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Expression and Regulation of the Antimonite, Arsenite, and Arsenate Resistance Operon of *Staphylococcus xylosus* Plasmid pSX267

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The arsenate, arsenite, and antimonite resistance region of the *Staphylococcus xylosus* plasmid pSX267 was subcloned in *Staphylococcus carnosus*. The sequenced DNA region revealed three consecutive open reading frames, named *arsR*, *arsB*, and *arsC*. Expression studies in *Escherichia coli* with the bacteriophage T7 RNA polymerase-promoter system yielded three polypeptides with apparent molecular weights of 8,000, 35,000, and 15,000, which very likely correspond to ArsR, ArsB, and ArsC, respectively. ArsB was distinguished by its overall hydrophobic character, suggesting a membrane association. The arsenate, arsenite, and antimonite resistance was shown to be inducible by all three heavy metal ions. Inactivation of the first gene, *arsR*, resulted in constitutive expression of resistance. Similar results were obtained with transcriptional fusions of various portions of the *ars* genes with a lipase reporter gene, indicating a function of ArsR as a negative regulator of a putative promoter in front of *arsR*. The inactivation of *arsR* also resulted in reduction of resistance to arsenite and antimonite, while arsenate resistance was unaffected. The three *ars* genes conferred arsenite resistance in *E. coli* and arsenite as well as arsenate resistance in *Bacillus subtilis*.

Plasmid-mediated resistance to arsenate, arsenite, and antimonite salts in *Staphylococcus aureus* was first described by Novick and Roth (29). The resistance genes are normally found on plasmids encoding penicillinase and various heavy metal resistance determinants, such as resistance to Cd^{2+} and Hg^{2+}. The arsenical resistance genes on plasmid pI258 are composed of at least two loci (23), which are clustered (28). Studies on the arsenical resistance operon of the *Escherichia coli* conjugative R-factor plasmid R773 and the *S. aureus* plasmid pI258 revealed that the resistance determinants from both organisms are inducible by arsenate, arsenite, or antimonite. The mechanism of plasmid-mediated arsenate resistance in both *E. coli* and *S. aureus* is based on an efflux system (25, 42), which, at least in *E. coli*, functions as an ATPase. It was demonstrated that, at least in *E. coli*, arsenite is translocated similarly to arsenate (32).

Arsenate and arsenite resistances are common in other staphylococcal species. An investigation of a collection of *Staphylococcus xylosus* strains isolated from soybean oil meal showed that more than 90% were arsenate and arsenite resistant and that the resistance genes were plasmid encoded. One strain, *S. xylosus* DSM 20267, harboring a 29.5-kb plasmid, designated pSX267, was studied in more detail. In searching for a pSX267-associated resistance to different antibiotics, no indication of plasmid-borne resistance to any of 63 antibacterial drugs tested was detected in this strain. Heteroduplex analysis between pSX267 and pI258 revealed only high DNA homology within the arsenical resistance genes and the replication region (14).

The arsenical resistance genes of pSX267 have been subcloned in *Staphylococcus carnosus* by using pC194. The resulting plasmid, pCA43 (21), confers resistance to chloramphenicol, arsenate, arsenite, and antimonite. Insertional inactivation studies showed that the arsenical resistance determinants are composed of at least two genes, one of which encodes both the arsenite and antimonite resistances. Arsenate resistance occurs only when both genes are intact (21).

In this report, we present the DNA sequence of the arsenical resistance genes of the *S. xylosus* plasmid pSX267 and the results of gene expression studies in *E. coli*. Furthermore, we provide evidence for the negative regulation of the putative operon at the transcriptional level by the first gene, *arsR*, of a three-gene operon and for the induction of resistance by all three heavy metal ions. In addition, it is shown here that the staphylococcal resistance genes confer arsenite resistance in *E. coli* and arsenite and arsenate resistance in *Bacillus subtilis* 1A280.

MATERIALS AND METHODS

Bacterial strains. The following strains were used as cloning hosts: *S. carnosus* TM300 (39); *E. coli* JM83 (48); *E. coli* HMS174 (7); *E. coli* K38 (34); and *B. subtilis* 1A280 (2), a deletion mutant (*asaA*Δ2) of *B. subtilis* 168-2 (*trpC2 leu-2*), obtained from the *Bacillus* Genetic Stock Center of Ohio State University.

Plasmids. *S. carnosus* plasmid pC194 (17) is a *Staphylococcus* vector plasmid. pCA43 is a pC194 replicon containing the arsenical resistance genes of *S. xylosus* DSM 20267 (21). pLipP1 is a pC194 replicon containing the *S. hyicus* lipase gene (*lip*) (12). pLipPS1 is a derivative of pLipP1 which contains an *S. carnosus* DNA fragment which leads to a 30- to 40-fold enhancement of *lip* transcription (22). pT181mcs (5) was constructed by cloning a *Pvu*II fragment with the multiple cloning site of pUC18 (53) into the *Nde*I site of pT181 (20). *E. coli* plasmids pUC8 (48), pUC19 (53), pT7-5, pT7-6 (45), pGP1-2 (45), and pACYC184 (8, 31) were used.

DNA preparation and transformation. *S. carnosus* plasmid DNA was prepared by the cleared lysate method (27). *E. coli*

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supercoiled plasmid DNA was prepared by the modified alkaline lysis method (6). *E. coli* was transformed with $CaCl_2$ (11), *B. subtilis* was transformed by the competence method (3), and *S. carnosus* was transformed by protoplast transformation (13) or electroporation (4).

DNA sequence analysis. Some of the DNA was sequenced chemically (24), by the A+G modification procedure of Gray et al. (15). Restriction fragments for Maxam-Gilbert sequencing were 5'-end labeled with [γ-^{32}P]ATP (0.1 TBq/μmol; New England Nuclear) by using T4 polynucleotide kinase (Bethesda Research Laboratories). The remaining sequence was determined by enzymatic sequencing of supercoiled plasmid DNA (10, 37). Subclones for plasmid sequencing were generated on the basis of the restriction map of pCA43 and pCA44 (Fig. 1A). Deoxy- and dideoxynucleotides were purchased from Pharmacia, Klenow enzyme was from Boehringer (Mannheim, Germany), and [α-^{32}P]dATP (111 TBq/mmol) was from Amersham.

Expression studies with the T7 RNA polymerase-promoter system. The T7 expression system was used as described by Tabor and Richardson (45). The *ars* genes or portions thereof were cloned into the T7 promoter-containing vector pT7-5 or pT7-6 (Fig. 1B). The resulting recombinant plasmids were used to transform *E. coli* K38 containing plasmid pGP1-2, carrying the gene for T7 RNA polymerase under heat-inducible control of the *c*I857 repressor. Expression was measured by labeling the induced gene products with [^{35}S]-methionine (51.8 TBq/mmol, 0.37 GBq/ml; in vivo cell-labeling grade; Amersham). The labeled polypeptides were separated on 15% polyacrylamide gels with 0.1% sodium dodecyl sulfate (SDS) (38) and visualized by autoradiography.

Enzyme assays. Lipase activity was determined spectrophotometrically at 405 nm by monitoring hydrolysis of the substrate *p*-nitrophenylcaprylate (1.3 mg/ml) (50). The buffer consisted of 20 mM Tris-Cl (pH 9.0), 10 mM $CaCl_2$, and 0.1% (vol/vol) Triton X-100.

Chemicals. The following heavy metal salts were used: arsenate ($Na_2HAsO_4 \cdot 7H_2O$), arsenite ($NaAsO_2$), and antimonite [potassium antimonyl tartrate; $K(SbO)C_4H_4O_6$]. Enzymes for molecular cloning were obtained from Boehringer, BRL (Eggenstein, Germany), or Pharmacia (Sweden). *p*-Nitrophenylcaprylate was obtained from Sigma (Deisenhofen, Germany).

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 2 has been submitted to GenBank under accession number M80565.

RESULTS

Nucleotide sequence of the *ars* genes. pCA43 is a recombinant plasmid consisting of the vector pC194 and a DNA fragment containing the arsenical resistance genes from pSX267 (21). pCA44 is a deletion product of pCA43 lacking the pSX267-derived 1.2-kb *Pvu*II-*Sca*I fragment (Fig. 1A). The nucleotide sequence of the *ars* region of pCA43 is shown in Fig. 2. It comprises 2,546 nucleotides.

The sequence contains the three open reading frames *arsR*, *arsB*, and *arsC*. *arsR*, starting at position 350 and ending at position 664, comprises 315 bp, corresponding to a protein of 104 amino acids. *arsB* overlaps by 1 bp with *arsR*, beginning at nucleotide 664 and ending at base 1953. *arsB* encodes a protein of 429 amino acids. *arsC* starts at position 1971, ends at 2367, and encodes a gene product of 131 amino acids. All three open reading frames are preceded by putative ribosome-binding sequences (40). The quality of the

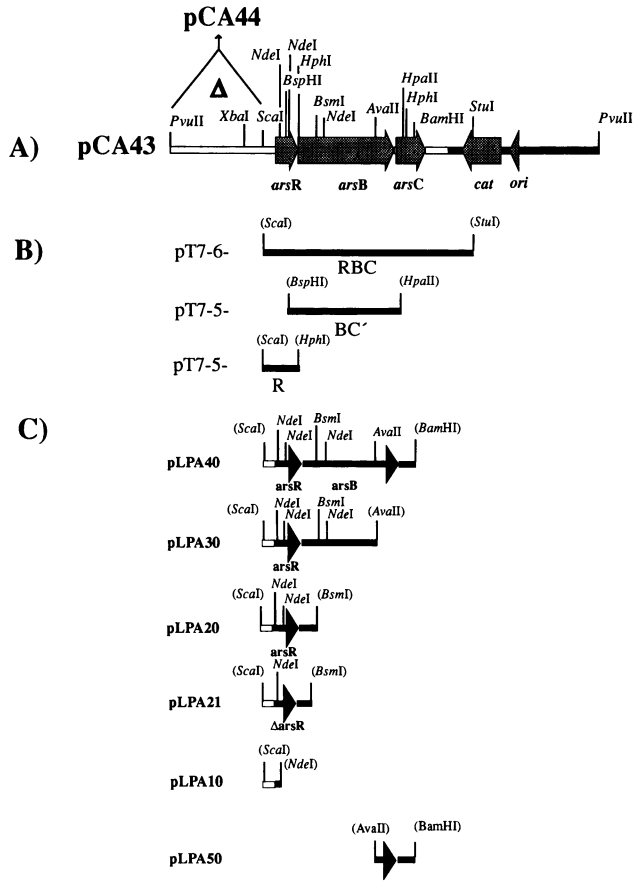

FIG. 1. Genetic maps of (A) the staphylococcal plasmids pCA43 and pCA44, (B) various pT7 subclones, and (C) *ars* DNA fragments inserted into pPS11. (A) The section corresponding to the vector pC194 is indicated by the solid black segment. The remaining area corresponds to the pSX267 fragment. The genes *arsR*, *arsB*, *arsC*, and *cat* and the orientation of the plasmid replication origin (*ori*) are designated by arrows. pCA44 is derived from pCA43 by deletion of the 1.2-kb *Pvu*II-*Sca*I fragment. (B) For gene expression studies in *E. coli*, pT7-6RBC, pT7-5BC', and pT7-5R were constructed. The RBC fragments were cut at the indicated restriction sites and inserted at the *Sma*I site of pT7-6 or pT7-5 (45) by blunt-end ligation. (C) Genetic maps of *ars* DNA fragments inserted into pPS11. The arsenical resistance gene fragments were isolated after digestion of pCA43 (21) with the restriction enzymes shown in parentheses and inserted into the *Bam*HI site of pPS11 by blunt-end ligation after treatment of the fragments with Klenow enzyme, yielding the indicated plasmids. pLPA21 was constructed from pLPA20 by deleting the 57-bp *Nde*I fragment as described for pCA44ΔR22.

Shine-Dalgarno (SD) sequences was estimated by calculating the free energies (46) of base-pairing between the putative SD sequence and the 3' end of staphylococcal 16S rRNA (26). In terms of free energy (ΔG), the ribosome-binding sites are characterized as follows: SD1 (*arsR*), $\Delta G = -18.8$ kcal; SD2 (*arsB*), $\Delta G = -14.3$ kcal; and SD3 (*arsC*), $\Delta G = -12.0$ kcal. The G+C content of the arsenical resistance genes is 31%, which is consistent with the low G+C content of staphylococcal DNA.

Immediately following the three consecutive stop codons of *arsC*, there exists an inverted repeat with a relatively high G+C content ($\Delta G = -14.0$ kcal), followed by a T-rich region which may function as a transcription termination structure

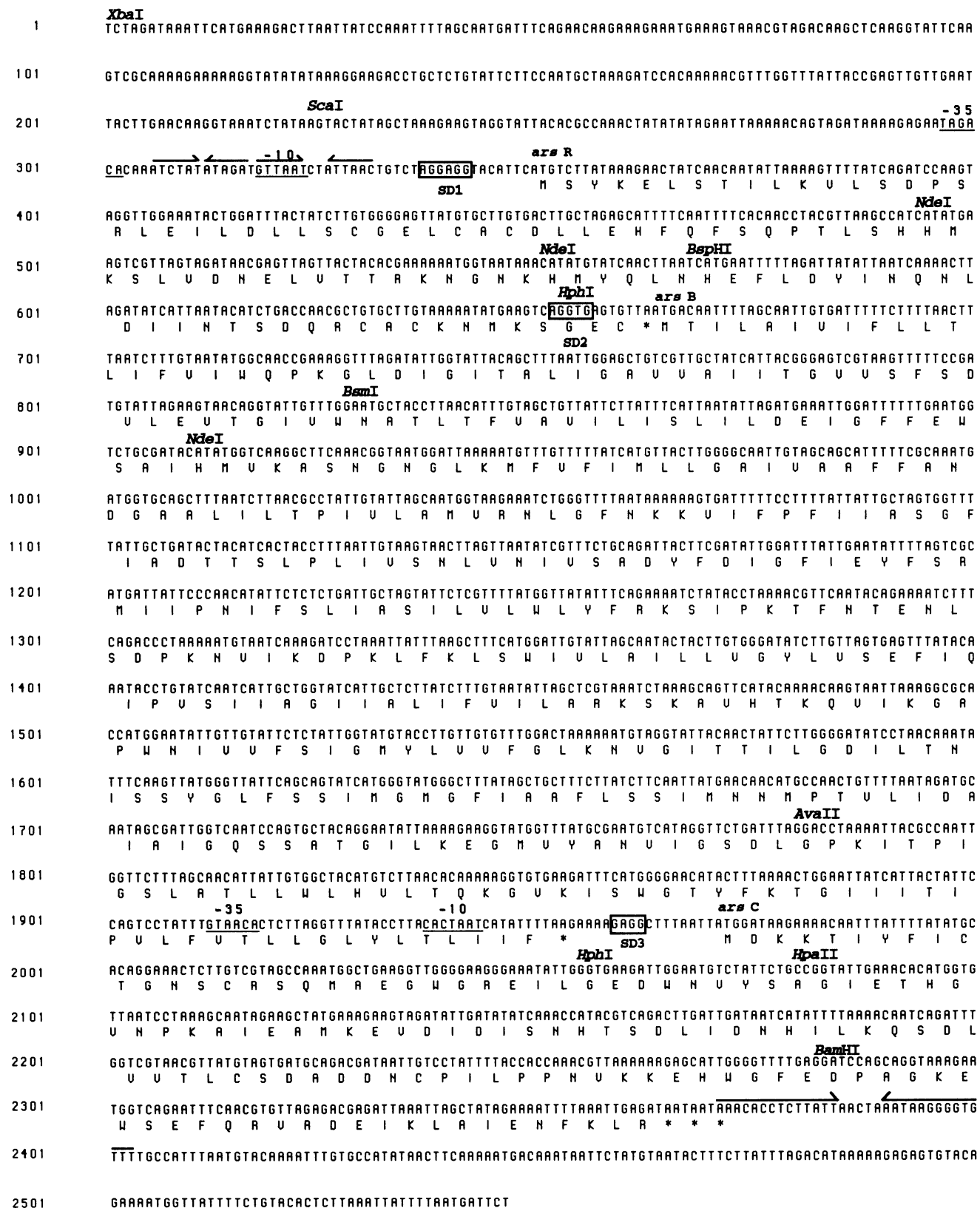

FIG. 2. Nucleotide sequence of the *arsRBC* genes. The amino acid sequences of the putative gene products are shown below the DNA sequence. Stop codons are marked by asterisks. Shine-Dalgarno (SD) sequences are boxed and numbered 1 to 3. Inverted repeats are indicated by arrows. Putative promoter sequences are underlined and marked with −10 and −35. Some restriction sites are also shown.

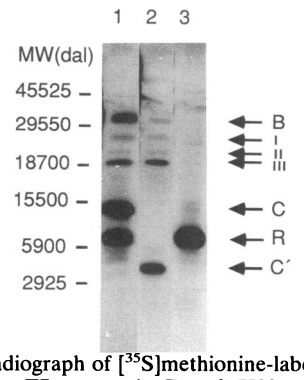

FIG. 3. Autoradiograph of [^{35}S]methionine-labeled polypeptides expressed with the T7 system in *E. coli* K38 containing plasmid pGP1-2 and either pT7-5R, pT7-5BC', or pT7-6RBC. The locations of all *ars*-encoded polypeptides are indicated by arrows. Lanes: 1, pT7-6RBC; 2, pT7-5BC'; 3, pT7-5R. Arrows labeled B, C, and R indicate the putative full-length ArsB, ArsC, and ArsR proteins, respectively; the arrow labeled C' indicates truncated ArsC protein. The bands representing putative degradation products of ArsB or polypeptides having different translational start sites in *E. coli* are labeled I, II, and III. Positions of molecular mass markers (in daltons) are indicated on the left.

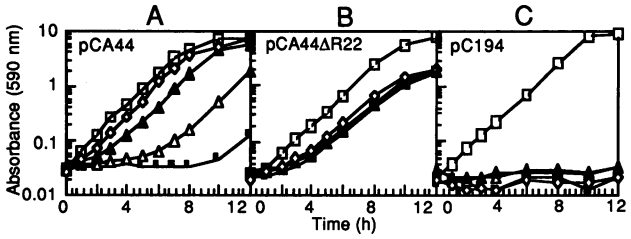

FIG. 4. Inducible and constitutive expression of arsenate resistance in various *S. carnosus* clones. (A) *S. carnosus*(pCA44), wild-type *ars* determinant; (B) *S. carnosus*(pCA44ΔR22); (C) *S. carnosus*(pC194), sensitive vector control. Incubation conditions: (□) grown in LB medium alone (control); (■) grown without arsenate to mid-log phase and then in LB medium (1:100) containing 3 mM arsenate; grown with 0.03 mM (△), 0.1 mM (▲), or 1 mM (◇) arsenate to mid-log phase and then in LB medium (1:100) containing 3 mM arsenate.

(30). All these features suggest that the *ars* genes are arranged in an operon.

Computer analysis of the putative *ars* gene products. ArsB has a calculated molecular weight of 46,569 and shows an overall hydrophobic character, with the hydrophobic domains distributed evenly throughout the protein (data not shown). In contrast to ArsB, ArsR (11,867 Da) and ArsC (14,889 Da) appear to be hydrophilic proteins, suggesting a cytoplasmic location.

Comparison of the staphylococcal gene products with the corresponding *E. coli* R773 gene products yielded the following percentages of identical amino acids: ArsR, 30%; ArsB, 58%; and ArsC, 18%. In the *S. xylosus* sequence, no gene corresponding to the *E. coli* R773 *arsA* gene was found.

Expression studies of the *arsRBC* genes. The *arsRBC* gene products were identified with the *E. coli* expression system based on the T7 RNA polymerase-promoter system (45). The *ars* fragments inserted into the pT7 vectors are shown in Fig. 1B; they were transferred into *E. coli*, and expression of *arsR*, *arsB*, and *arsC* was detected by autoradiography (Fig. 3). Plasmid pT7-5R, containing only *arsR*, led to the expression of an 8-kDa protein; plasmid pT7-5BC', containing the complete *arsB* and a portion of *arsC*, led to the expression of a small polypeptide of approximately 5 kDa and four weakly expressed polypeptides of 35, 27, 21, and 19 kDa. The expression of the fragment containing all three *ars* genes (pT7-6RBC) revealed three predominant polypeptides of 35, 15, and 8 kDa. In addition, three fainter bands of 27, 21, and 19 kDa, corresponding to the bands obtained with pT7-5BC', were also visible.

Inactivation of *arsR* leads to constitutive expression of the *ars* system. *S. carnosus*(pCA44) was grown in the absence of heavy metal ions, and then the cells were used to inoculate a second culture with inhibitory amounts of arsenate (Fig. 4A). Exponential growth started after about 8 h. When cells were precultivated in the presence of various subinhibitory concentrations of arsenate, growth showed shorter lag phases, depending on the amount of arsenate ions. This indicates that *ars* expression is inducible by subinhibitory arsenate concentrations. Similar results were obtained with arsenite and antimonite (data not shown). These results are consistent with those obtained by Silver et al. (41). If *arsR* encodes a regulatory protein, its inactivation should lead to either constitutive expression or noninducibility of the arsenical resistance genes, depending on the negative or positive nature of the regulation, respectively. To inactivate *arsR* in pCA44, the 57-bp *Nde*I fragment (Fig. 2) within *arsR* was deleted by partial digestion with *Nde*I and religation (partial digestion was necessary because there is a third *Nde*I site within *arsB*; Fig. 2). The resulting plasmid was designated pCA44ΔR22. The *Nde*I deletion in *arsR* did not shift the reading frame, but it should inactivate ArsR. In contrast to *S. carnosus*(pCA44), the growth curve of uninduced *S. carnosus*(pCA44ΔR22) exhibited no lag phase, indicating that inactivation of *arsR* led to constitutive expression of the arsenical resistance genes (Fig. 4B). *S. carnosus*(pC194) was sensitive to arsenical compounds (Fig. 4C).

Lipase gene expression under transcriptional control of *ars* promoters. To determine the position and strength of the *ars* operon promoters, the *Staphylococcus hyicus* lipase gene (*lip*) was placed under the transcriptional control of various fragments of the *ars* system. The lipase gene encodes an exoprotein whose activity can be easily determined (12, 49). A series of promoter search plasmids were constructed (unpublished data) by using the *S. hyicus* lipase gene cloned in *S. carnosus* on plasmid pLipP1 (12) or pLipPS1 (22), of which pPS11 is one example. The lipase gene on pPS11 is silent unless a promoter fragment is inserted in the correct orientation at the *Bam*HI site. Figure 1C shows the various *ars* DNA fragments cloned into the *Bam*HI site of pPS11, yielding pLPA10 through pLPA50.

In order to study the *trans* effect of the *arsR* gene product, we constructed pTAR10. The same *ars* fragment as indicated for pLPA20 was cloned at the *Sma*I site of pT181mcs (5), which is compatible with pPS11. The results on lipase induction are summarized in Table 1. When the lipase gene was fused to the intact *arsR*, its expression was inducible by arsenite (Table 1). When the lipase gene was fused to the disrupted *arsR*, lipase expression was constitutive (Table 1, plasmids pLPA21 and pLPA10). Lipase production was also constitutive when the gene was under control of the proposed weak *arsC* promoter (Table 1, plasmid pLPA50). A pronounced *trans* effect of the *arsR* gene product of pTAR10 was observed with the *ars-lip* fusions when *arsR* was inactivated (Table 1). In the absence of arsenite, the presence of pTAR10 led to a marked decrease in lipase activity in *arsR*

TABLE 1. Extracellular lipase activity of *S. carnosus* containing various plasmids under inducing and noninducing conditions

Plasmid(s)	Lipase activity[a] (mU/mg [dry wt])	
	− Arsenite	+ Arsenite
pLPA40/pT181mcs[b]	50	550
pLPA40/pTAR10	59	740
pLPA30/pT181mcs	6	150
pLPA30/pTAR10	6	100
pLPA20/pT181mcs	8	120
pLPA20/pTAR10	6	140
pLPA21/pT181mcs	150	130
pLPA21/pTAR10	11	240
pLPA10/pT181mcs	450	300
pLPA10/pTAR10	6	76
pLPA50/pT181mcs	8	6
pLPA50/pTAR10	7	6
pPS11	0	ND[c]
None	0	ND

[a] Lipase activity was detected after 15 h of growth; 10 μM arsenite was used for induction.
[b] pT181mcs is present as a control; its absence has no influence on inducibility.
[c] ND, not determined.

mutant strains. The approximately 100-times-weaker *arsC* promoter was unaffected by the presence of pTAR10 (Table 1). In the control strains *S. carnosus*(pPS11) and *S. carnosus* without a plasmid, no lipase activity was detectable in the culture supernatant (Table 1). These results confirm that the promoter in front of *arsR* is negatively regulated by ArsR and introduce for the first time the possibility of a weak constitutive promoter in front of the *arsC* gene. Potential −35 and −10 sequences for this promoter are indicated in Fig. 2.

Involvement of the three *ars* genes in arsenite, arsenate, and antimonite resistance in *S. carnosus*. The influence of the various *ars* genes on arsenite, arsenate, and antimonite resistances in *S. carnosus* was studied (Table 2). Maximal resistance to all three oxyanions was only observed when all three genes were present, as in pCA44. Inactivation of *arsR* (pCA44ΔR22) caused a decrease in the resistance level with antimonite and arsenite; the resistance to arsenate was unaffected. When *arsC* was inactivated (pLPA40), the clone became sensitive to arsenate but still exhibited reduced resistance to antimonite and arsenite. *arsR* alone (pLPA20) did not confer resistance.

TABLE 2. Involvement of *arsR*, *arsB*, and *arsC* in antimonite, arsenite, and arsenate resistance in *S. carnosus*[a]

Plasmid	Intact gene(s)	MIC (mM)		
		Antimonite	Arsenite	Arsenate
pCA44	*arsR arsB arsC*	0.06	1.5	3.5
pCA44ΔR22	*arsB arsC*	0.03	0.5	3.5
pLPA40	*arsR arsB*	0.02	0.3	0.2
pLPA20	*arsR*	0.006	0.07	0.2
None		0.006	0.07	0.2

[a] MICs were determined by growing cells in 5 ml of LB medium with various concentrations of oxyanions for 8 h and then measuring turbidity.

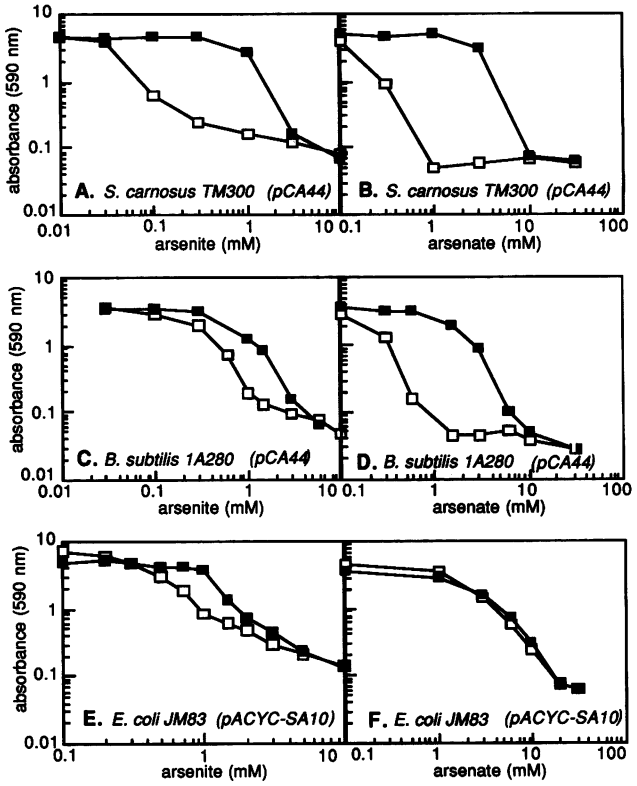

FIG. 5. Effect of the staphylococcal *ars* resistance system on arsenite and arsenate resistance in *S. carnosus*(pCA44), *E. coli* JM83(pACYC-SA10), and *B. subtilis* 1A280(pCA44). Cultures (5 ml) in LB were inoculated 1:100 with an overnight culture and grown for 8 h, and turbidity was measured for strains with (■) and without (□) the indicated plasmid.

Expression studies of staphylococcal arsenical resistance genes in *E. coli* and *B. subtilis*. Since the staphylococcal plasmid pCA44 does not replicate in *E. coli*, the *ars* genes were cloned into an *E. coli* vector. The *Sca*I-*Stu*I fragment of pCA43 was inserted by blunt-end ligation into the *Bam*HI-digested low-copy-number plasmid pACYC184, forming recombinant plasmid pACYC-SA10 (data not shown). Since *B. subtilis* 168 exhibits a natural high tolerance to arsenite and arsenate (1), the presence of pCA44 (which replicates in *B. subtilis*) caused only a slightly increased resistance to arsenite and arsenate (data not shown). We therefore used the arsenate-sensitive *B. subtilis* deletion mutant 1A280 (2). The effect of the staphylococcal *ars* resistance system on arsenite and arsenate resistance in *S. carnosus* TM300 (pCA44), *E. coli* JM83(pACYC-SA10), and *B. subtilis* 1A280 (pCA44) is shown in Fig. 5. *E. coli* JM83 exhibited a naturally high tolerance to arsenite and arsenate. The presence of pACYC-SA10 led to a slight but reproducible increase in arsenite resistance, while arsenate resistance was unaffected. The presence of pCA44 in *B. subtilis* 1A280 led to increased arsenite and arsenate resistance.

DISCUSSION

The nucleotide sequence of the *ars* operon of *E. coli* plasmid R773 (9, 19, 35) contains four open reading frames, *arsR*, *arsA*, *arsB*, and *arsC*, which encode putative proteins

of 117, 583, 429, and 141 amino acids, respectively. The *E. coli* ArsA protein exhibits two regions of homology to a highly conserved sequence in adenylate-binding proteins (9, 43). Rosen et al. (33) demonstrated that the *E. coli* ArsA exhibits an arsenite- and antimonite-dependent ATPase activity. The arsenite and arsenate efflux system is energized by ATP hydrolysis (25, 32). In contrast to the *E. coli* R773 *ars* operon, the staphylococcal arsenical resistance genes do not include the ATPase gene *arsA*. The same genetic organization as for the *S. xylosus ars* genes was found in the *S. aureus* pI258 *ars* operon, which was sequenced by Ji and Silver (18). The pI258 *ars* genes are highly homologous to the pSX267 genes, and they also include no gene corresponding to the R773 *arsA* (18).

The *E. coli* and *S. xylosus* ArsB proteins are identical in size and exhibit an overall amino acid similarity of 58%. Furthermore, the hydropathy plots are nearly superimposable (data not shown). The computer-aided analysis of ArsB yielded an extremely hydrophobic character, with the hydrophobic domains distributed evenly throughout the protein, indicating a location in the cytoplasmic membrane (data not shown); the *E. coli* plasmid R773-encoded ArsB has been identified as an integral inner membrane protein, and there is evidence that it functions as the channel of an arsenite pump (36, 47). The extensive homology of both proteins indicates an equivalent function in these gram-positive and gram-negative species.

The ArsC proteins of *S. xylosus* and *E. coli* are similar in size (131 versus 141 amino acids) and exhibit weak sequence homology (18% identical amino acids). The *E. coli* ArsC has a postulated function in changing the specificity of the extrusion pump from arsenite to arsenate (9). This is in agreement with our findings (Table 2). Therefore, we postulate that both ArsC proteins act similarly.

In the expression studies with the *E. coli*-T7 system, we obtained three dominant polypeptides of 35, 15, and 8 kDa and three fainter bands (Fig. 3). The size of the 15-kDa polypeptide is in excellent agreement with the expected molecular weight of ArsC (14,889). When part of the *arsC* gene was deleted, the 15-kDa band disappeared and a smaller peptide of about 5 kDa was expressed (Fig. 3, lane 2). Therefore, ArsC appears to be the 15-kDa polypeptide.

The smaller (mobility equivalent to 8 kDa) polypeptide, obtained with pT7-5R (which contains only the *arsR* gene), may correspond to ArsR, which has a calculated molecular weight of 11,867. The difference in the molecular weights calculated for ArsR and the detected polypeptide may be due to either an abnormal running behavior of the polypeptide in SDS-polyacrylamide gel electrophoresis (PAGE) or a specific degradation of ArsR in *E. coli*.

The T7 expression results also indicate that ArsB corresponds to the 35-kDa polypeptide, since this peptide is obtained only with constructs carrying *arsB*. The three fainter bands may represent either degradation products or polypeptides having different translational start sites in *E. coli*. The difference between the calculated molecular mass of ArsB (46,569 kDa) and the mobility of the expressed protein (35 kDa) may be due to the same reasons as discussed in the case of ArsR. In the case of the *E. coli* ArsB, a similar difference in calculated molecular weights and mobility on SDS-PAGE was observed (36).

In the case of pT7-6RBC, the densitometrically calculated level of expressed ArsB is about three times higher than with pT7-5BC′ (Fig. 3, lane 1 and 2). The difference in expression may be due to translational coupling (44) of the overlapping *arsR* and *arsB* genes. The SD sequence (SD2) in front of *arsB* seems to be weak (at least in *E. coli*), as can be seen from the weak expression of ArsB. Therefore, the more efficient translation of *arsR*, which is expressed at very high levels in the T7 system, coupled with the translation of *arsB* might cause the observed increase in expressed ArsB with pT7-6RBC.

In the induction studies, it was shown that ArsR negatively regulates expression of arsenite, arsenate, and antimonite resistance. When *arsR* was inactivated by deleting the *Nde*I fragment in the middle of the gene (pCA44ΔR22), inducibility of the arsenical resistance genes was lost (Fig. 4B). This result was confirmed by a comparison of the *ars-lip* fusions of plasmids pLPA20 and pLPA21 (Table 1). The studies with the transcriptional fusion plasmids (pLPA40 to pLPA10; Fig. 1C) indicate that regulation of the arsenical resistance genes occurs at the transcriptional level. The promoter regulated by ArsR is located in front of *arsR*, as demonstrated by the lipase expression studies with pLPA20, pLPA21, and pLPA10.

These results demonstrate that ArsR acts as a repressor protein which apparently becomes inactivated by arsenite, arsenate, or antimonite ions. The six cysteine residues of ArsR might be target sites for the binding of these heavy metal ions.

When we screened the staphylococcal *ars* sequences for promoter sites with the *E. coli* promoter consensus sequence (16), we found two potential −10 and −35 regions, one in front of *arsR* and the other in front of *arsC* (Fig. 2). There is a common 5′-ACACNAATC-3′ sequence in both promoters, which is to some extent also present in other staphylococcal promoters (51). No potential promoter sequences were found in front of *arsB*. The *arsC* promoter appears to be weak when its expression on pLPA50 is compared with the expression of the *arsR* promoter on pLPA10 or pLPA21 (Table 1). The *arsC* promoter was not influenced by *arsR*. The high lipase activity found with induced *S. carnosus* (pLPA40) (Table 1) cannot be explained simply by the additive promoter strengths of the *arsR* and *arsC* promoters. The differences in lipase expression could be explained by differential mRNA stabilities of the various transcriptional fusions or by influences on promoter activity by the recombinant DNA sequences of the *ars-lip* fusion sites.

As shown in Fig. 2, there are several inverted repeat sequences upstream of the −35 and around the −10 proposed regions of the *arsR* promoter which represent potential operator sites.

The transcriptional control of the staphylococcal *ars* genes seems not to be very efficient. Uninduced *S. carnosus* clones containing either pLPA30 or pLPA20 without pTAR10 exhibit a relatively high background lipase activity. The low-level constitutive *ars* expression may allow the cells to react immediately when suddenly exposed to toxic concentrations of the arsenicals. In addition, ArsR must be produced continually in order to maintain autogenous transcriptional regulation; the subsequent genes in the operon (*arsB* and *arsC*) would be cotranscribed with *arsR*.

The staphylococcal ArsR exhibits similarities in both size and conserved sequences (30% identical amino acids) to the regulatory protein ArsR of the *E. coli* plasmid R773 (35, 52). We provide evidence that the *S. xylosus* ArsR acts as a negative regulator of the *arsR* promoter.

Since there is no gene present in the staphylococcal *ars* operon corresponding to *E. coli arsA*, the question remains how arsenical efflux is energized by staphylococci. There are in principle two possibilities: staphylococci may possess a chromosomally encoded ATPase subunit which is involved

in the efflux system, or the necessary energy is mediated by a different system, such as the proton motive force. Attempts to clone a staphylococcal chromosomally encoded ATPase in *E. coli* containing the staphylococcal arsenical resistance genes by selecting for increased arsenate resistance failed (unpublished results). If the staphylococcal *ars* operon confers arsenite and arsenate resistance in *E. coli*, the efflux of these heavy metal ions must be at least independent of ArsA. As can be seen in Fig. 5, the staphylococcal *ars* operon definitely confers arsenite resistance in *E. coli* JM83, to approximately the same arsenite concentration as occurs in *S. carnosus* TM300(pCA44). Arsenate resistance was not detectable with the pSX267 *ars* operon in *E. coli* JM83, which exhibits a relatively high level of natural arsenate resistance. Therefore, arsenate resistance provided by the pSX267 operon might hardly be detectable in the *E. coli* strain. We extended our *ars* expression studies to *B. subtilis*. As shown in Fig. 5, the presence of plasmid pCA44 in *B. subtilis* 1A280 led to both arsenite and arsenate resistance. These results are probably less conclusive than those obtained with *E. coli*, since we do not know which gene in the arsenate-sensitive mutant 1A280 is affected. However, we can say that the staphylococcal *ars* operon is able to confer arsenate and arsenite resistance in unrelated genera.

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