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Genetic Evidence for an Activator Required for Induction of Colicin-Like Bacteriocin 28b Production in *Serratia marcescens* by DNA-Damaging Agents

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Bacteriocin 28b production is induced by mitomycin in wild-type *Serratia marcescens* 2170 but not in *Escherichia coli* harboring the bacteriocin 28b structural gene (*bss*). Studies with a *bss-lacZ* transcriptional fusion showed that mitomycin increased the level of *bss* gene transcription in *S. marcescens* but not in the *E. coli* background. A *S. marcescens* Tn*5* insertion mutant was obtained (*S. marcescens* 2170 *reg*::Tn*5*) whose bacteriocin 28b production and *bss* gene transcription were not increased by mitomycin treatment. Cloning and DNA sequencing of the mutated region showed that the Tn*5* insertion was flanked by an SOS box sequence and three genes that are probably cotranscribed (*regA*, *regB*, and *regC*). These three genes had homology to phage holins, phage lysozymes, and the Ogr transcriptional activator of P2 and related bacteriophages, respectively. Recombinant plasmid containing this wild-type DNA region complemented the *reg*::Tn*5* regulatory mutant. A transcriptional fusion between a 157-bp DNA fragment, containing the apparent SOS box upstream of the *regA* gene, and the *cat* gene showed increased chloramphenicol acetyltransferase activity upon mitomycin treatment. Upstream of the *bss* gene, a sequence similar to the consensus sequence proposed to bind Ogr protein was found, but no sequence similar to an SOS box was detected. Our results suggest that transcriptional induction of bacteriocin 28b upon mitomycin treatment is mediated by the *regC* gene whose own transcription would be LexA dependent.

Serratia marcescens has been shown to produce bacteriocins upon induction with DNA-damaging agents (50). These bacteriocins have been classified into two groups (23): fraction 1 bacteriocins are active against *Escherichia coli* but not against *S. marcescens*, and fraction 2 bacteriocins are active against *S. marcescens* but not against *E. coli* (50). Bacteriocins belonging to fraction 1 are simple polypeptides that resemble colicins (50). Only colicin-like bacteriocins L and 28b have been studied in some detail. Bacteriocin L from *S. marcescens* JF246 has been isolated and characterized, and the effects of this bacteriocin on the incorporation of labelled leucine and thymidine and on the cellular levels of ATP in *E. coli* were similar to those produced by pore-forming colicins (17, 18, 40). On the other hand, the bacteriocin 28b structural gene (*bss*) has been cloned and sequenced, and the predicted amino acid sequence of the C-terminal part of this bacteriocin has been shown to have a high degree of similarity to the C-terminal domains of pore-forming colicins (56). The two bacteriocins are very closely related, if not the same, and similar bacteriocins are produced by most *S. marcescens* biotypes (21).

Colicin production is induced by mitomycin and other DNA-damaging agents (38). Determinants for colicin production studied so far are encoded by either small high-copy-number colicinogenic plasmids (type I) or large low-copy-number colicinogenic plasmids (type II) (38). An important feature of these plasmids is that they confer on their host the property of being specifically insensitive (immune) to the plasmid-encoded colicin (39). The gene responsible for colicin immunity is always located downstream of the colicin structural gene (38). A third gene responsible for colicin release has been found downstream from the immunity gene in all genetic determinants coding for colicins located in type I, but not in type II, colicinogenic plasmids (12, 33, 38, 45). These colicinogenic genes form an operon, and SOS boxes have been located in the main operon promoter region of all colicins studied (35, 38, 52). The LexA protein has been shown to bind to these SOS boxes and to repress colicinogenic gene transcription (14, 29, 30). Besides the LexA protein, other regulatory colicin controls have been found. The cyclic AMP receptor protein-cyclic AMP complex is involved in cloacin DF13 and colicin E1 transcription regulation (42, 52, 53). Colicin E1, E2, E3, K, and D production increases in anaerobic conditions (32), and the Fnr protein has been shown to mediate this effect in colicin E1 (15).

Despite the homology found between bacteriocin 28b and the pore-forming colicins, bacteriocin 28b is chromosomally encoded and no immunity or release genes are found downstream from the structural gene (21, 55). In this work we present results suggesting that bacteriocin 28b production by *S. marcescens* upon mitomycin induction is mediated by a transcriptional activator whose own transcription is SOS dependent.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were grown in Trypticase soy broth (TSB) or Trypticase soy agar (TSA) supplemented with ampicillin (100 μg/ml and 2 mg/ml for *E. coli* and *S. marcescens* strains, respectively), chloramphenicol (50 μg/ml), kanamycin (25 μg/ml), or tetracycline (12.5 μg/ml), when needed.

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TABLE 1. Bacterial strains, plasmids, and bacteriophages used in this study

Strain, plasmid, or bacteriophage	Relevant characteristic(s)[a]	Source or reference
Bacterial strains		
S. marcescens		
2170	Wild-type strain	37
2170 *reg*::Tn*5*	Bacteriocin 28b regulatory mutant	This work
SM6	Wild-type strain	25
SM6 *nucB*::Tn*5*	*recA* mutant	3
E. coli		
MC1061	*araD139* Δ(*ara-leu*)*7696* Δ(*lac*)*174 galU galK hsdR2* (r_K^- m_K^+) *mcrB1 rpsL*	Stratagene
XL1blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q} *ZΔM15* Tn*10*	Stratagene
NM554	*recA13 araD139* Δ(*ara-leu*)*7696* Δ(*lac*)*174 galU galK hsdR2 rpsL mcrA mcrB*	Stratagene
Plasmids and cosmids		
pBA289	Ap^r Cm^r pBR328 containing *bss* gene	56
pJE256	Tet^r Cm^r mini-F	13
pJEBAC1	Cm^r pJE256 containing *bss* gene	This work
pBA391	Ap^r pBluescript SK containing *bss* gene	56
pRS550	Ap^r Km^r *lacZYA t14*	46
pACYC184	Tet^r Cm^r *ori* of P15A	8
pSF921	Km^r Ap^r pRS550 containing *bss-lacZ* transcriptional fusion	This work
pSF931	Cm^r pACYC184 containing *bss-lacZ* transcriptional fusion	This work
pUA44	Ap^r pUC9 containing *recA* gene from *E. coli*	J. Barbé
pBluescript SK	Ap^r *ori* of ColE1	Stratagene
pBluescript KS	Ap^r *ori* of ColE1	Stratagene
pLT101	Ap^r pBluescript SK containing 1.3-kb *Bam*HI-*Pst*I fragment including *recA* promoter and first 79 *recA* codons	This work
pLT102	Ap^r Km^r pRS550 containing *recA-lacZ* transcriptional fusion	This work
pLT103	Cm^r pACYC184 containing *recA-lacZ* transcriptional fusion	This work
Supercos 1	Ap^r Km^r, cloning cosmid	Stratagene
Supercos 2	Ap^r Km^s Supercos 1 derivative	This work
CosPD3	Ap^r Km^r Supercos 2 containing 40-kb chromosomal *S. marcescens* 2170 *reg*::Tn*5 Sau3A* insert	This work
pKR941	Ap^r Km^r pBluescript SK with 4-kb *Bam*HI-*Bam*HI fragment containing 1.1-kb *S. marcescens* chromosomal DNA and adjacent 2.9 kb of Tn*5* (left side)	This work
pBRK6	Ap^r Cm^r pBR328 with 5.1-kb *Bam*HI-*Bam*HI fragment containing 2.67 kb of Tn*5* (right side) and adjacent 2.43 kb of *S. marcescens* chromosomal DNA	This work
pKSA10	Ap^r pBluescript KS containing last 101 *regB* codons and complete *regC* gene under control of *lac* promoter	This work
pSKA01	Ap^r pBluescript SK containing the same insert as pKS10 but in opposite orientation	This work
pGEM-T	Ap^r *ori* of ColE1	Promega
pGPR001	Ap^r pGEM-T with 282-bp fragment (from nucleotides 120 to 402) containing putative *reg* promoter under control of *lac* promoter	This work
pGPR100	Ap^r pGEM-T containing the same insert as pGPR001 but in opposite orientation	This work
pSKCAT	Ap^r pBluescript SK containing a promoterless *cat* gene	Our laboratory
pPRCAT	Ap^r pGPR100 containing a putative *reg* promoter-*cat* transcriptional fusion	This work
pBR328	Cm^r Ap^r Tet^r, *ori* of ColE1	48
pOP950	Ap^r pGEM-T containing *regA, regB, and regC* genes and wild-type putative *reg* promoter	This work
pOP951	Ap^r pBR328 containing *regA, regB, and regC* genes and wild-type putative *reg* promoter	This work
Bacteriophages		
λ467 (λ::Tn*5*)	λ*b221 rex*::Tn*5 c*I857 *O*am29 *P*am80	11
3M	*S. marcescens* generalized transducing phage	41

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tet^r, tetracycline resistance.

General DNA methods. DNA manipulations were carried out essentially as previously described (43). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers. Recombinant clones were selected on TSA plates containing the appropriate antibiotics.

Plasmid constructions. The small-copy-number plasmid pJEBAC1 containing the bacteriocin 28b structural gene (*bss*) was constructed by ligation of a 5.8-kb *Hin*dIII-*Bgl*II fragment from pBA289 to pJE256 double digested with *Hin*dIII and *Bam*HI.

Plasmid pSF921, containing a *bss-lacZ* transcriptional fusion, was constructed by ligation of an *Eco*RI-*Eco*RI fragment from pBA391, containing 330 bp upstream of and 546 bp downstream of the initiation codon of the *bss* gene, to *Eco*RI-digested pRS550. A 8.6-kb *Hin*dIII-*Sal*I fragment, containing the *bss-lacZ* transcriptional fusion, was ligated to *Hin*dIII-*Sal*I double-digested pACYC184 to obtain pSF931.

Plasmid pLT103, containing a *recA-lacZ* transcriptional fusion, was constructed by ligation of a 1.3-kb *Bam*HI-*Pst*I fragment from plasmid pUA44, containing the promoter region and the first 79 codons of the *E. coli recA* gene, to *Bam*HI-*Pst*I double-digested pSK to obtain pLT101. A 1.3-kb *Eco*RI-*Bam*HI fragment, containing the promoter region and the first 79 codons of *recA*, was ligated to *Eco*RI-*Bam*HI double-digested pRS550 to obtain plasmid pLT102. A 9.3-kb *Hin*dIII-*Sal*I fragment containing the *recA-lacZ* transcriptional fusion was ligated to *Hin*dIII-*Sal*I double-digested pACYC184 to obtain pLT103.

The kanamycin resistance gene of Supercos 1 vector DNA (Stratagene) was deleted by *Pvu*II digestion and ligation to obtain kanamycin-sensitive Supercos 2.

Plasmid pKR941, containing *S. marcescens* 2170 chromosomal DNA adjacent

TABLE 2. Oligonucleotide primers

Primer	Oligonucleotide	Use
pTn5-4	5′-GGTTCCGTTCAGGACGCTA-3′	Sequencing
pID-1	5′-TAAAAGCCTGCAGATCTT-3′	Sequencing
pID-2	5′-ATGGATTTGGTCGACGAT-3′	Sequencing
pID-3	5′-CTGGGTTTACGTAAACGG-3′	Sequencing
pID-4	5′-AGACGCTTACCATCAATG-3′	Sequencing
pIR-1	5′-CGAGCCAGGCGAGAAACA-3′	Sequencing, PCR
pIR-2	5′-GGCGCTGCACTGGTAAGG-3′	Sequencing
pIR-3	5′-CACATTGAAAGCGAAAGC-3′	Sequencing
pIR-4	5′-CAGTGCATCATAAGAACC-3′	Sequencing
pIR-5	5′-CACGTTGCATTTGAGAGG-3′	Sequencing
pDR-1	5′-GCGCCGAAGAACACCGAC-3′	Sequencing
pPCR1	5′-TCGAAGCCATCATTGATG-3′	PCR

to the left *reg*::Tn*5* junction, was obtained by ligation of a 4.0-kb *Bam*HI-*Bam*HI DNA fragment from recombinant cosmid CosPD3 to *Bam*HI-digested pSK. The ligation mixture was transformed into *E. coli* XL1blue and plated on TSA containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml).

Plasmid pBRK6, containing *S. marcescens* 2170 chromosomal DNA adjacent to the right Tn*5*::*reg* junction, was obtained by ligation of a 5.1-kb *Bam*HI-*Bam*HI DNA fragment from recombinant cosmid CosPD3 to *Bam*HI-digested pBR328. Upon transformation, clones containing plasmid pBRK6 were identified by colony hybridization with a 1.15-kb *Bam*HI-*Bgl*II Tn*5*-labelled fragment as a probe.

Plasmid pKSA10, containing the last 101 *regB* codons and the complete *regC* gene under the control of the *lac* promoter, was constructed in two steps. A 1.1-kb *Pst*I-*Pst*I fragment from plasmid pBRK6 was ligated to *Pst*I-digested pSK to obtain pSKA01 (P_{lac}←*regC* Δ*regB*). A 0.9-kb *Bam*HI-*Cla*I fragment from pSKA01 was ligated to *Bam*HI-*Cla*I double-digested pKS to obtain pKSA10 (P_{lac}→Δ*regB regC*).

Plasmid pGPR100 (P_{lac}←P_{reg} Δ*regA*) was constructed by ligation of a 282-bp DNA fragment obtained by PCR amplification of *S. marcescens* 2170 DNA to plasmid pGEM-T. The 282-bp insert contains the first 34 codons of *regA* and 179 bp upstream of that *regA* start codon (P_{reg}); the 179-bp DNA fragment has similarity to *E. coli* promoters and contains an SOS box sequence. Plasmid pGPR001 contains the same 282-bp fragment inserted in the opposite orientation (P_{lac}→P_{reg} Δ*regA*).

Plasmid pPRCAT, containing a P_{reg}-*cat* operon fusion, was constructed by ligation of a 0.8-kb *Cla*I-*Pst*I fragment, containing the *cat* gene without its promoter region from pSKCAT, to *Cla*I-*Pst*I double-digested pGPR100.

Plasmid pOP950, containing the *regA*, *regB*, and *regC* genes and 179 bp of the wild-type upstream region, was constructed as follows. A 2.3-kb *Eco*RV-*Bam*HI fragment from pBRK6 containing the *regA*, *regB*, and *regC* genes from *S. marcescens* 2170 was purified, treated with the Klenow enzyme, and ligated to plasmid pGPR100 digested with *Eco*RV. This construction (pOP950) was successfully transformed only into *E. coli* $RecA^-$ strains and was unstable in $RecA^+$ strains. To obtain plasmid pOP951, we subcloned the complete construction (P_{reg}→*regA regB regC*) as a 2.4-kb *Aat*II-*Sma*I restriction fragment into pBR328 digested with *Aat*II and *Eco*RV. This new plasmid was maintained in both $RecA^-$ and $RecA^+$ strains.

Isolation of *S. marcescens reg*::Tn*5* mutant. Tn*5* transposon mutagenesis of *S. marcescens* 2170 with lambda 467 phage (λ::Tn*5*) was carried out as previously described (37). Kanamycin (25 μg/ml)-resistant colonies were screened for lower levels of bacteriocin production upon mitomycin treatment by using the agar overlay test (40). The mutation was transferred by 3M phage transduction to a clean *S. marcescens* 2170 background as previously described (41).

Bacteriocin production and sensitivity assay. Bacteriocin production was determined in TSB medium, as previously described (56). Mitomycin (Boehringer Mannheim) was added to a final concentration of 1 μg/ml in induction experiments. Cells were harvested 2.5 h later, washed, and sonicated in 0.05 M phosphate buffer (pH 8.0) containing 1 M NaCl. Bacteriocin 28b production was tested by the spot test (40) with a suitable *E. coli* sensitive strain. A unit of bacteriocin activity (AU) was defined as the reciprocal of the last dilution still able to inhibit the growth of *E. coli* in the spot test.

CAT and β-galactosidase enzyme assays. Cells containing transcriptional fusion plasmids were grown as described for bacteriocin 28b production. Chloramphenicol acetyltransferase (CAT) was assayed by using an enzyme-linked immunosorbent assay kit following the instructions of the manufacturer (Boehringer Mannheim). β-Galactosidase was assayed by the method described by Miller (34). Protein concentrations were determined by the Lowry method (31).

Construction of an *S. marcescens* 2170 *reg*::Tn*5* genomic library. *S. marcescens* 2170 *reg*::Tn*5* genomic DNA was isolated and partially digested with *Sau*3A as described by Sambrook et al. (43). Supercos 2 was first digested with *Xba*I, dephosphorylated, digested with *Bam*HI, and then ligated to *Sau*3A genomic DNA fragments. DNA packaging by using Gigapack Gold II (Stratagene) and infection of *E. coli* NM554 were carried out following the manufacturer's instructions.

PCR amplification and DNA sequencing. Primers used for PCR and DNA sequencing (Table 2) were purchased from Pharmacia. Double-stranded DNA sequencing was performed with 5′-[α-^{35}S]deoxyadenosine thiotriphosphate (NEN-Dupont), by using the Sanger dideoxy-chain termination method (44), according to the instructions included in the T7 DNA sequencing kit (Pharmacia LKB Biotechnology). Compressions were resolved by using the deaza T7 sequencing mixes (Pharmacia LKB Biotechnology).

DNA and protein sequence analyses. The DNA sequence was translated in all six frames, and all open reading frames (ORFs) greater than 100 bp were inspected. Deduced amino acid sequences were compared with those of DNA translated in all six frames from the nonredundant GenBank version 76 and EMBL version 34 databases by using the BLAST network service at the National Center of BioTechnology Information (1). Multiple sequence alignments and determination of possible terminator sequences were done by using the PileUp and Terminator programs from the Genetics Computer Group package in a VAX 4300. Hydropathy profiles were calculated according to the method of Kyte and Doolite (27). The TopPredII program (10) was used to construct models of P21 S protein and *regA* gene product disposition in the cytoplasmic membrane.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article have been submitted to GenBank and have been assigned accession no. U31763.

RESULTS

Mitomycin treatment induces both bacteriocin 28b production and bacteriocin 28b structural gene (*bss*) transcription in *S. marcescens* but not in *E. coli*. Mitomycin treatment (1 μg/ml) increased the bacteriocin 28b production about 10-fold in cultures of wild-type *S. marcescens* 2170 but not in *E. coli* MC1061 harboring the bacteriocin 28b structural gene (*bss*) in plasmid pJEBAC1 (Table 3).

To test the effect of mitomycin on *bss* gene transcription, a *bss-lacZ* transcriptional fusion was constructed. As shown in Table 3, mitomycin treatment increased the β-galactosidase activity about 16-fold in cultures of *S. marcescens* 2170 but not in an *E. coli* MC1061 ($RecA^+$) background.

These results suggested an SOS-dependent regulation of *bss* gene transcription in *S. marcescens* 2170, similar to that described for colicins. However, the lack of mitomycin inducibility of bacteriocin 28b production and β-galactosidase activity in *E. coli* MC1061 and the absence of an SOS box in the 330-bp sequence upstream of the *bss* gene (Fig. 1) (54) suggested the

TABLE 3. Effect of mitomycin on bacteriocin 28b production determined by bacteriocinogenic specific activity and β-galactosidase activity of a *bss-lacZ* transcriptional fusion

Strain	Bacteriocinogenic sp act (AU/mg)		β-Galactosidase activity[a] (Miller units)	
	Mitomycin		Mitomycin	
	−	+	−	+
S. marcescens 2170	26.2	273.8	≤25	≤25
+ pSF931 (*bss-lacZ* transcriptional fusion)	ND[b]	ND	250	4,125
S. marcescens 2170 *reg*::Tn*5*	163.8	189.3	≤25	≤25
+ pSF931 (*bss-lacZ* transcriptional fusion)	ND	ND	420	430
E. coli MC1061	NDe[c]	NDe	NDe	NDe
+ pJEBAC1 (containing *bss*)	152	145	NDe	NDe
+ pSF931 (*bss-lacZ* transcriptional fusion)	NDe	NDe	215	210

[a] Values are the means for three independent experiments.
[b] ND, not determined.
[c] NDe, no activity detected.

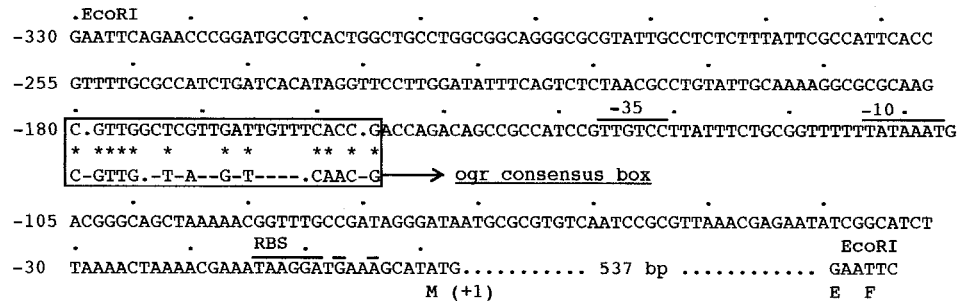


FIG. 1. Nucleotide sequence of 330-bp upstream region *bss* initiation codon showing the putative −10 and −35 *bss* promoter regions (54, 56) (EMBL nucleotide sequence no. X62454). Nucleotides (−180 to −156) similar to the proposed consensus sequence for phage P2 Ogr and phage P4 Ogr-like δ controlled promoters (20, 51) are shown inside the box. Asterisks denote identical nucleotides.

existence of regulatory proteins other than LexA and RecA linking SOS induction to *bss* gene expression in *S. marcescens.*

Isolation and characterization of an *S. marcescens* regulatory mutant. To study *bss* gene transcription regulation, *S. marcescens* 2170 cultures were mutagenized by using transposon Tn*5*. Kanamycin-resistant mutants were screened for the absence of or low levels of bacteriocin production by the agar overlay method upon mitomycin treatment. One mutant, termed *S. marcescens* 2170 *reg*::Tn*5*, that did not show increased levels of bacteriocin 28b production or of β-galactosidase activity, when harboring the *bss-lacZ* transcriptional fusion, upon mitomycin treatment was found. Both the levels of bacteriocin production and β-galactosidase activities were higher in the mutant than in the uninduced wild-type strain (Table 3). Thus, the behavior of the *S. marcescens reg*::Tn*5* mutant in response to mitomycin treatment resembled that found in the *E. coli* MC1061 background.

Both *S. marcescens* 2170 *reg*::Tn*5* and its parent strain showed similar levels of methyl methanesulfonate sensitivity and of *recA* gene transcription upon mitomycin induction, as determined with a reporter *E. coli recA-lacZ* transcriptional fusion in plasmid pLT103 (data not shown). Furthermore, the *E. coli recA* gene on plasmid pUA44 was not able to complement the mutant phenotype. These results strongly suggested that neither RecA nor LexA functions were altered in the *S. marcescens* 2170 *reg*::Tn*5* mutant.

Cloning and DNA sequencing of the regulatory mutation. A genomic library of the *S. marcescens* 2170 *reg*::Tn*5* chromosomal DNA was constructed and introduced into *E. coli* NM554. Kanamycin (25 μg/ml)-resistant clones were selected, and recombinant DNA was isolated and characterized by restriction enzyme digestion. Two *Bam*HI-*Bam*HI fragments from recombinant cosmid CosPD3, with sizes of 4 and 5.1 kb, hybridized with a 3.2-kb *Hin*dIII-*Hin*dIII Tn*5* internal probe (2) in Southern blot experiments. The 4-kb DNA fragment was subcloned in pSK to obtain pKR941. The 5.1-kb DNA fragment was unstable in *E. coli* when subcloned in pSK. Plasmid pBRK6, containing the 5.1-kb DNA fragment in pBR328, could be stably maintained only when *E. coli* clones were grown on TSA plates containing 0.2% glucose or other sugars such as maltose, suggesting the presence of lethal genes when overexpressed in TSA without 0.2% sugar.

With oligonucleotide pTn5-4 (Table 2), matching the Tn*5* ends, the nucleotide sequence of Tn*5* flanking DNA was initiated from pKR941 and pBRK6 and extended up to 1,436 bp by using sequence-derived oligonucleotides (Table 2 and Fig. 2). Analysis of this sequence showed only a potential promoter region, located upstream of the Tn*5* insertion point, similar to the *E. coli* promoter regions (24). The sequence TACTGTATGCATATACAGTA, highly similar to the SOS box consensus sequence (57), was found overlapping the proposed −10 region, suggesting that this promoter-like region could be LexA regulated. Three potential ORFs were found downstream from the Tn*5* insertion point (Fig. 2). The beginning of ORF2 (*regB*) overlapped the end of ORF1 (*regA*). ORF3 (*regC*) was found 68 bp downstream from ORF2. Putative ribosome-binding sites were found between 5 and 13 nucleotides upstream of these genes (Fig. 2). Only a potential Rho-independent terminator was identified downstream of the *regC* gene. These results suggest that the *regA*, *regB*, and *regC* genes are cotranscribed.

Analysis of RegA-, RegB-, and RegC-deduced amino acid sequences. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of plasmid pOP951 gene products, by using an *E. coli* S30 coupled transcription/translation system (Amersham) and [^{35}S]methionine (Amersham), revealed polypeptides with sizes of about 10, 19, and 8 kDa (data not shown) in close agreement to the sizes of the expected *regB*, *regA*, and *regC* gene products.

Computer database searching showed similarities among the deduced 88-amino-acid *regA* gene product, the *S. marcescens* SM6 deduced *nucD* gene product (GenBank U11698), and the bacteriophage P21 S protein (7). Amino acid alignment of these three proteins (Fig. 3A) showed that RegA and NucE are nearly identical, with a difference of only two amino acids. A lower degree of similarity was found between the RegA and S proteins (Fig. 3A). Bacteriophage P21 S protein belongs to a class of phage proteins known as holins (58). Models for the disposition of the S protein and the *regA* gene product in the cytoplasmic membrane showed that both proteins appeared to have two transmembrane helical domains and charge-rich N- and C-terminal domains presumed to be cytoplasmic anchors. These similarities suggest similar roles for both proteins.

The deduced 179-amino-acid *regB* gene product was nearly identical to the deduced amino acid sequence from the *S. marcescens* SM6 *nucD* gene (GenBank U11698) and was also similar to the family of bacteriophage lysozymes. Residues universally conserved in the T4 lysozyme gene family (58) were found in the RegB protein (Fig. 3B).

The deduced 75-amino-acid *regC* gene product was similar to the *S. marcescens* SM6 *nucC* gene product (GenBank nucleotide sequence accession no. U11698), the P2 bacteriophage Ogr transcriptional activator protein, and related proteins from bacteriophages P4, Φ-R73, and 186 (9) (Fig. 3C). These phage proteins are involved in transcriptional activation of bacteriophage late expression genes (9). A Cys4-coordinated Zn^{2+} DNA binding domain has been described for the Ogr protein (28), and a similar four-cysteine-residue motif was

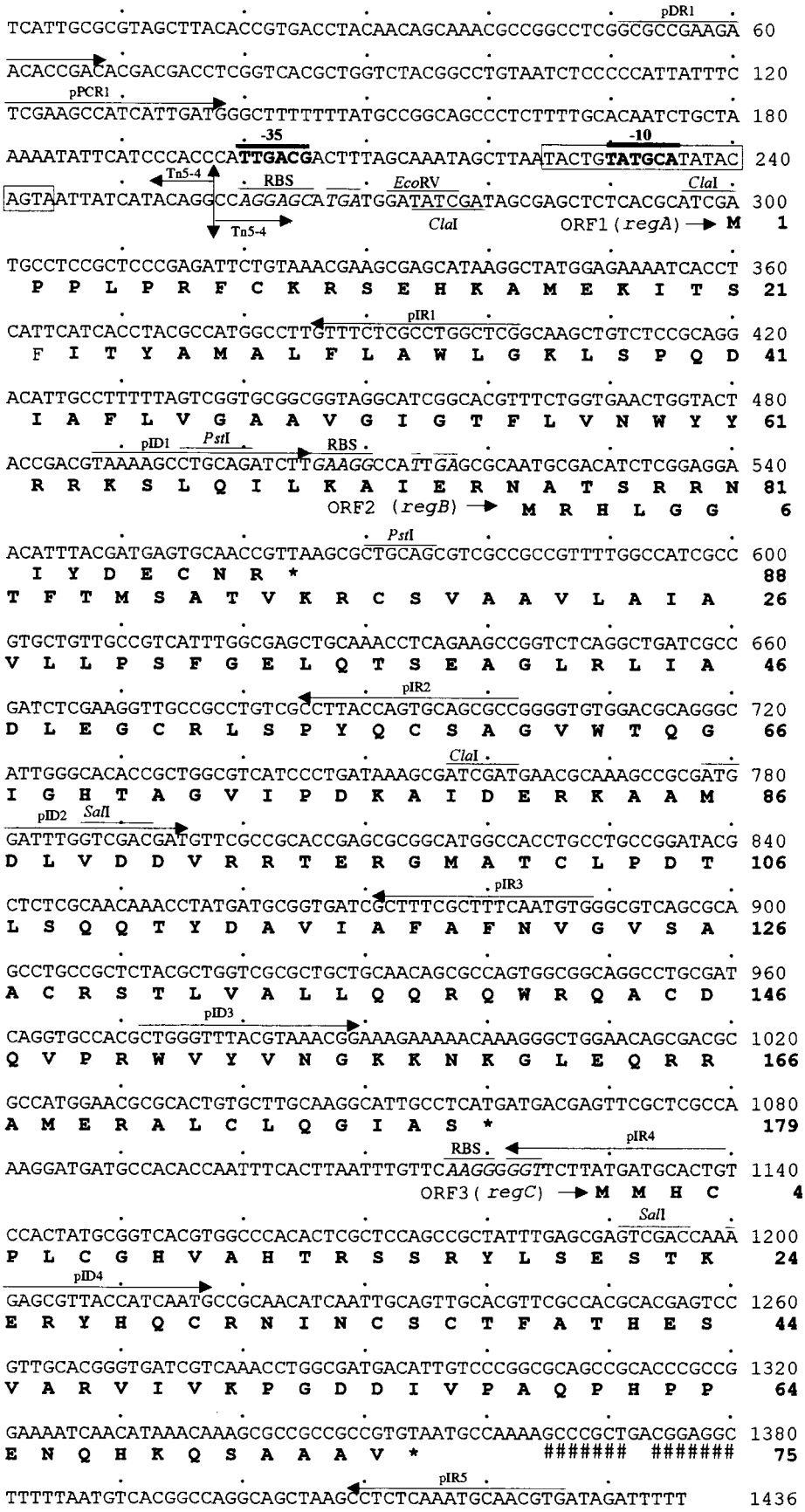
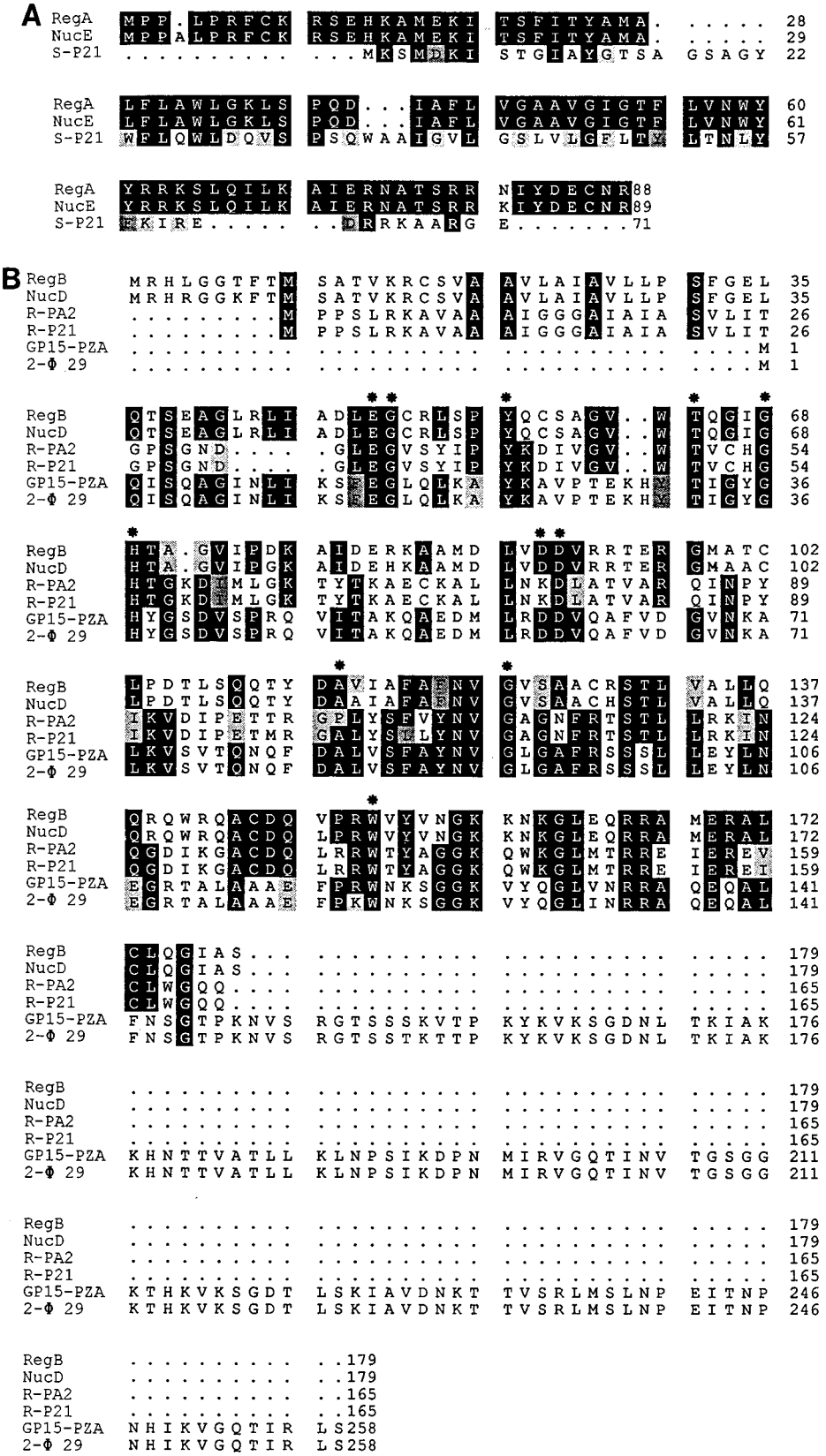


FIG. 2. DNA nucleotide sequence of *reg* region. The deduced *regA*, *regB*, and *regC* gene products are indicated below the nucleotide sequence. Potential ribosome-binding sites (RBS) and putative −35 and −10 promoter regions are shown in bold letters. A sequence with a high degree of similarity to the SOS consensus sequence (57) is boxed. Arrows indicate oligonucleotides used for sequencing and/or PCR. Putative Rho-independent terminators (#) are also indicated.


A
RegA MPP.LPRFCK RSEHKAMEKI TSFITYAMA. 28
NucE MPPALPRFCK RSEHKAMEKI TSFITYAMA. 29
S-P21 ....MKSMDKI STGIAYGTSA GSAGY 22
RegA LFLAWLGKLS PQD...IAFL VGAAVGIGTF LVNWY 60
NucE LFLAWLGKLS PQD...IAFL VGAAVGIGTF LVNWY 61
S-P21 WFLQWLDQVS PSQWAAIGVL GSLVLGFLTY LTNLY 57
RegA YRRKSLQILK AIERNATSRR NIYDECNR88
NucE YRRKSLQILK AIERNATSRR KIYDECNR89
S-P21 RKIRE..... ..DRRKAARG E.....71
B
RegB MRHLGGTFTM SATVKRCSVA AVLAIAVLLP SFGEL 35
NucD MRHRGGKFTM SATVKRCSVA AVLAIAVLLP SFGEL 35
R-PA2 ....M PPSLRKAVAA AIGGGAIAIA SVLIT 26
R-P21 ....M PPSLRKAVAA AIGGGAIAIA SVLIT 26
GP15-PZA ....M 1
2-Φ 29 ....M 1
RegB QTSEAGLRLI ADLEGCRLSP YQCSAGV..W TQGIG 68
NucD QTSEAGLRLI ADLEGCRLSP YQCSAGV..W TQGIG 68
R-PA2 GPSGND.... .GLEGVSYIP YKDIVGV..W TVCHG 54
R-P21 GPSGND.... .GLEGVSYIP YKDIVGV..W TVCHG 54
GP15-PZA QISQAGINLI KSTEGLQLKA YKAVPTEKHW TIGYG 36
2-Φ 29 QISQAGINLI KSTEGLQLKA YKAVPTEKHW TIGYG 36
RegB HTA.GVIPDK AIDERKAAMD LVDDVRRTER GMATC 102
NucD HTA.GVIPGK AIDEHKAAMD LVDDVRRTER GMAAC 102
R-PA2 HTGKDTMLGK TYTKAECKAL LNKDLATVAR QINPY 89
R-P21 HTGKDTMLGK TYTKAECKAL LNKDLATVAR QINPY 89
GP15-PZA HYGSDVSPRQ VITAKQAEDM LRDDVQAFVD GVNKA 71
2-Φ 29 HYGSDVSPRQ VITAKQAEDM LRDDVQAFVD GVNKA 71
RegB LPDTLSQQTY DAVIAFAENV GVSAACRSTL VALLQ 137
NucD LPDTLSQQTY DAAIAFAENV GVSAACHSTL VALLQ 137
R-PA2 IKVDIPETTR GPLYSFVYNV GAGNFRTSTL LRKIN 124
R-P21 IKVDIPETMR GALYSTLYNV GAGNFRTSTL LRKIN 124
GP15-PZA LKVSVTQNQF DALVSFAYNV GLGAFRSSSL LEYLN 106
2-Φ 29 LKVSVTQNQF DALVSFAYNV GLGAFRSSSL LEYLN 106
RegB QRQWRQACDQ VPRWVYVNGK KNKGLEQRRA MERAL 172
NucD QRQWRQACDQ LPRWVYVNGK KNKGLEQRRA MERAL 172
R-PA2 QGDIKGACDQ LRRWTYAGGK QWKGLMTRRE IEREV 159
R-P21 QGDIKGACDQ LRRWTYAGGK QWKGLMTRRE IERET 159
GP15-PZA EGRTALAAAE FPRWNKSGGK VYQGLVNRRA QEQAL 141
2-Φ 29 EGRTALAAAE FPKWNKSGGK VYQGLINRRA QEQAL 141
RegB CLQGIAS... 179
NucD CLQGIAS... 179
R-PA2 CLWGQQ.... 165
R-P21 CLWGQQ.... 165
GP15-PZA FNSGTPKNVS RGTSSSKVTP KYKVKSGDNL TKIAK 176
2-Φ 29 FNSGTPKNVS RGTSSTKTTP KYKVKSGDNL TKIAK 176
RegB 179
NucD 179
R-PA2 165
R-P21 165
GP15-PZA KHNTTVATLL KLNPSIKDPN MIRVGQTINV TGSGG 211
2-Φ 29 KHNTTVATLL KLNPSIKDPN MIRVGQTINV TGSGG 211
RegB 179
NucD 179
R-PA2 165
R-P21 165
GP15-PZA KTHKVKSGDT LSKIAVDNKT TVSRLMSLNP EITNP 246
2-Φ 29 KTHKVKSGDT LSKIAVDNKT TVSRLMSLNP EITNP 246
RegB 179
NucD 179
R-PA2 165
R-P21 165
GP15-PZA NHIKVGQTIR LS258
2-Φ 29 NHIKVGQTIR LS258

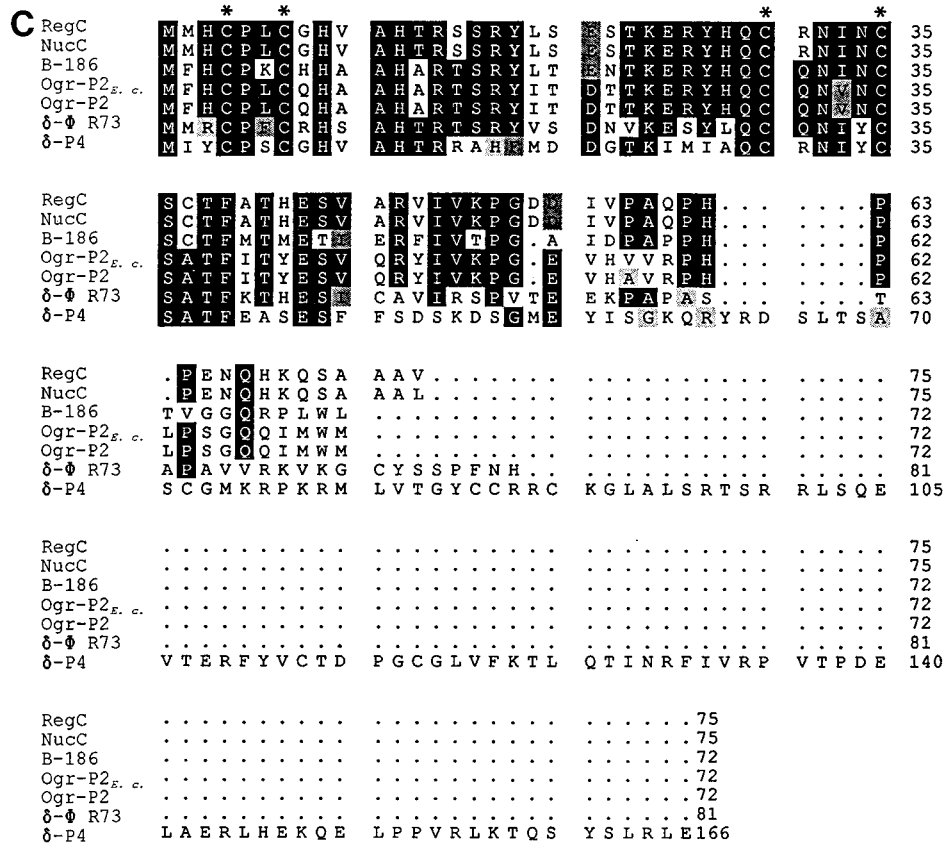


FIG. 3. Amino acid alignment among deduced *regA*, *regB*, and *regC* gene products and similar proteins. White letters in black boxes denote identical residues; black letters in grey boxes denote similar residues. (A) Amino acid alignment among deduced *regA* gene product, *S. marcescens* SM6 deduced *nucE* gene product (GenBank nucleotide sequence accession no. U11698), and phage P21 protein S (7). (B) Amino acid alignment among deduced *regB* gene product, *S. marcescens* SM6 deduced *nucD* gene product (GenBank nucleotide sequence accession no. U11698), phage PA2 protein R (6), phage P21 protein R (7), phage PZA protein GP15 (36), and phage Φ29 protein 2 (19). Asterisks denote residues universally conserved in the T4 lysozyme gene family. (C) Amino acid alignment among deduced *regC* gene product, *S. marcescens* SM6 deduced *nucC* gene product (GenBank nucleotide sequence accession no. U11698), phage B protein 186 (26), phage P2 protein Ogr in *E. coli* (*E. c.*) (4, 47), phage P2 protein Ogr (5), phage Φ-R73 protein δ (49), and phage P4 protein δ (22). Asterisks denote conservated Cys residues, probably defining a zinc finger domain.

found in the RegC protein (Fig. 3C). The similarity among RegC, Ogr, and Ogr-like proteins and the presence upstream from the *bss* gene of a sequence similar to the proposed consensus sequence of late expression phage genes activated by the Ogr and Ogr-like δ proteins (20, 51) (Fig. 1) suggested that the RegC protein could be involved in the *bss* gene transcriptional regulation.

Role of *regC* in *bss* gene expression. To test the above hypothesis, recombinant plasmids containing the last 101 codons of the *regB* gene and the complete *regC* gene were used. Expression of the *regC* gene was under the control of the vector *lac* promoter or dependent on possible promoters present upstream of the *regC* gene in pKSA10 or pSKA01, respectively. β-Galactosidase activity was determined from cultures with and without 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) induction of *S. marcescens* 2170, *S. marcescens* 2170 *reg*::Tn*5*, *E. coli* MC1061(RecA$^+$), and *E. coli* XL1blue (RecA$^-$), harboring a *bss-lacZ* transcriptional fusion in pSF931 and either pKSA10 or pSKA01. Thirty- to 40-fold increases in β-galactosidase activities were found in both the mutant and parent *S. marcescens* strains when the *regC* gene was under the control of the vector *lac* promoter (plasmid pKSA10) but not when the *regC* gene was in an orientation opposite that of the *lac* promoter (pSKA01). Similar results were found in *E. coli* MC1061 (RecA$^+$) and XL1blue (RecA$^-$), suggesting that the *regC* gene product was able to induce *bss* gene transcription in both *S. marcescens* and *E. coli* backgrounds (Fig. 4). No increase in β-galactosidase activity was observed in IPTG-treated cultures, suggesting that *regC* basal level transcription from the *lac* promoter in plasmid pKSA10 produced enough RegC protein to saturate the *bss-lacZ* reporter system.

RegC expression is SOS regulated. Analysis of the nucleotide sequence shown in Fig. 2 suggested that the *regA*, *regB*, and *regC* genes were cotranscribed under the control of a LexA-repressed promoter. To test this hypothesis, recombinant plasmids pOP950 and pOP951 containing the *regA*, *regB*, and *regC* genes and the 179-bp wild-type DNA fragment upstream from the *regA* gene were used. Plasmid pOP950, based in high-copy-number vector pGEM-T, was successfully transformed in *E. coli* XL1blue (RecA$^-$) but not in *E. coli* MC1061 (RecA$^+$). Plasmid pOP951 was transformed into both RecA$^+$ and RecA$^-$ strains of *E. coli* and *S. marcescens* harboring a *bss-lacZ* transcriptional fusion on plasmid pSF931. Cultures of these strains treated with mitomycin showed a twofold increase in β-galactosidase activity only in the RecA$^+$ background, and not in the RecA$^-$ background, of both *E. coli* (Table 4) and *S. marcescens* (data not shown). Furthermore, plasmid pOP951 complemented the mutant *S. marcescens reg*::Tn*5* (Table 4). These results suggested that *regC* gene transcription was de-

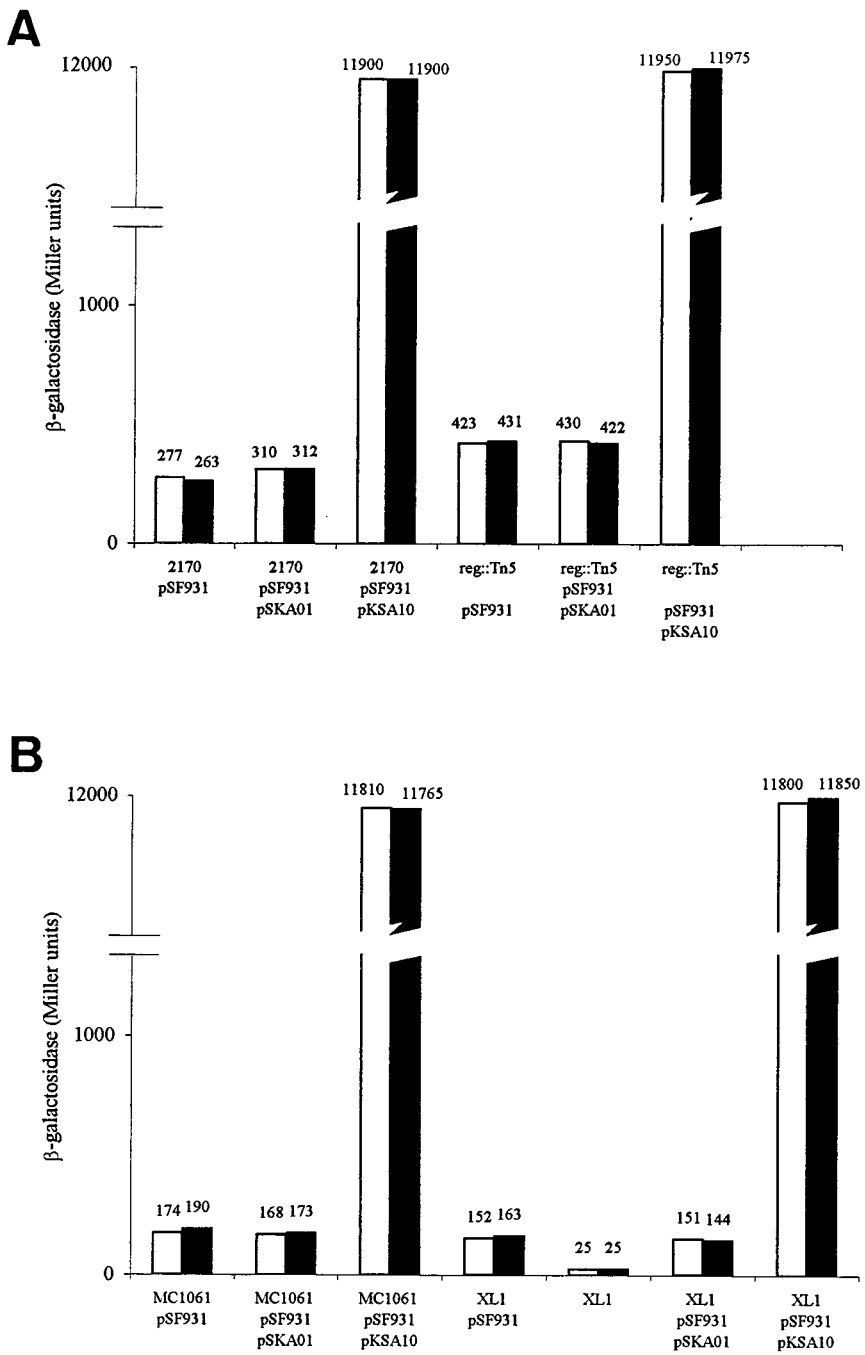


FIG. 4. Effect of multicopy plasmids containing *regC* gene (plasmids pKSA10 and pSKA01) on β-galactosidase activity of a *bss-lacZ* transcriptional fusion (on plasmid pSF931) in *S. marcescens* (A) and *E. coli* (B) strains. Cultures with (closed bars) or without (open bars) IPTG induction.

pendent on a LexA-controlled promoter located upstream of the *regA* gene.

To test the SOS-dependent inducibility of the possible *reg* promoter, plasmid pPRCAT, containing a transcriptional fusion between a 159-bp wild-type DNA fragment (nucleotides 121 to 279 in Fig. 2) containing an SOS-like sequence and the *cat* gene, was used. Twofold-higher levels of CAT activity (Table 5) were obtained in both *E. coli* and *S. marcescens* $RecA^+$ strains harboring plasmid pPRCAT upon mitomycin treatment. The levels of CAT activity were lower in $RecA^-$ strains of both *E. coli* and *S. marcescens* harboring plasmid pPRCAT, and no increase in CAT activity was found with mitomycin treatment (Table 5). This is the behavior expected for SOS-controlled genes, and similar results have been previously found with a *recA-lacZ* transcriptional fusion in several gram-negative bacteria (16). These results suggest that the *regA* upstream region contains a LexA-regulated promoter.

DISCUSSION

The data reported in this work show that both bacteriocin 28b production and bacteriocin 28b gene transcription, deter-

TABLE 4. Effect of plasmid pOP951 (containing the wild-type *reg* region) on β-galactosidase activity of a *bss-lacZ* transcriptional fusion (on plasmid pSF931) in *E. coli* and *S. marcescens*

Strain	β-Galactosidase activity[a] (Miller units)	
	Without mitomycin	With mitomycin
E. coli MC1061 (pSF931, pOP951) (Rec^+)	9,410	16,838
E. coli XL1blue (pSF931, pOP951) (Rec^-)	1,023	983
S. marcescens 2170 (pSF931, pOP951)	11,155	22,592
S. marcescens 2170 *reg*::Tn*5* (pSF931, pOP951)	10,998	21,931

[a] Values are the means for three independent experiments.

mined with a *bss-lacZ* transcriptional fusion, are increased in *S. marcescens* upon mitomycin treatment. This behavior is similar to that found with other bacteriocins and colicins (38). For all the colicin structural genes hereto characterized, transcription is directly repressed by the LexA protein, this repression being abolished by induction of the SOS system (38). Concerning bacteriocin 28b, on the basis of (i) the lack of an SOS-like sequence in the promoter region of the *bss* gene, (ii) the isolation of an uninducible *S. marcescens reg*::Tn*5* mutant phenotypically different from *recA* and *lexA* mutants, and (iii) the uninducibility of bacteriocin 28b production and *bss* gene transcription in *E. coli* MC1061 ($RecA^+$), we suggest that mitomycin induction of the bacteriocin 28b production is not based on direct LexA repression of the *bss* gene transcription.

DNA sequence analysis showed that the *S. marcescens reg*::Tn*5* mutant phenotype is due to a Tn*5* insertion in a promoter-like region controlling the apparently cotranscribed *regA*, *regB*, and *regC* genes. The deduced amino acid sequence of the RegC protein is highly similar to that of the Ogr protein, a transcriptional activator for the bacteriophage P2 late expression genes (20). Furthermore, features similar to a proposed consensus DNA sequence for Ogr and Ogr-like δ protein DNA interaction (20, 51) are found in the promoter region of the *bss* gene (Fig. 1).

Evidence for a role of the *regC* gene in *bss* gene transcription activation was provided by the effect of multicopy plasmids, harboring the *regC* gene, on the β-galactosidase activity levels of a *bss-lacZ* transcriptional fusion. High levels of apparent *bss* gene transcription were obtained in the regulatory mutant *S. marcescens* 2170 *reg*::Tn*5*, the 2170 parent strain, and both $RecA^+$ and $RecA^-$ *E. coli* strains when the *regC* gene was expressed from the *lac* promoter. A multicopy plasmid containing 179 bp of wild-type DNA and the downstream apparently cotranscribed *regA*, *regB*, and *regC* genes was able to complement the *S. marcescens* 2170 *reg*::Tn*5* mutant and to induce a twofold increase in *bss* gene transcription only in $RecA^+$ strains of *E. coli*, thus suggesting that *regC* gene expression was under SOS control. Furthermore, an SOS sequence was found 64 bp upstream of the *regA* gene. A transcriptional fusion between this DNA region and the *cat* gene produced a twofold increase in CAT activity upon mitomycin treatment only in $RecA^+$ strains of both species, suggesting that the SOS box-containing sequence behaves as a LexA-controlled promoter. These results suggest that under normal growth conditions, the LexA protein would repress *regA*, *regB*, and *regC* gene expression and upon mitomycin induction the *regC* gene would be transcribed, leading to RegC-mediated transcription activation of the *bss* gene. This working model will be useful to define future experiments to test the proposed bacteriocin 28b regulation mechanism. Since in the mutant strain the Tn*5* insertion point is located 15 bp downstream of the SOS-like sequence, the nearly twofold difference in basal *bss* gene transcription between the mutant *S. marcescens reg*::Tn*5* and wild-type *S. marcescens* 2170 is probably due to *regC* transcription from a distal Tn*5* promoter.

TABLE 5. Effect of mitomycin on CAT activity of $RecA^+$ and $RecA^-$ strains of *E. coli* and *S. marcescens* harboring plasmid pPRCAT (a transcriptional fusion between the proposed *reg* promoter and *cat* gene)

Strain	RecA	CAT (U)[a]		
		Without mitomycin	With mitomycin	Induction factor
E. coli MC1061 (pPRCAT)	+	5,690	10,306	1.8
E. coli XL1blue (pPRCAT)	−	737	752	1.02
S. marcescens SM6 (pPRCAT)	+	1,918	3,875	2.07
S. marcescens SM6 *nucB*::Tn*5* (pPRCAT)	−	617	631	1.02

[a] Values are the means for three independent experiments.

It has been shown that *E. coli* K-12 and B have a chromosomal *ogr* gene, probably originated by an aberrant P2 prophage excision event (4, 47). No SOS-like sequences have been found in this chromosomal *ogr* promoter, explaining the lack of mitomycin inducibility of the *bss* gene transcription in *E. coli*, even if this *ogr* gene product could interact with the *bss* gene promoter.

The *regA* and *regB* gene products are similar to phage P21 holin S and bacteriophage lysozymes, respectively. Most bacteriophages use two proteins, a holin and a muramidase, to lyse infected cells during lytic growth (58). The genes coding for these two proteins are often adjacent, cotranscribed, and usually overlap (58), an organization similar to that found for the *regA* and *regB* genes. It has been proposed that the phage holins insert in the cytoplasmic membrane allowing the muramidase enzyme to reach the periplasm, where the enzyme will degrade the murein. Thus, the *regA* and *regB* genes could be remnants of an *S. marcescens* prophage. The probable holin and lysozyme natures of *regA* and *regB* gene products, respectively, would account for the difficulties found in subcloning *regA regB*-containing DNA fragments and suggest that the presence of 0.2% sugar in TSA culture medium protected pBRK6 harboring cells from osmotic lysis due to RegA and RegB protein production.

Upon standard mitomycin induction about one-third of the bacteriocinogenic activity was found bound to the *S. marcescens* cell surface, and higher levels of mitomycin induction (>1 μg/ml) caused culture lysis, although no gene involved in bacteriocin 28b release has been found nearby the *bss* gene in the *S. marcescens* chromosome (55). Bacteriocinogenic activity was detected only in the cytoplasmic fraction in *E. coli* strains harboring the *bss* gene on a recombinant plasmid (54, 55). Since a holin-lysozyme system is used by most bacteriophages to release its progeny from the infected cells (58), it is tempting to speculate that the *regA* and *regB* gene products could be somehow involved in bacteriocin 28b release in *S. marcescens.*

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ADDENDUM IN PROOF

After this article was submitted for publication, an article reporting the sequences for *nucE, nucD,* and *nucC* genes, nearly identical to *regA, regB,* and *regC* genes, was accepted (S. Jin, Y. Chen, G. E. Christie, and M. J. Benedik, J. Mol. Biol., in press). In this work, *nucC* is shown to be a positive regulatory gene for *Serratia marcescens* extracellular nuclease.

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