-ml capacity) containing 60 ml of the above-described medium were purged with nitrogen gas for 4 min at a pressure of 0.75 atm prior to being autoclaved. Aerobic cultures were subcultured (0.5 ml) in 60 ml of the above-described medium and grown for 16 to 20 h to stationary phase (optical density at 600 nm [OD₆₀₀] of ~2), and 2 ml was used to inoculate 60 ml of the same medium.

Anaerobic growth of *S. aureus* colonies on plates was carried out in a sealed anaerobic jar (Oxoid) equipped with an AnaeroGen (Oxoid) sachet and by employing an Anaerostest indicator strip (Merck) for verifying anaerobic conditions.

DNA manipulations. Standard procedures were employed for PCR amplification, restriction enzyme digestion, ligation, Southern blotting, and other DNA manipulations (46). Preparation of plasmids and electroporation of competent cells were done as described previously (46). *S. aureus* genomic DNA was prepared as described previously (40).

RNA extraction. Total RNA was isolated from *S. aureus* exponential-phase cultures grown in TSB medium at 37°C, as described previously (27). Cells (50 mg [wet weight]) were lysed in 0.3 ml of TES buffer (100 mM Tris-HCl [pH 7.5], 8 mM EDTA, 150 mM NaCl) containing 100 μg ml⁻¹ of lysostaphin (Sigma), and RNA was extracted using 1.5 ml of RNazol B (Tel-test). For reverse transcription reactions, residual DNA was removed by treatment with RQ1 RNase-free DNase (Promega). RNA concentrations were determined by A₂₆₀ measurements, and RNA integrity was analyzed by agarose-formaldehyde gel electrophoresis (46).

Northern blot analysis. *S. aureus* total RNA (5 μg) was electrophoresed in an agarose-formaldehyde gel and transferred onto NytranN nylon membranes (Schleicher & Schuell) as previously described (36). Internal DNA fragments of *arcA*, *arcB*, *arcC*, *arcD*, *arcR*, *lctE*, *pflB*, and *txb* were amplified by PCR and labeled with the PCR DIG Probe Synthesis kit (Roche). Primers used for the preparation of probes are listed in Table 2. Chemiluminescent detection was carried out according to the manufacturer's instructions.

Construction of the *arcR* deletion mutant. Deletion of the *S. aureus arcR* gene was performed by homologous recombination in RN4220. A ~1.1-kb DNA fragment from the *arcR* upstream region, containing the last 39 bp of *arcD* and

TABLE 2. Oligonucleotide primers used for construction by PCR of DNA probes

Primer	Primer sequence ^a	Description
arcR_FOR	5'-CATATGACAGAAAACCTTTATTTTGGG-3'	Amplification of <i>arcR</i> gene
arcR_REV	5'-GGATCCTTAAACACATACATCATTTG-3'	Amplification of <i>arcR</i> gene
arcA_for	5'-GACCCAAAATACCTTTATTTATG-3'	Amplification of 233-bp probe in <i>arcA</i> regulatory region
arcA_rev	5'-CCAGGACGCTTAAGTAACAC-3'	Amplification of 233-bp probe in <i>arcA</i> regulatory region
lctE_for	5'-CACTGGCGAAGTACGAAGAC-3'	Amplification of 210-bp probe in <i>lctE</i> regulatory region
lctE_rev	5'-AAAGGTCATGTGTCATCCGC-3'	Amplification of 210-bp probe in <i>lctE</i> regulatory region
adh1_for	5'-TGTCTTAGATTGATTGGGAG-3'	Amplification of 215-bp probe in <i>adh1</i> regulatory region
adh1_rev	5'-GACATAATCGATATGCTAACG-3'	Amplification of 215-bp probe in <i>adh1</i> regulatory region
lukM_for	5'-ATTAATGACTTTGTACACAC-3'	Amplification of 211-bp probe in <i>lukM</i> regulatory region
lukM_rev	5'-GCACATGATAATGATGACGC-3'	Amplification of 211-bp probe in <i>lukM</i> regulatory region
srrA_for	5'-GTCATTTAGCAGAACATGGG-3'	Amplification of 157-bp probe in <i>srrAB</i> regulatory region
srrA_rev	5'-ACAGGTCATACCTCCCACAC-3'	Amplification of 157-bp probe in <i>srrAB</i> regulatory region
nrdD_for	5'-ACATGTCGAAATGACGGACG-3'	Amplification of 221-bp probe in <i>nrdD</i> regulatory region
nrdD_rev	5'-AACCCGTTAATGCTTCTTCG-3'	Amplification of 221-bp probe in <i>nrdD</i> regulatory region
arcA mut_for	5'-TATGTGAATATAATGGGATGTAAGCGTTTGAAG-3'	Mutagenesis
arcA mut_rev	5'-AACGCTTACATCCCATTATATTCACATAAAG-3'	Mutagenesis

^a The mismatched bases for the mutations of primers used for mutagenesis are underlined.

arcC and 80 bp of the *arc-arcR* intergenic region, was amplified by PCR using forward primer 1250 (with a native *PacI* restriction site [underlined]) (5'-TCG GGTTAATTAAGTTATTGATGGG-3') and reverse primer 1251 (with an added *EcoRI* restriction site [underlined]) (5'-TCTCGAATTCCTTGCAAAG TGTCAGCAGAC-3'). The fragment was cut with *PacI* and *EcoRI*, purified from an agarose gel, ligated with the ~8.25-kb *PacI-EcoRI* fragment of pMUTIN-4 (51) to give pUP, and introduced into *E. coli* XL1-Blue by electroporation. A ~1.1-kb DNA fragment from the *arcR* downstream region, containing 328 bp of the *arcR-clfB* intergenic region and 793 bp of *clfB*, was amplified by PCR using forward primer 1252 (with an added *EcoRI* restriction site [underlined]) (5'-TCTCGAATTCGTTGAACATGAGGTCTAACG-3') and as reverse primer 1569 (with an added *EheI* restriction site [underlined]) (5'-TCTC GGCGCCAACTATCTGGTAACTTCGCTG-3'). The fragment was cut with *EcoRI* and *EheI*, purified, and inserted into the ~4.9-kb *EcoRI-EheI* fragment of pUP to create pUP-DW. Digestion of pUP-DW with *EcoRI* and ligation with the 2.27-kb *EcoRI-EcoRI* Ω km-2 cassette yielded pUP_Km_DW, with two possible orientations of the cassette. Restriction analysis identified an isolate in which the *arcC* and the kanamycin cassette possess the same orientation and was introduced into *S. aureus* RN4220 by electroporation. Transformants were selected on TSB plates containing erythromycin and kanamycin. The expected single integration event resulting from recombination between pUP_Km_DW and the host chromosomal region was confirmed by PCR and Southern analysis (data not shown). To select for spontaneous segregation of the wild-type *arcR* allele, one transformant was propagated for 100 generations in TSB liquid medium containing kanamycin but lacking erythromycin, and colonies were screened for a loss of the erythromycin marker on TSB plates containing kanamycin. One kanamycin-resistant, erythromycin-sensitive clone was chosen for further analysis by PCR and DNA sequencing to confirm the desired deletion-substitution in the *arcR* gene. The *arcR* substitution-deletion ($\Delta arcR$) was introduced into *S. aureus* SH1000 (*rsbU*⁺) by ϕ 11 phage transduction, and its presence was verified by PCR using forward primer 1250 and reverse primer 1252 (5'-AGAATAATCCACGCTCC-3') and by DNA sequencing.

Cloning, expression, and purification of ArcR. The *S. aureus arcR* gene was amplified from genomic DNA by PCR using the primers listed in Table 2. The 714-bp fragment was purified using the QIAquick gel extraction kit (QIAGEN), ligated into the pGEM-T Easy vector (Promega), and introduced into *E. coli* XL1-Blue by electroporation, and transformants were selected on LB plates containing ampicillin, IPTG (isopropyl- β -D-thiogalactopyranoside) (0.5 mM), and X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) (80 μ g/ml). After overnight incubation at 37°C, white colonies were picked and tested for the presence of the *arcR* gene by colony PCR. Positive clones were used for plasmid isolation and sequencing. pGEMT-Easy:*arcR* was cut with restriction endonucleases *NdeI* and *BamH*, ligated into the pET28a(+) expression vector, cut with the same enzymes, and electroporated into *E. coli* XL1-Blue. The resulting plasmid, pET28a(+):*arcR*, contains *arcR* fused in frame at its 3' end to six histidine codons. To overexpress ArcR, pET28a(+):*arcR* was introduced into *E. coli* BL21(DE3) cells, and cultures were grown to an OD₅₅₀ of 0.4 and treated with 0.5 mM IPTG for 3 h to induce synthesis. ArcR was purified by Ni²⁺-CAM affinity chromatography according to the manufacturer's instructions (Sigma). Purified proteins were dialyzed against buffer containing 50 mM Tris-HCl (pH 8), 300 mM NaCl, and 5 mM dithiothreitol using a PD-10 desalting column (Amersham Biosciences). Recovery of recombinant protein was monitored by the Bradford method (Sigma) using bovine serum albumin as a standard (6) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

DNA binding assays. Electrophoretic gel mobility shift assays (EMSAs) were performed with DNA probes containing the *arcA*, *lctE*, *adh1*, *lukM*, *srrA*, and *nrpD* upstream regulatory regions. Probes were generated by PCR using primers listed in Table 2 and purified using the QIAquick gel extraction kit (QIAGEN). DNA fragments were labeled at the 3' end with digoxigenin (DIG)-dUTP using the Terminal Transferase kit (Roche, Mannheim, Germany). Binding reactions were carried out in a final volume of 20 μ l containing 3 fmol labeled DNA probe, 20 mM Tris-HCl (pH 8.5), 5% (vol/vol) glycerol, 1 mM MgCl₂, 40 mM KCl, 1 mM dithiothreitol, purified wild-type or mutant ArcR (0.2 to 1.6 μ g protein) or crude cell extracts (0.4 to 6 μ g protein), 1 μ g sonicated salmon sperm DNA, and 0.1 μ g poly-L-lysine. The mixture was incubated for 30 min at 37°C and placed on ice, and 5 μ l of loading buffer was added to each sample. Protein-DNA complexes were monitored on a pre-electrophoresed 6% polyacrylamide gel in 0.5 \times TBE running buffer (89 mM Tris base [Sigma], 89 mM boric acid, 2 mM EDTA) at room temperature for 2 h. Gels were contact blotted for 8 h onto a Hybond-N+ membrane (Amersham Biosciences). Cross-linking of oligonucleotides was carried out using a Stratalinker and baking at 120°C for 30 min. Chemiluminescent detection was performed by adding alkaline phosphatase conjugated with an anti-DIG antibody. The membrane was washed at room

temperature in washing buffer and equilibrated in detection buffer. One milliliter of alkaline phosphatase chemiluminescent substrate (CSPD, ready to use; Roche Applied Science) was added to allow the visualization of the hybrids (5 min at room temperature). The membrane was exposed to X-ray film (FUJI) for 15 to 30 min at 37°C.

Preparative two-dimensional (2D) gels and protein identification by mass spectrometry. Cytoplasmic proteins were prepared from 50-ml cultures of cells grown to an OD₅₅₀ of 0.7 under anaerobic conditions. Cells were harvested by centrifugation for 10 min at 7,000 \times g at 4°C, washed twice with ice-cold Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and resuspended in Tris-EDTA buffer containing 4 mM sodium azide. Cells were disrupted using a Ribolyser (Thermo Electron Corporation) for 30 s at 6.5 m/s. Cell debris and insoluble and aggregated proteins were removed by centrifugation for 25 min at 21,000 \times g at 4°C, and the supernatant was centrifuged for 45 min at 4°C. The protein concentration was determined using Roti-Nanoquant (Roth, Karlsruhe, Germany). The protein solution was stored at -20°C.

Preparative 2D gel electrophoresis was performed using an immobilized pH gradient (IPG) technique as described previously (5, 20). Proteins were separated in the first dimension on IPG strips (GE Healthcare, Piscataway, NJ) with a pH range of 4 to 7, and proteins were separated in the second dimension on 12.5% polyacrylamide gels. Proteins were stained with colloidal Coomassie brilliant blue (9), and the gels were scanned with a Scanner X-Finity Ultra (Quato Graphic). For protein identification, Coomassie-stained proteins were cut from the gel using a spot cutter (Proteome Work) (30). Trypsin digestion of proteins, spotting of peptide samples onto matrix-assisted laser desorption/ionization targets, and matrix-assisted laser desorption/ionization-time of flight mass spectrometry analyses were performed as described previously (20, 30).

RESULTS

S. aureus ArcR is a Crp/Fnr-like transcriptional regulator.

To identify whether *S. aureus* contains Crp/Fnr-like transcriptional regulators, the complete genomes of nine strains, NCTC 8325, N315, Mu50, MW2, MRSA252, MSSA476, COL, USA300, and RF122 (a bovine isolate), were screened with a BLAST algorithm (1) using the *E. coli* Fnr and Crp protein sequences and the *Bacillus subtilis* Fnr-like protein sequences as queries. A single ORF possessing limited but significant sequence conservation was detected in all strains. The *S. aureus* 234-amino-acid ORF SAOUHSC_02964 (NCTC 8325) has a calculated molecular mass of 27,428 Da. It is closely related to *Staphylococcus epidermidis* ORFs SE2214 (strain ATCC 12228) and SERP2246 (strain RP62A), with which it shares approximately 60% sequence identity. Among the Crp family of proteins, the *S. aureus* ORF is most similar to the *B. licheniformis*, *Bacillus cereus* ATCC 10987, and *L. sakei* ArcR proteins, which have been reported to regulate the expression of the ADI pathway genes (34, 56). Figure 1C shows a sequence alignment of the *S. aureus* ORF with *B. licheniformis* ArcR and *E. coli* Crp (also called CAP, for catabolite-activating protein). The *S. aureus* ORF shares overall 19% identity and 45% similarity with *B. licheniformis* ArcR but is much less similar to *E. coli* Crp. In this work, it is designated ArcR. Multiple alignments of the deduced amino acid sequences of 18 ArcR proteins revealed that they share remarkably low overall similarity, suggesting that they differ in their response to various environmental signals (see the supplemental material).

Organization of genes in the *S. aureus* ADI operon. The *S. aureus* ADI metabolic pathway genes are organized in an operon, *arcABDC* (Fig. 1A). The *arcR* gene is located 98 bp downstream of *arcC* and is oriented in the same direction as the gene cluster. A second putative regulatory gene, *argR*, encoding an ArgR/AhrC repressor, is positioned upstream of *arcA*. *S. epidermidis* contains two *arc* gene clusters. Similar *arc*

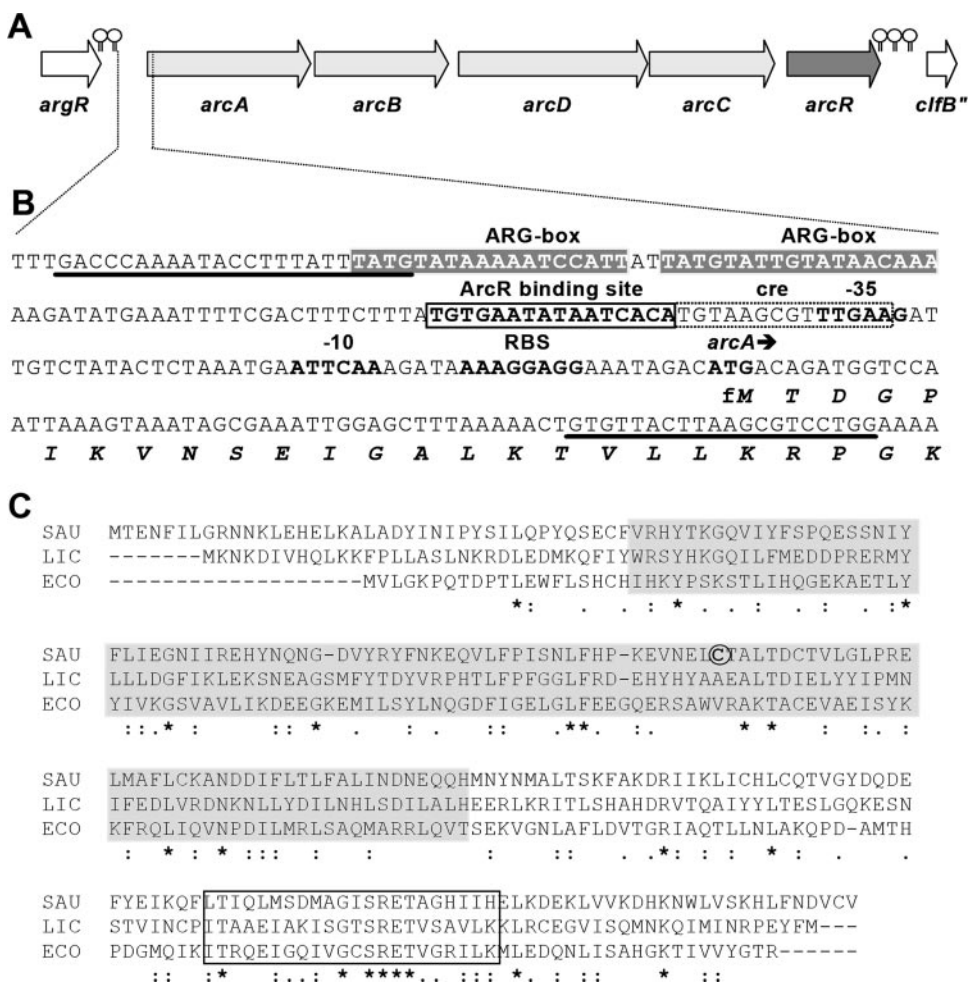


FIG. 1. Organization of the *S. aureus* arc operon. (A) Schematic representation of the *S. aureus* RN4220 (NCTC 8325) *arcABDCR* gene cluster. (B) Nucleotide sequence of the *arcA* regulatory region. A putative ArcR 16-bp binding site is shown framed and in boldface type; a putative *cre* motif is indicated by a hyphenated box; two putative ARG boxes are highlighted in gray. The predicted *arcA* ribosomal binding site (RBS) and ATG initiation codon are shown in boldface type. Positions of primers used for PCR amplification of the *arcA* promoter probe in DNA binding studies are underlined. The putative -10 and -35 promoter elements are indicated by boldface type. The DNA sequence is reported under GenBank accession number AJ566750. (C) Alignment of the *S. aureus* ArcR (SAU), *B. licheniformis* ArcR (LIC), and *E. coli* Crp (ECO) deduced protein sequences. Predicted cyclic nucleotide binding and HTH domains are highlighted in gray and in a rectangular box, respectively. Identical amino acid residues are shown by asterisks, highly conserved residues are shown by colons, and weakly conserved residues are shown by dots. The cysteine that was mutated in this work is circled.

gene clusters occur in other gram-positive and gram-negative bacteria. We noted the presence of a second *arc* gene cluster but lacking *arcA* in *S. aureus*. A somewhat different ADI gene organization occurs in community-acquired methicillin-resistant *S. aureus* strain USA300, where it is termed the arginine catabolic mobile element and appears to have been acquired horizontally from *S. epidermidis* or other coagulase-negative staphylococci (15).

Domain structure of ArcR. *S. aureus* ArcR contains the two structural domains that are characteristic of members of the Crp family of regulatory proteins. Sequence alignment of *S. aureus* ArcR with the *B. licheniformis* ArcR and *E. coli* Crp shows that that the N-terminal portion possesses a cyclic nucleotide binding domain (PF00027; IPR000595) (Fig. 1C). In *E. coli*, this domain is composed of about 120 amino acids that form a network of α -helices and an antiparallel β -barrel structure (37, 48). Figure 1C shows that the *S. aureus* ArcR cyclic

nucleotide binding domain contains several key structural residues that are conserved in Crp-related proteins. The ArcR C-terminal portion contains a well-conserved 22-amino-acid helix-turn-helix (HTH) motif. It possesses several residues, including Arg201 and Glu202, which correspond to residues that are known from crystal structure and mutagenesis studies of *E. coli* Crp to be involved in protein-DNA interactions (42). Sequence comparison of the cyclic nucleotide binding and HTH domains in 18 ArcR proteins is presented in the supplemental material.

ArcR is necessary for utilization of arginine as an energy source for growth under anaerobic conditions. To assess the role of *S. aureus* ArcR in generating energy for growth via the anaerobic ADI pathway, we constructed a deletion mutant of the *arcR* gene. The wild type (SH1000) and a kanamycin substitution-replacement mutant (Δ *arcR*) were plated onto TSB medium lacking glucose and containing 50 mM arginine as the

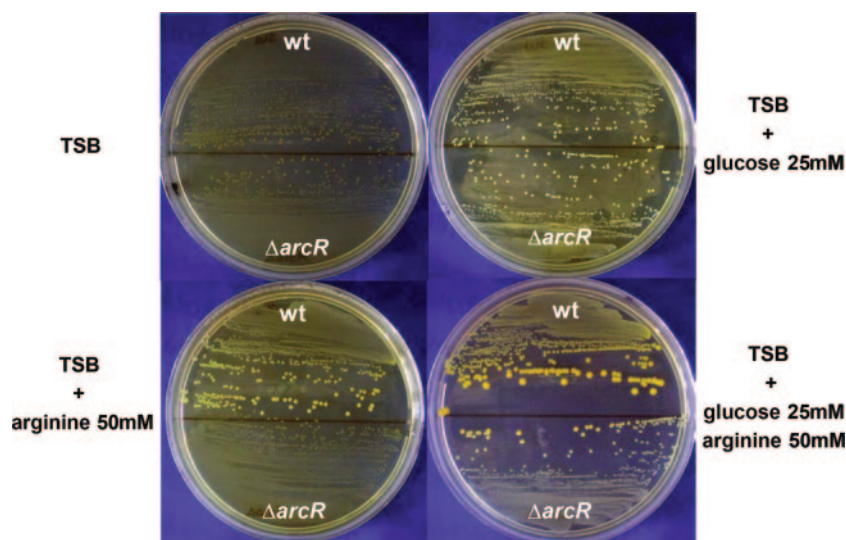


FIG. 2. Growth of *S. aureus* SH1000 and the *arcR* deletion mutant under anaerobic conditions. Cultures were plated onto TSB solid medium supplemented with 25 mM glucose or 50 mM arginine or both as the main energy source and incubated for 72 h at 37°C. wt, wild type.

main energy source. Plates containing glucose (25 mM) or glucose plus arginine served as controls. Plates were incubated for 72 h under aerobic and anaerobic conditions. Under aerobic conditions, no significant difference was found in the growth of the wild-type and mutant strains under any of the conditions (data not shown). Under anaerobic conditions, significant differences in growth occurred depending on the energy sources (Fig. 2). In the absence of supplements, wild-type and mutant strains exhibited limited growth. When glucose was added, the growth of both strains was improved. If the main energy source was arginine, growth of the *arcR* deletion mutant was considerably impaired compared with that of the wild type. When glucose and arginine were both present, the growth of the parent strain was further enhanced, whereas the growth of the *arcR* mutant was similar to that in medium containing glucose alone. These observations establish that under anaerobic conditions, ArcR is necessary for the utilization of arginine as an energy source for growth.

ArcR is a positive regulator of transcription of *arcABDCR* genes under anaerobic conditions. Northern blot analysis was performed to assess the role of ArcR in regulating the transcription of the *arcABDC* genes. Initially, we showed in SH1000 cells that *arcABDC* and *arcR* are cotranscribed. Total RNA was extracted from aerobically and anaerobically grown cultures of SH1000 grown in TSB medium, supplemented with glucose, and hybridized with *arcA*, *arcB*, *arcC*, *arcD*, and *arcR* PCR-labeled probes. Under anaerobic conditions, a ~5.8-kb transcript was detected with each of the probes but only at the stationary growth phase (Fig. 3). The transcript corresponds in size to that expected for the cotranscription of the *arcABDC* and *arcR* genes, showing that they form an operon. We suppose that the inability to detect the ~5.8-kb transcript during exponential growth is most likely due to glucose catabolic repression, which is relieved when, at the stationary phase, the medium becomes depleted of glucose. In contrast, under aerobic conditions, we were unable to detect the ~5.8-kb transcript with any of the probes at any stage of growth.

While the ~5.8-kb transcript is the major *arc* transcript made under anaerobic conditions, several smaller transcripts were also detected, suggesting a partial termination of transcription or processing of the full-length transcript (Fig. 3). Alternatively, some of the minor transcripts may arise from an additional promoter located within the *arc* operon. Thus, a ~1.3-kb transcript was detected in total RNA isolated from anaerobic cultures using *arcC* and *arcR* probes but was not detected with *arcA*, *arcB*, and *arcD* probes, and a ~0.7-kb transcript was detected with the *arcC* probe in each case, independent of the growth phase. Sequence analysis identified a putative promoter in the 3' end of the *arcC* gene, which, if functional, would generate the ~1.3-kb transcript terminating at the *arcR* terminator signal. The ~0.7-kb transcript is likely the result of processing of the ~1.3-kb transcript.

To assess the role of ArcR in the transcription of *arcABDCR*, parallel experiments were carried out with the *arcR* deletion mutant. In contrast to the wild-type strain, we were unable to detect full-length ~5.8-kb transcripts in total RNA isolated from anaerobic cultures employing *arcA*, *arcB*, *arcC*, *arcD*, and *arcR* probes at any growth phase (Fig. 3). Hence, under anaerobic conditions, ArcR positively controls the transcription of the *arcABDCR* operon. Confirmation that *arcABDCR* is transcribed solely under anaerobic conditions and depends on a functional ArcR was obtained by reverse transcription-PCR analysis (data not shown). None of the smaller transcripts mentioned above that were detected in the wild type by the *arcR* probe were detected in the *arcR* deletion mutant. However, the *arcC* probe detected two transcripts of ~0.7 kb and ~2.4 kb (Fig. 3). These transcripts were also detected in RNA isolated from the *arcR* deletion mutant under aerobic conditions (Fig. 3). The putative promoter in *arcC* most likely accounts for the two transcripts. It is predicted to generate the ~0.7-kb transcript through termination at a stem-loop structure in the upstream region of the kanamycin cassette, while the ~2.3-kb transcript is predicted to arise from termination at the kanamycin terminator signal. Since both

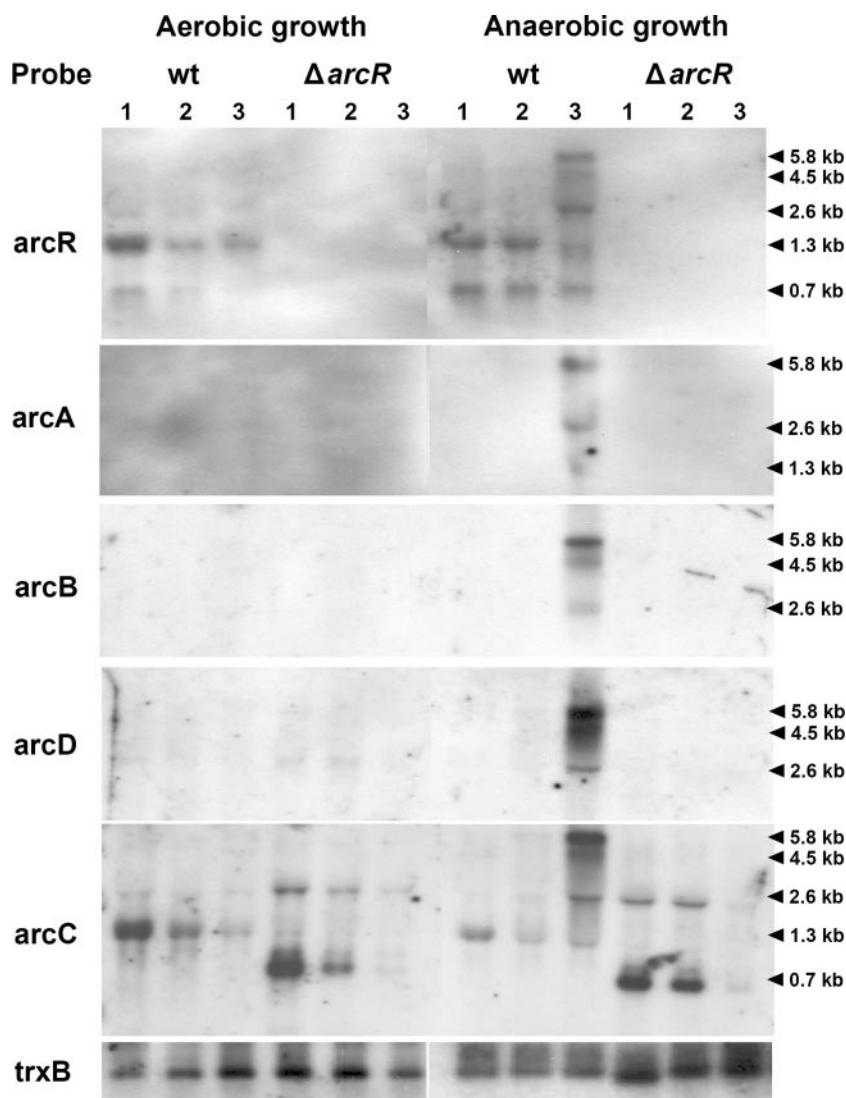


FIG. 3. Northern blot analysis of *arcABDC* and *arcR* genes under aerobic and anaerobic conditions. Total RNA was isolated from cultures of wild-type (wt) *S. aureus* SH1000 and the *arcR* deletion strain grown under aerobic and anaerobic conditions and hybridized with *arcR*, *arcA*, *arcB*, *arcD*, and *arcC* probes and a control *trxB* probe. Lanes 1, 2, and 3 indicate sampling times. Lanes for aerobic growth are as follows: 1, exponential phase with an OD_{600} of ~ 0.4 ; 2, late exponential phase with an OD_{600} of ~ 2.0 ; 3, stationary phase with an OD_{600} of ~ 4.0 . Lanes for anaerobic growth are as follows: 1, exponential phase with an OD_{600} of ~ 0.3 ; 2, late exponential phase with an OD_{600} of ~ 0.8 ; 3, stationary phase with an OD_{600} of ~ 1.1 .

transcripts were detected under aerobic and anaerobic conditions in the wild type and the *arcR* deletion mutant (see below), the putative *arcC* promoter is independent of the oxygen level and independent of the ArcR regulator.

Similar experiments were carried out to determine whether ArcR controls the expression of genes encoding lactate dehydrogenase (*lctE*) and pyruvate formate-lyase (*pflBA*), genes that are expressed under conditions of anaerobic growth. As described below, both sets of genes contain a consensus Crp binding site in their upstream regulatory regions and, for this reason, were considered to be likely to be regulated by ArcR. Northern blot analysis performed with *lctE* and *pflB* probes revealed the presence of ~ 1.2 -kb and ~ 3 -kb transcripts, respectively, corresponding to the expected full-size *lctE* and *pflBA* transcripts. However, unlike *arcABDCR*, no significant

difference was found in their transcription under aerobic and anaerobic growth conditions, nor was there any appreciable difference in *lctE* and *pflBA* transcription between the wild type and the *arcR* deletion mutant. Similar results were obtained using *S. aureus* RN4220 and its *arcR* mutant (data not shown).

Glucose and arginine exert reciprocal effects on *arcABDCR* transcription. To assess the effect of glucose and arginine on the transcription of the *arc* operon, total RNA was isolated from aerobically and anaerobically grown exponential cultures of SH1000 and SH1000 Δ *arcR* grown to an OD_{550} of 0.4 in media containing or lacking glucose and/or arginine. Northern blot analysis performed with *arcA* and *arcR* probes showed that full-length ~ 5.8 -kb transcripts were readily detected under anaerobic conditions in medium containing arginine and lack-

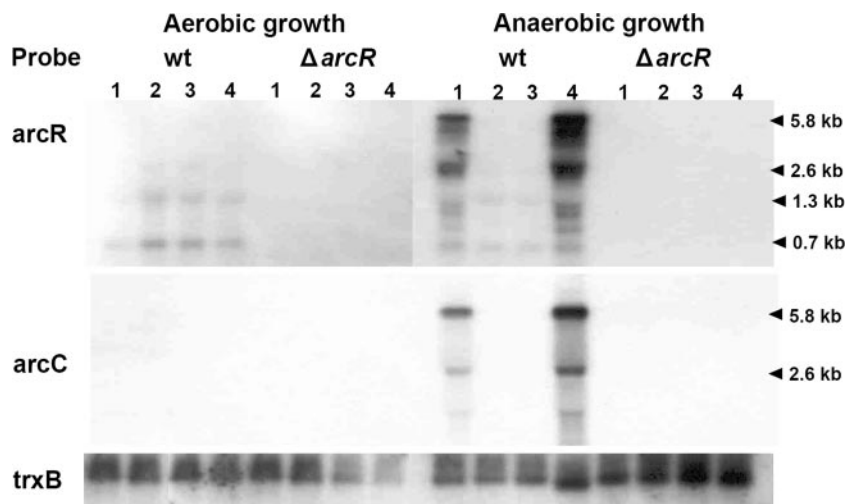


FIG. 4. Northern blot analysis of *arcABDC* and *arcR* transcripts in wild-type (wt) SH1000 and the *arcR* deletion strain in different growth media under aerobic and anaerobic conditions. Total RNA isolated from cultures of wild-type SH1000 and the *arcR* deletion mutant grown in TSB medium to an OD_{550} of 0.4 was hybridized with *arcR* and *arcA* probes and a control *trxB* probe. The lanes indicate different growth media. Lanes: 1, TSB medium (without glucose); 2, TSB medium plus 25 mM glucose and 50 mM arginine; 3, TSB medium plus 25 mM glucose; 4, TSB medium plus 50 mM arginine.

ing glucose (Fig. 4). Transcription was perceptibly weaker when arginine was omitted from the medium. In the presence of glucose (with or without arginine), *arcABDCR* transcription was strongly reduced, showing that the *arc* operon is subject to catabolic repression. In the *arcR* deletion mutant, we were unable to detect full-length *arcABDCR* transcripts using *arcA* or *arcR* probes under any of the growth conditions tested (Fig. 4). As described above, no full-length *arc* transcripts were detected under aerobic conditions in both parent and mutant strains with either probe; two minor ~0.7-kb and ~1.3-kb transcripts were detected in the wild type by the *arcR* probe under aerobic and anaerobic conditions but were not detected in the *arcR* deletion mutant.

Synthesis of ArcA, ArcB, and ArcC under anaerobic conditions requires a functional ArcR. Further evidence that ArcR positively regulates the expression of the *arc* operon under anaerobic conditions was obtained from proteome analyses. *S. aureus* SH1000 and the *arcR* deletion mutant were grown anaerobically in TSB medium to late exponential phase (OD_{540} of 0.8), and cytoplasmic proteins were prepared as described in Materials and Methods. Figure 5 shows that the amounts of ArcA, ArcB, and ArcC catabolic proteins were markedly reduced in the *arcR* mutant. Surprisingly, ArcA, ArcB, and ArcC were the only proteins whose amounts were significantly affected by the elimination of ArcR, and the amounts of other proteins encoded by genes known to be expressed specifically in anaerobic conditions, such as lactate dehydrogenase and alcohol dehydrogenase, were not appreciably different in the wild-type and *arcR* mutant strains.

ArcR binds to the regulatory region of the *arc* *ABDCR* operon. The HTH domain in Crp regulatory proteins recognizes the symmetric sequence TGTGA-N₆-TCACA (8). *B. licheniformis* ArcR recognizes a similar target (34). In a screen of the Mu50 genome, we identified 15 copies of Crp-like binding sites, one in the upstream regulatory region of *arcA* and the others in similar locations of genes involved in anaerobic me-

tabolism. To determine if *S. aureus* ArcR controls the expression of the *arc* operon by binding to the *arc* promoter region, EMSAs were performed by employing crude cell extracts and purified recombinant His-ArcR proteins. DNA binding reactions were carried out with a DIG-labeled 232-bp PCR-amplified *arcA* fragment containing the Crp consensus binding motif TGTGA-N₆-TCACA. Incubation of wild-type crude cell extracts with labeled DNA probe resulted in a shift in the mobility of the fragment, with it migrating more slowly than the free fragment, presumably due to the formation of a DNA-protein complex (Fig. 6A). Increasing the amount of cell extract led to the formation of higher-order complexes and a further reduction in mobility of the fragment. When binding was carried out with cell extracts from the *arcR* deletion mutant, a slight change in mobility of the fragment occurred but did not change with increasing amounts of extract.

When purified His-tagged ArcR was used in DNA binding reactions, a single DNA-protein complex was formed, the amount of which increased with increasing amounts of ArcR in the reaction mixture. The specificity of the reaction was shown in competition experiments in which the addition of a large excess of cold probe in the binding reaction mixture resulted in the disappearance of the signal (Fig. 6C). Control reactions employing an unrelated His-tagged NrdR protein had no effect on probe mobility (Fig. 6D). Also, ArcR did not bind to an unrelated 221-bp *nrdD* control promoter probe that lacks Crp binding site DNA. These results demonstrate that ArcR binds to the upstream regulatory region of the *arcA* promoter.

Multiple Crp consensus binding sites are present in the *S. aureus* genome and occur predominantly in the regulatory regions of genes required for anaerobiosis. Numerous putative Crp-like binding sites were identified in the *S. aureus* genome and are listed in Table S1 of the supplemental material. Fifteen binding sites contain the identical sequence motif of the con-

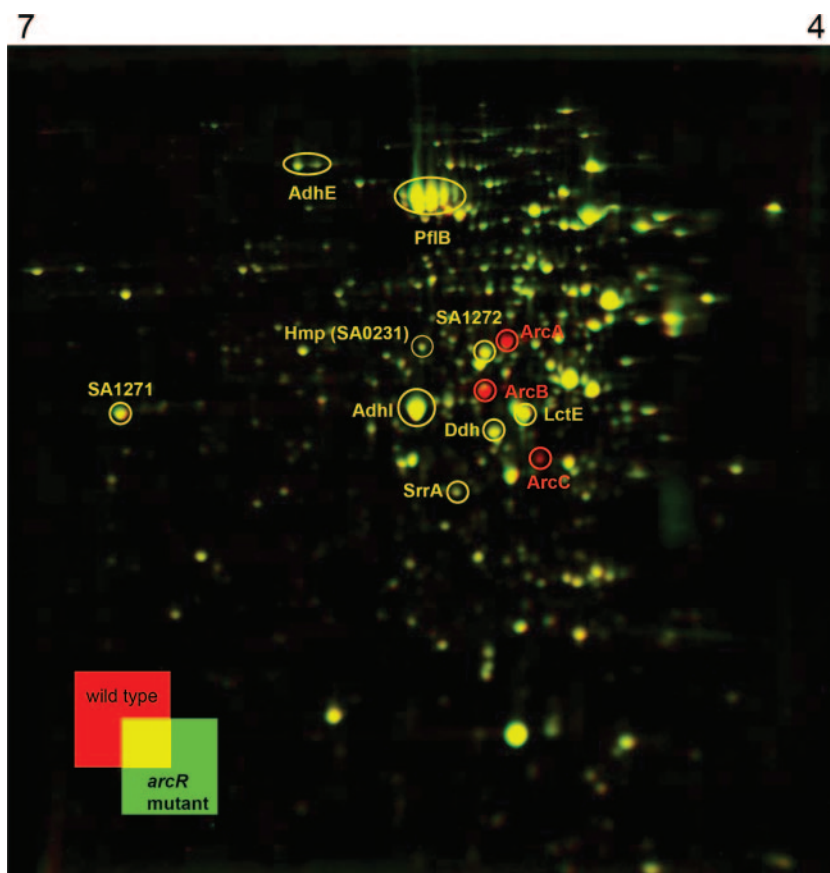


FIG. 5. Effect of ArcR on the synthesis of cytoplasmic proteins under anaerobic conditions. Shown is a false-colored dual-channel image of 2D gels of cytoplasmic proteins extracted from the wild type (SH1000) (red) and the *arcR* deletion mutant grown to an OD_{540} of 0.8 in TSB medium under anaerobic conditions. Three hundred micrograms of protein of crude cell extracts was separated on 2D gels using IPG strips in the pH range of 4 to 7. Proteins were stained with colloidal Coomassie brilliant blue. Proteins whose amounts were positively affected by ArcR appear in red, and proteins not affected by ArcR appear in yellow. Spots of interest are labeled with their protein names, as annotated for *S. aureus* N315.

sensus Crp binding site, another 14 contain a single change, and another 5 contain two changes in either one of the two half-sites of the consensus motif. Many of these sites occur in front of genes that are thought to be required for anaerobic growth. For example, potential Crp binding sites were found in the upstream regions of genes encoding the two-component systems SrrAB (50, 53) and VicRK (18), for the cytochrome oxidase homolog CtaB, for the aerolysin/leukocidin toxin LukM, and for various other functions. Most of these sequences were found within intergenic regions and positioned in front of genes coding for putative anaerobic regulatory and enzymatic systems, suggesting that they may be transcriptionally regulated by ArcR.

ArcR binds to the regulatory regions of *adh1*, *lctE*, *srrAB*, and *lukM*. To assess whether *S. aureus* ArcR potentially controls the expression of the genes encoding alcohol dehydrogenase (*adh1*), lactate dehydrogenase (*lctE*), and aerolysin/leukocidin toxin (*lukM*), each of which contains a Crp consensus binding site in the upstream regulatory region, and the two-component signal transduction system *srrAB*, which contains a single change in the Crp motif, we examined the ability of ArcR to bind to DNA fragments containing the regulatory regions. EMSAs showed that *S. aureus* wild-type cell extracts

caused similar shifts in mobility of the *adh1*, *srrA*, and *lukM* probes, whereas cell extracts of the *arcR* deletion mutant had no discernible effect on mobility (Fig. 7). The ability of ArcR to bind to the *adh1*, *srrA*, and *lukM* regulatory regions was confirmed by using purified His-ArcR, which formed, in each case, a single DNA-protein complex (data not shown). Unexpectedly, while cell extracts caused a mobility shift with the *lctE* DNA fragment, this was not observed with purified ArcR, suggesting that additional cellular factors might be necessary for binding.

ArcR binding to the *lctE* regulatory region is cAMP dependent. Crp proteins typically form a complex with cyclic AMP (cAMP) that facilitates binding to its target promoter region. To determine whether cAMP influences the binding of ArcR to the *lctE* regulatory region, 0.5 mM cAMP was included in DNA binding reactions. EMSAs showed that cAMP significantly increased the formation of an ArcR-DNA complex (Fig. 8A). The specificity of ArcR binding to the *lctE* DNA fragment was shown by competitive inhibition of binding by the addition of an excess of unlabeled fragment (Fig. 8B). In contrast, cAMP had little or no effect on stimulating ArcR binding to the *arcA*, *adh1*, and *srrAB* DNA fragments (data not shown).

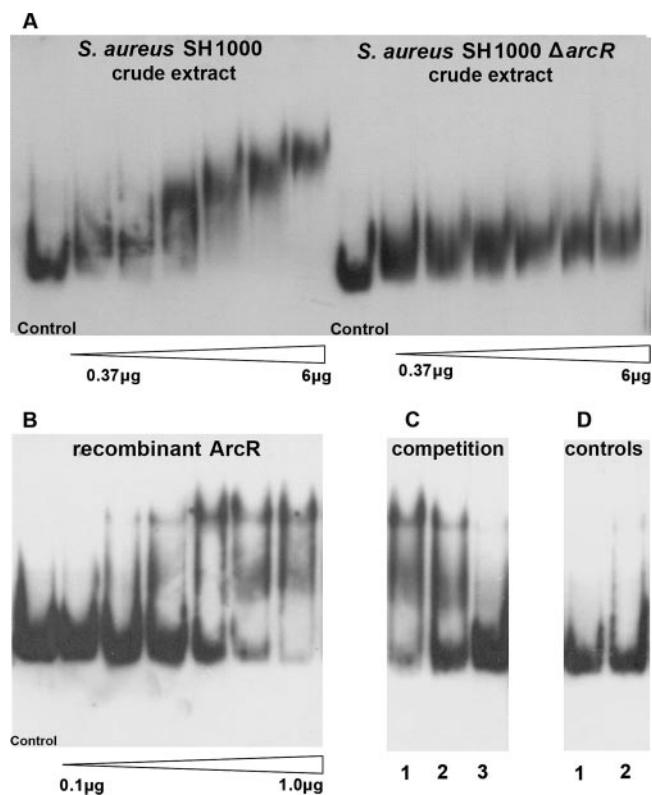


FIG. 6. EMSAs of ArcR binding to the *arc* operon regulatory region. (A) Crude cell extracts prepared from *S. aureus* SH1000 and the *arcR* deletion mutant (0.37 μ g to 6 μ g protein) incubated with a 232-bp DIG-labeled *arcA* fragment (3 fmol). (B) Purified recombinant His-ArcR (0.1 to 1.0 μ g protein) incubated with the 232-bp *arcA* fragment (3 fmol). (C) Competition assay. His-ArcR (0.8 μ g protein) and a DIG-labeled *arcA* fragment (3 fmol) were incubated in the presence of increasing amounts of unlabeled *arcA* fragment. Lane 1, 3 fmol unlabeled fragment; lane 2, 30 fmol unlabeled fragment; lane 3, 300 fmol unlabeled fragment. (D). Control assays. Lane 1, purified His-NrdR (5 μ g) incubated with a DIG-labeled *arcA* fragment (3 fmol); lane 2, purified His-ArcR (1 μ g) incubated with a DIG-labeled *nrdD* fragment (3 fmol).

DISCUSSION

A systematic search of the *S. aureus* genome databases identified a single ORF homologous to ArcR, a member of the Crp-Fnr family of transcriptional regulators. ArcR was shown in several bacteria to govern expression of the ADI pathway in response to arginine, providing a source of ATP for energy under anaerobic conditions (21, 44, 56) and generating ammonia to protect against the deleterious effects of acidic environments (10). In this study, we show that *S. aureus* ArcR is not essential for growth under aerobic conditions but is necessary to support growth under anaerobic conditions when arginine is the sole energy source. The significance of the *S. aureus* ADI system for anaerobic growth is also indicated by studies that showed that *hemB* mutants that are deficient in citric acid cycle and electron transport pathways can compensate to produce ATP by upregulating the ADI pathway (29). Similarly, transcription of the ADI pathway genes was reported to be stimulated in an *agr*- and cell density-dependent manner (19), thereby enabling anaerobic growth in the presence of arginine.

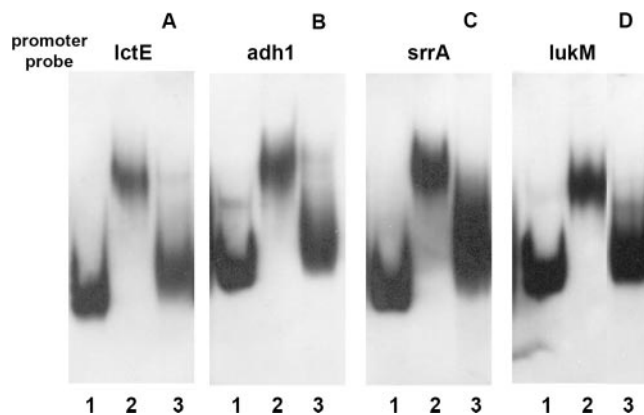


FIG. 7. EMSAs of ArcR binding to the *lctE*, *adh1*, and *lukM* regulatory regions. Crude cell extracts prepared from *S. aureus* SH1000 and the *arcR* deletion mutant were incubated with DIG-labeled DNA fragments (3 fmol). (A) *lctE* fragment (210 bp). (B) *adh1* fragment (215 bp). (C) *srrA* fragment (157 bp). (D) *lukM* fragment (211 bp). Lane 1, negative control (DNA probe only); lane 2, SH1000 crude extract (6 μ g); lane 3, *S. aureus* crude extract of *arcR* deletion mutant (6 μ g).

Recent studies suggest that *S. aureus* ArcR may play an important role in anaerobic biofilm formation, since the ADI and urease pathway genes are significantly induced in biofilm cells compared to planktonic conditions and possibly are the basis for survival under anoxic conditions (3, 45).

The *S. aureus arcR* and *arcABDC* genes are coexpressed and form an operon. Full-length transcripts were detected only under anaerobic conditions. In medium containing glucose, *arcABDC* transcription was observed only in the stationary state, indicating that the operon is subject to catabolite control and that expression is induced when glucose is depleted. In contrast, in the absence of glucose, arginine strongly enhanced the transcription of *arc* genes. Glucose and arginine therefore have reciprocal effects on regulating the expression of the *arc* genes: arginine acts as a positive regulator, and glucose acts as

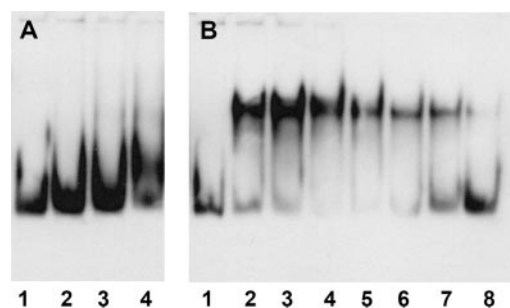


FIG. 8. EMSAs of His-ArcR binding to the *lctE* regulatory region in the absence and presence of cAMP (0.5 mM). (A) Incubation of His-ArcR with the *lctE* fragment (3 fmol). His-ArcR protein concentrations were as follows: lane 1, 0 μ g; lane 2, 0.1 μ g; lane 3, 0.2 μ g; lane 4, 4 μ g. (B) Incubation of His-ArcR and the *lctE* fragment (3 fmol) in the presence of cAMP (0.5 mM). His-ArcR protein concentrations were as follows: lane 1, 0 μ g; lane 2, 0.1 μ g; lane 3, 0.2 μ g; lane 4, 0.4 μ g; lane 5, 0.5 μ g. Lanes 6 to 8 show a competition assay. His-ArcR (0.4 μ g) and the DIG-labeled *lctE* fragment (3 fmol) were incubated in the presence of increasing amounts of unlabeled *lctE* fragment. Lane 6, 3 fmol; lane 7, 30 fmol; lane 8, 300 fmol.

a negative regulator. Similar observations were reported previously for other bacteria (13, 14). To determine whether ArcR is essential for the expression of *arc* genes under conditions of anaerobic growth, we deleted *arcR* and showed that the expression of the *arcABDC* gene cluster was abolished. Thus, under conditions when arginine is the main source of carbon for energy, ArcR is essential for anaerobic growth. Proteomic analysis confirmed that Arc proteins are synthesized under the control of ArcR under anaerobic conditions. ArcA, ArcB, and ArcC were all detected under anaerobic conditions in the wild type but were absent in the *arcR* deletion mutant. ArcD, which encodes the arginine-ornithine antiporter, was not detected, presumably because it is a membrane protein. Unexpectedly, the expression of several other proteins encoded by genes implicated in anaerobic metabolism, *pflB*, *hmp*, *lctE*, *ddh*, and *srrA*, was not significantly different in the wild-type and mutant strains even though all were recently shown to be transcriptionally upregulated in the transition from aerobic to anaerobic growth (22). Thus, while the regulatory regions of these genes have a consensus Crp motif and bind ArcR (see below), their activation during anaerobiosis is likely to be mediated by a Crp-like activator other than ArcR. Alternatively, ArcR may exert a much less pronounced effect on inducing the expression of these genes compared to the *arc* genes under the physiological conditions employed in these experiments.

Crp regulatory proteins act by binding to specific sequences in the promoter regions of the genes that they control (31, 38). The consensus Crp DNA binding sequence is TGTGA-N₆-TCACA. We assumed that since *S. aureus* ArcR is a Crp-like protein, it would bind to a similar motif in the *arc* promoter region. The *arc* promoter region contains a consensus Crp binding site to which ArcR binds. Possibly, *S. aureus* ArcR functions in conjunction with ArgR to activate the expression of the *arc* operon. A putative *argR* gene coding for an ArgR repressor and two putative ARG boxes are located immediately upstream of the *arcA* gene.

Close inspection of the *S. aureus* genome revealed the existence of numerous additional Crp-like binding sites, some which are identical to the consensus site and others which differ by just one or two bases (see Table S1 in the supplemental material). Interestingly, nearly all of the Crp-like sites are located in front of genes thought to have a role in anaerobiosis. Some of these sites show strong similarity to *B. subtilis* Fnr and Rex binding sites (26). Michel et al. (38) previously showed that the expression of many of these genes, and that of other genes presumed to be involved in anaerobic growth, is affected in a ClpP protease mutant. Several of the genes containing Crp-like binding sites were chosen as candidates to determine if ArcR is able to bind to their promoter regions. Promoter probes for four of these genes, *lctE*, *adh1*, *srrAB*, and *lukM*, all exhibited binding with cell extracts, and with the exception of *lctE*, all showed binding with purified ArcR. Plausibly, additional cellular factors might be necessary for ArcR binding to the *lctE* promoter region.

Crp is a DNA binding protein that assists RNA polymerase in binding to promoters to activate transcription. Crp binds cAMP and adopts a conformation that stimulates its interaction with RNA polymerase to establish active transcription complexes at Crp-dependent promoters (23). The ArcR N-terminal region contains a predicted cyclic nucleotide binding

domain, and we therefore tested whether cAMP stimulates the binding of ArcR to the *lctE* promoter probe. cAMP significantly improved the formation of a complex between ArcR and the *lctE* probe. However, we failed to detect an effect of cAMP on Crp binding with any of the other probes. We analyzed the *lctE* promoter probe in more detail and found that it contains two sets of Crp-like binding sites, whereas each of the other promoter regions contains a single Crp-like binding site. cAMP stimulation of ArcR binding to the *lctE* promoter may involve a cooperative interaction between two Crp molecules. Lactate dehydrogenase is a key enzyme in anaerobic fermentation. Plausibly, the *lctE* gene is subject to glucose catabolic repression. Under anaerobic conditions, when glucose is limiting, the binding of cAMP to ArcR results in a conformational change that enhances its ability to bind to the *lctE* regulatory region and thus to activate transcription, allowing the anaerobic degradation of alternative carbon sources to obtain energy.

Catabolite repression of the *arc* operon has been reported previously for various species such as oral streptococci (16), *L. sakei* (55), and *Streptococcus suis* (25). In these bacteria, the expression of the ADI pathway genes is induced in the presence of arginine and repressed by glucose. A recent study shows that CcpA (catabolite control protein A) is involved in the catabolic repression of transcription of the *S. gordonii arc* operon (54). A similar mechanism may operate on the *S. aureus arc* operon, since we noted the presence of a putative *cre* sequence upstream of *arcA*, and a *ccpA* ortholog in *S. aureus* was recently described (49). In *S. gordonii*, ArcR, which is closely related to ArgR transcriptional regulators, appears to recognize high-affinity and low-affinity binding sites in the *arc* promoter region (54). In this respect, the *S. aureus* and *S. gordonii* ArcR proteins differ from *E. coli* Crp, which binds to two symmetrical sites. The presence of what may be high- and low-affinity ArcR binding sites in the *S. aureus arcA* promoter region may allow the fine-tuning of the regulation of the ADI pathway in response to different environmental signals.

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