

## Development of a Thermally Regulated Broad-Spectrum Promoter System for Use in Pathogenic Gram-Positive Species

David A. Schofield,\* Caroline Westwater, Brian D. Hoel, Phillip A. Werner,  
James S. Norris, and Michael G. Schmidt

Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, South Carolina 29403

Received 10 February 2003/Accepted 25 March 2003

**Selectively regulating gene expression is an essential molecular tool that is lacking for many pathogenic gram-positive bacteria. In this report, we describe the evaluation of a series of promoters regulated by the bacteriophage P1 temperature-sensitive C1 repressor in *Enterococcus faecium*, *Enterococcus faecalis*, and *Staphylococcus aureus*. Using the *lacZ* gene to monitor gene expression, we examined the strength, basal expression, and induced expression of synthetic promoters carrying C1 operator sites. The promoters exhibited extremely low basal expression and, under inducing conditions, gave high levels of expression (100- to 1,000-fold induction). We demonstrate that the promoter system could be modulated by temperature and showed rapid induction and that the mechanism of regulation occurred at the level of transcription. Controlled expression with the same constructs was also demonstrated in the gram-negative bacterium *Escherichia coli*. However, low basal expression and the ability to achieve derepression were dependent on both the number of mismatches in the C1 operator sites and the promoter driving *c1* expression. Since the promoters were designed to contain conserved promoter elements from gram-positive species and were constructed in a broad-host-range plasmid, this system will provide a new opportunity for controlled gene expression in a variety of gram-positive bacteria.**

The overuse of antibiotics has contributed to the emergence and increasing prevalence of antibiotic-resistant bacteria. Gram-positive cocci such as *Staphylococcus aureus* and enterococci are the leading cause of hospital-acquired infections (39). *S. aureus* causes a variety of infections ranging from localized skin suppuration to life-threatening septicemia. Alarming, *S. aureus* isolates resistant to vancomycin, the last effective antibiotic, are emerging worldwide (22). Enterococcus species are a leading cause of urinary tract infection, nosocomial infection, and surgical-wound infection (39). *Enterococcus faecalis* is responsible for the majority of enterococcal infections (26, 41) and, for the time being, usually remains sensitive to at least one antibiotic. In contrast *Enterococcus faecium*, which causes fewer infections, is more likely to be resistant to all antibiotics.

The recent emergence of antibiotic-resistant gram-positive bacteria has highlighted the need for genetic studies addressing the mechanism of bacterial pathogenesis. Regulated promoters are essential for the functional analysis of genes through expression studies (1) and reverse genetics. However, only a few regulated promoters are available for use in enterococci and streptococci. The tetracycline-regulated promoter system has been shown to function in *Streptococcus pneumoniae* (47), *Bacillus subtilis* (11), and *S. aureus* (1, 25). In addition, the xylose-inducible promoter system has been used for *B. subtilis* and staphylococci (27, 52, 56). However, the levels of regulation achieved with these systems are below those obtained for gram-negative bacteria (33), and tight basal expression is achieved at the expense of reduced inducibility (11, 56). The most studied regulated promoter from gram-

positive bacteria is the *nisA* promoter, derived from the *Lactococcus lactis* *nisin* gene cluster. For regulated expression, the system requires coexpression of histidine protein kinase NisK and response regulator NisR (6). Induction is achieved by the addition of subinhibitory levels of the lantibiotic nisin. Controlled gene expression over a 1,000-fold range in *L. lactis* has been demonstrated (4). However, more-modest regulation has been demonstrated in heterologous hosts (6) such as *E. faecalis* (20-fold), *Streptococcus agalactiae* (10-fold), and *Streptococcus pyogenes* (60-fold).

One of the reasons why there are fewer regulated promoters for gram-positive species may be the more stringent control of promoter usage in gram-positive species than in gram-negative species. Multiple conserved regions, in addition to the  $-35$  and  $-10$  hexamers, have been identified in promoters from gram-positive species (14, 20, 50, 51). Consequently, well-characterized promoters from gram-negative species such as  $P_{tac}$  and  $P_{trc}$  are inactive in gram-positive hosts even though they contain consensus  $-35$  and  $-10$  hexamers (38).

The temperate bacteriophage P1 can infect and lysogenize many gram-negative species (55). Stable lysogeny is maintained by the action of the components of the tripartite immunity system (17). The C1 repressor protein acts as a central regulator by controlling the expression of a variety of genes (3, 7, 18, 19) by binding to C1 asymmetric operator (7) sites (consensus sequence, ATTGCTCTAATAAATTT). A bacteriophage P1-derived promoter in conjunction with the temperature-sensitive C1 repressor (40) has been used to regulate gene expression in gram-negative bacteria (45). In this report we demonstrate that the P1 temperature-sensitive C1 repressor can be used to control gene expression by using synthetic promoters in the pathogenic gram-positive species *E. faecium*, *E. faecalis*, and *S. aureus*. We compare the strengths of the promoters in different species and show that the promoters

\* Corresponding author. Mailing address: Department of Microbiology and Immunology, BSB 201, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29403. Phone: (843) 792-7703. Fax: (843) 792-2464. E-mail: schofida@musc.edu.



FIG. 1. Construction of the temperature-sensitive C1-regulated promoter system. (A) Topography and sequences of the promoters. Determined based on compilation analysis, the conserved promoter nucleotides from gram-positive bacteria are in boldface (14, 20). The synthetic promoters (Pro1, -2, and -3) consist of two partially overlapping C1 operators (top and bottom strands; underlined sequences). Pro1 carries two C1 operator sites that match the 17-bp consensus (7, 18), while Pro2 and Pro3 deviate from the consensus by 1 and 5 nucleotides, respectively (large font). Pro2 differs from Pro1 by a single nucleotide in the  $-10$  hexamer (G versus the consensus T). Pro3 differs from Pro2 by 2 nucleotide changes in the spacer region (AT versus the consensus TG). ProA and ProB, which drive *c1* expression, differ in the nucleotide spacer sequence between the  $-35$  and  $-10$  hexamers. (B) Map of the reporter plasmid and its relevant features. The *lacZ* reporter gene was placed under the transcriptional control of a C1-regulated promoter (Pro1, -2, or -3; arrows denote direction). To control gene expression and to aid the binding of the repressor to its operator site, the temperature-sensitive C1 repressor and Bof modulator were cloned 3' of *lacZ* and placed under the transcriptional control of either ProA or -B. The reporter construct contains the p15A origin of replication, the origin of replication derived from pGB354, and the chloramphenicol (Cm) resistance markers from pACYC184 and pGB354 (53).

exhibit extremely low basal expression and that the control of regulation occurs at the level of transcription. Since the promoter system was constructed in a broad-host-range plasmid and contained conserved promoter elements, the system will provide a new opportunity for controlled gene expression in gram-positive bacteria.

#### MATERIALS AND METHODS

**Strains and media.** The bacterial strains used in this study were *E. coli* DH5 $\alpha$  ( $\phi$ 80*lacZ* $\Delta$ M15 *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR*  $\Delta$ (*lacZYA-argF*)U169; obtained from Gibco-BRL), *S. aureus* RN4220 (restriction-deficient strain kindly provided by Jean Lee, Channing Laboratory, Boston, Mass.), *E. faecalis* ATCC 47077 (designation OG1RF), and *E. faecium* ATCC 12952. The following growth media (Difco) were used: Luria-Bertani broth for *E. coli*, tryptic soy broth for *S. aureus*, brain heart infusion broth for *E. faecalis*, and Todd-Hewitt broth for *E. faecium*.

**Reporter plasmid construction.** The reporter plasmids were constructed in the gram-negative and gram-positive species shuttle vector pAM401 (53). The *lacZ* gene was amplified by PCR with pBHR*lacZ* (45) as the template and the upstream primer 5'-AGGACGGTCTGACTAAGGAGGTGAAAAGTATGGTCTTTTACAAGCTCG and downstream primer 5'-TCCTCCGCATGCTCCCCCTGCCCCGTTAT, which contained *SalI* and *SphI* restriction sites (underlined) for cloning into the *SalI* and *SphI* sites of pAM401. The upstream primer also contained a ribosome binding site (RBS; 5'-TAAGGAGG) positioned 8 bp upstream of a start codon (boldface) to initiate translation. The C1-regulated promoters (Fig. 1A; Pro1, -2, and -3) were obtained by annealing complementary oligonucleotides that contained partial and full *SalI* overhangs (5' and 3', respectively). The promoters were cloned (in the same orientation as *lacZ*) into the *SalI* site of the *lacZ* construct, thereby re-creating the 3' *SalI* site only. To increase the number of cloning sites, the oligonucleotides also contained a *SpeI* site at the 5' end. To reduce readthrough from cryptic promoters into the 5' end of the expression cassette, the TL<sub>17</sub> transcriptional terminators (54) were cloned into the *SpeI* site. To prevent "runaway" transcription, the terminators were also cloned at the 3' end of the expression cassette (*EcoRV* site). To control gene expression, the coding sequences for the *c1* repressor and *bof* modulator were inserted initially into the cloning vector pBluescript II SK(+) (Stratagene). The forward PCR primers used to amplify *c1* and *bof* contained both an RBS and restriction endonuclease site. To incorporate both of these features, *c1* and *bof* were amplified by a seminested-PCR strategy. *c1* was amplified by using the

thermosensitive P1 mutant as the template (40) (kindly provided by Michael Yarmolinsky, National Institutes of Health, Bethesda, Md.) with the forward primer 5'-TAAGGAGGTGAAAAGTATGATAAATTATGTCTACGGC and the reverse primer 5'-CTAGCTGAATTCCTATTGCGCGCTTTCGGGGTTG. After 10 amplification cycles, an aliquot (1  $\mu$ l) was reamplified with the forward nested primer 5'-CGCAGTGAATTCCTAAGGAGGTGAAAAGTATG and the same reverse primer. The forward primers each contained an RBS upstream of the start codon (boldface), and the reverse primer and the latter forward primer contained *EcoRI* restriction sites (underlined sequences) for cloning into the corresponding sites of pBluescript II SK(+). Similarly, the forward primer 5'-TAAGGAGGTGAAAAGTATGAAAAGCGGATACTACACAG, reverse primer 5'-GTAGTAGCATGCGGTGAGCAACAGCCAT, and nested forward primer 5'-GCTAGGAAGCTTTAAGGAGGTGAAAAGTATG were used to amplify *bof* with bacteriophage P1 DNA as the template. The *bof* primers contained *HindIII* and *SphI* sites (underlined). However, *bof* was cloned 3' of *c1* into the *HindIII* and *HincII* sites of pBluescript II SK(+). To drive expression of *c1* and *bof*, complementary oligonucleotides containing promoter elements (Fig. 1A; ProA and -B) were cloned upstream of *c1* and *bof* into the *BamHI* and *PstI* sites of pBluescript II SK(+). The promoter *c1.bof* fragments with *BamHI/SphI* overhangs were then cloned into the corresponding sites of pDAS101, pDAS111, and pDAS121 to create the final reporter constructs (Fig. 1B and Table 1).

**Transformation.** *E. coli* was transformed as described by Sambrook et al. (42). *E. faecalis* and *E. faecium* were electroporated as described by Friesenegger et al. (9) except cells were resuspended at 1/100 of their original culture volume. *S. aureus* was electroporated by the method described by Lee (31). Chloramphenicol was used to select for plasmids at the following concentrations: 25  $\mu$ g/ml, *E. coli*; 20  $\mu$ g/ml, *E. faecalis*; 5  $\mu$ g/ml, *E. faecium*; 15  $\mu$ g/ml, *S. aureus*.

**RNA extraction and slot blot hybridizations.** RNA was extracted from *E. faecium*, *E. faecalis*, and *S. aureus* by using the Qiagen RNeasy kit according to the manufacturer's instructions with the following modification: to break open the bacterial cells, the samples were vortexed continuously for 10 min in the presence of acid-washed glass beads (212 to 300  $\mu$ m). RNA (up to 10  $\mu$ g) was vacuum blotted onto Duralon UV membranes (Stratagene) with a slot blot apparatus (42). Two identical RNA blots were prepared for each species. Both membranes were probed with a <sup>35</sup>S-tailed (Roche) oligonucleotide complementary to either *lacZ* (5'-CGCTCAGGTCAAATTCAGACGGCAAACGA) or a conserved region of 16S rRNA (5'-CCAACATCTCACGACGACGCTGACGACAA). Hybridization was performed in a mixture containing 1 $\times$  Denhardt's solution, 4 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50  $\mu$ g of poly(A)/ml, 500  $\mu$ g of salmon sperm/ml, 10% dextran sulfate, and 45% formamide at 37°C. Washing was performed at 37°C at a final stringency of 0.5 $\times$

TABLE 1. Plasmids used in this study

Plasmid	Description and relevant characteristics	Origin or reference
pAM401	Gram-positive and -negative species shuttle vector	53
pDAS100	pAM401 containing <i>lacZ</i> and transcriptional terminators	This study
pDAS101	pDAS100 with Pro1 driving <i>lacZ</i>	This study
pDAS102	pDAS101 with ProA driving <i>c1</i> and <i>bof</i>	This study
pDAS103	pDAS101 with ProB driving <i>c1</i> and <i>bof</i>	This study
pDAS111	pDAS100 with Pro2 driving <i>lacZ</i>	This study
pDAS112	pDAS111 with ProA driving <i>c1</i> and <i>bof</i>	This study
pDAS113	pDAS111 with ProB driving <i>c1</i> and <i>bof</i>	This study
pDAS121	pDAS100 with Pro3 driving <i>lacZ</i>	This study
pDAS122	pDAS121 with ProA driving <i>c1</i> and <i>bof</i>	This study
pDAS123	pDAS121 with ProB driving <i>c1</i> and <i>bof</i>	This study

SSC and 0.1% sodium dodecyl sulfate (SDS). The membranes were visualized with a phosphorimager.

**Reporter gene assays.**  $\beta$ -Galactosidase ( $\beta$ -Gal) activity was assayed as described by Miller (35) except that the cells were permeabilized with 4 drops of chloroform and 2 drops of 0.1% SDS.

**Nucleotide sequence accession number.** The sequence of the reporter plasmid pDAS112 has been deposited in GenBank under accession no. AY230218.

## RESULTS AND DISCUSSION

**Rationale for and characteristics of the reporter gene constructs.** Controlled expression using a temperature-sensitive C1-regulated promoter system has been demonstrated previously for gram-negative bacteria (44, 45) by using a promoter responsible for driving *ban* gene expression in bacteriophage P1 (18). The *ban* promoter consisted of two overlapping C1 operator sites, and, although it did not contain consensus  $-35$  and  $-10$  hexamers, it was highly active in enteric gram-negative bacteria (43, 45). However, reporter gene analysis indicated that this promoter was inactive in the gram-positive species *E. faecalis* and *S. aureus* (data not shown). Functional promoters derived from gram-negative species are often inactive in gram-positive species (21, 30, 36, 38), which may reflect the fact that several conserved promoter elements (14, 20, 50, 51), in addition to the  $-35$  and  $-10$  hexamers, are required for activity. Consequently, compilation analysis of promoters from gram-positive species (14, 20) allowed three promoters (Pro1, -2, and -3) with conserved elements to be designed. The conserved elements consisted of the  $-35$  and  $-10$  hexamers, an A tract and T 5' of the  $-35$  hexamer, a TG dinucleotide 5' of the  $-10$  hexamer, and two A nucleotides 3' of the  $-10$  hexamer (Fig. 1A). The promoters differed by a single nucleotide within the  $-10$  hexamer (Pro1 versus Pro2) or by the addition of TG nucleotides (Pro2 versus Pro3; Fig. 1A). The promoters were also designed to contain two partially overlapping C1 operator sites (7). Placement of the C1 operators downstream of the  $-10$  hexamer resulted in only partial repression in the presence of C1 in *E. coli* (data not shown); consequently the operator on the top strand was placed between the  $-35$  and  $-10$  hexamers while the operator on the bottom strand completely covered the  $-10$  hexamer. In the presence of a repressor, this placement was expected to more effectively prevent transcription by exclusion of the RNA polymerase and/or by the masking of the promoter elements (18, 29). However, as a result of the optimization of promoter elements, Pro3 and Pro2 carried five and one mismatch to the consensus C1 operator sequence, respectively. Nevertheless, these operator sites were expected

to be effective since functional C1 binding sites containing mismatches to the consensus sequence have been identified throughout the P1 prophage (3, 43).

The amount of repressor produced is crucial to the effectiveness of a promoter system; small amounts of repressor can result in partial repression, while too much repressor results in the inability to achieve derepression (45, 46). Therefore, the *c1* gene was placed under the transcriptional control of two promoters (ProA and ProB; Fig. 1A) which have consensus  $-35$  and  $-10$  hexamers but which differ in their spacer sequences. Since variations in the spacer sequence have been shown to alter promoter strength by up to 400-fold (24), this was expected to provide differing amounts of C1 repressor. To enhance binding of the C1 repressor to its operator (49), the *bof* gene was cloned 3' of the *c1* gene. The Bof protein does not bind to C1 alone but binds to C1 operators by forming a C1-Bof-operator DNA ternary complex (48). To ensure efficient translation, the primers amplifying *lacZ* incorporated a contrived RBS derived from gram-positive species (6). This resulted in a 200-fold increase in  $\beta$ -Gal activity in *E. faecalis* (data not shown) compared to that with the *lacZ* RBS (GGA GG[N]<sub>6</sub>ATG) used for reporter gene studies of gram-negative bacteria (45); consequently, the former RBS was also incorporated into the forward primers amplifying *c1* and *bof*. At the permissive temperature, C1 binds to its operator sites and prevents transcription from the reporter gene, while at the nonpermissive temperature, C1 is thermally unstable, thereby allowing transcription to proceed.

The reporter plasmids were constructed in pAM401, which contains a p15A replicon derived from pACYC184 and a pGB354 replicon derived from the broad-host-range gram-positive-bacterium plasmid pIP501 (53). Consequently, the plasmid can be used for studies of enteric gram-negative bacteria, *Streptococcus* species (8, 28), *Enterococcus* species (9), *Streptococcus gordonii* (unpublished results), *L. lactis* (10), *Lactobacillus casei* (12), and *Pediococcus* species (13). The copy number of the pAM401 parental plasmid (pGB354) in streptococci has been reported to be approximately 50 copies/cell (2).

Previous work using the parent plasmid pAM401 has demonstrated transformation efficiencies of approximately  $10^5$  for *E. faecalis* and *E. faecium* (9). Although the transformation efficiencies were not measured in this report, it is expected that similar transformation efficiencies can be obtained for the larger reporter constructs since plasmids up to 25 kb can be

TABLE 2. Basal and induced activities from *lacZ* fusions to C1-regulated promoters in *E. coli* DH5 $\alpha$ <sup>a</sup>

Construct	Promoter driving:		Avg activity in Miller units ( $\pm$ SD)	
	<i>lacZ</i>	<i>c1</i>	Basal (31°C)	Induced (42°C)
pDAS100			4.2 (0.5)	6.0 (0.7)
pDAS101	Pro1		1,117.5 (223.4)	2,197.1 (77.9)
pDAS102	Pro1	ProA	ND	ND
pDAS103	Pro1	ProB	ND	ND
pDAS111	Pro2		15,478.9 (675.7)	10,899.2 (531.1)
pDAS112	Pro2	ProA	<0.25	15.9 (1.2)
pDAS113	Pro2	ProB	<0.25	5.7 (0.1)
pDAS121	Pro3		9,119.0 (272.4)	9,575.1 (666.2)
pDAS122	Pro3	ProA	2.8 (0.1)	2,386.1 (504.8)
pDAS123	Pro3	ProB	2.0 (0.3)	213.1 (11.1)

<sup>a</sup> Overnight cultures were diluted 1:100 and grown to an optical density at 600 nm (OD<sub>600</sub>) of approximately 0.1 at 31°C. The cultures were then divided equally and incubated at 31 or 42°C for 95 min prior to being assayed for  $\beta$ -Gal activity (OD<sub>600</sub> of approximately 0.6). The control strain carried a plasmid (pDAS100) containing a promoterless *lacZ* gene. Values are for multiple cultures ( $n = 3$ ) assayed in triplicate. A value of <0.25 indicates activity below the limits of detection for the assay. ND, not determined.

electroporated without any loss in transformation efficiency (34, 37). In our experiments, the relative transformation efficiency was slightly lower for *S. aureus* than for *E. faecalis* and *E. faecium* (data not shown).

Plasmid maintenance studies were performed to test the stability of pDAS122 in *S. aureus*, pDAS113 in *E. faecium*, and pDAS113 in *E. faecalis*. Cultures (in triplicate) harboring the plasmids were grown overnight in selective (chloramphenicol) liquid media and then plated on nonselective or selective solid media. The percentages of chloramphenicol-resistant colonies obtained for *E. faecalis*, *E. faecium*, and *S. aureus* were 89, 77, and 61%, respectively. Therefore, strains carrying the reporter constructs were always grown under selective pressure.

**Analysis of  $\beta$ -Gal activity from the temperature-sensitive C1-regulated promoters in *E. coli*.** Since *E. coli* is the preferred host for cloning and propagation of plasmids, it is important to know whether the regulated promoter is efficiently repressed in this host; this may be essential if, for example, the gene of interest encodes a toxic protein. Therefore, to demonstrate the functionality of the promoter system,  $\beta$ -Gal activity was measured initially in *E. coli*.  $\beta$ -Gal activity was measured by using three C1-regulated promoters driving *lacZ* at the permissive (31°C) and nonpermissive (42°C) temperatures. In the absence of C1, the activities of all three promoters were high, with Pro2 and Pro3 producing approximately 5- to 10-fold more Miller units than Pro1 (Table 2). This was most likely due to the nucleotide change from G to the consensus T within the -10 hexamers in Pro2 and Pro3 (Fig. 1A). Pro2 and Pro3 exhibited similar activities, indicating that the TG dinucleotide had little effect on promoter strength in *E. coli*. In the presence of C1 and at low temperature,  $\beta$ -Gal activity was significantly reduced, indicating that C1 can efficiently repress transcription from these promoters. In particular, the basal expression of Pro2 was much lower than that of Pro3, which was probably a reflection of the number of mismatches in the C1 operator sites (one and five mismatches, respectively) and hence the ability to more effectively repress transcription. Interestingly, the basal expression of Pro2 was also lower than that of the control vector, which contained a promoterless *lacZ* gene (pDAS100). This may be explained by the observation that, in *E. coli*, repressor-bound operators can prevent the formation of active complexes between RNA polymerase and promoters and also

terminate ongoing transcription (5). It also suggests that the terminators used in the plasmid construction were not 100% effective, leading to some promoter readthrough from the plasmid backbone. Little difference was observed in the basal levels of expression from the C1-regulated promoters when C1 was expressed from ProA or ProB, suggesting that adequate amounts of C1 were produced from both constructs to effectively repress transcription. At the nonpermissive temperature,  $\beta$ -Gal activity from the C1-regulated promoters significantly increased, although levels were still below levels obtained in the absence of C1 (Table 2). Nevertheless, the range of regulation was similar to that for the bacteriophage P1-derived C1-regulated promoter system in *E. coli* described previously (44).

**Analysis of  $\beta$ -Gal expression using the temperature-sensitive C1-regulated promoters in *E. faecium*, *E. faecalis*, and *S. aureus*.** The C1-regulated promoters in *E. faecium*, *E. faecalis*, and *S. aureus* were analyzed (Table 3). In the absence of C1, the activity of Pro1 was low to undetectable; therefore, constructs containing this promoter were not examined further. However, Pro2 expression was high, indicating that the 1-nucleotide difference between Pro1 and Pro2, in contrast to what was found for *E. coli*, was essential for activity in these species. The addition of the TG dinucleotide (Pro3) further increased the strength of the promoter. In the presence of C1 at the permissive temperature, the basal activity of Pro2 was reduced to the background level displayed by the control strain carrying the promoterless *lacZ* construct. This indicated that the Pro2 promoter was completely repressed in the presence of C1. In *E. faecalis* and *S. aureus*, the basal level of expression was below the limits of detection when *c1* was expressed from either ProA or ProB. In *E. faecium*, however, basal activity was slightly higher when ProB, instead of ProA, was used to drive *c1* expression, suggesting that the ability to repress transcription was dependent on the levels of C1 expressed.

Basal expression exhibited by Pro3 was generally higher than that exhibited by Pro2 and was more dependent on the promoter driving *c1* expression and, presumably, the concentration of repressor present. The higher basal expression may be a reflection of the increased number of mismatches in the C1 operator sites compared to that for Pro2 (five compared to one), leading to less-efficient binding of the C1 repressor (18).

TABLE 3. Basal and induced activities from *lacZ* fusions to C1-regulated promoters in *E. faecium*, *E. faecalis*, and *S. aureus*<sup>a</sup>

Species and construct	Promoter driving:		Avg activity in Miller units ( $\pm$ SD)	
	<i>lacZ</i>	<i>c1</i>	Basal (31°C)	Induced (42°C)
<i>E. faecium</i>				
pDAS100			1.3 (0.1)	0.6 (0.03)
pDAS101	Pro1		1.7 (0.4)	1.4 (0.35)
pDAS102	Pro1	ProA	ND	ND
pDAS103	Pro1	ProB	ND	ND
pDAS111	Pro2		1,769.9 (89.6)	3,849.2 (131.6)
pDAS112	Pro2	ProA	1.8 (0.3)	1.6 (0.1)
pDAS113	Pro2	ProB	3.4 (0.2)	640.3 (14.5)
pDAS121	Pro3		2,344.6 (165.1)	2,564.3 (387.7)
pDAS122	Pro3	ProA	227.6 (10.8)	699.0 (57.2)
pDAS123	Pro3	ProB	825.1 (16.3)	1,528.1 (65.4)
<i>E. faecalis</i>				
pDAS100			<0.25	<0.25
pDAS101	Pro1		<0.25	<0.25
pDAS102	Pro1	ProA	ND	ND
pDAS103	Pro1	ProB	ND	ND
pDAS111	Pro2		1,139.1 (23.6)	3,068.3 (119.7)
pDAS112	Pro2	ProA	<0.25	<0.25
pDAS113	Pro2	ProB	<0.25	269.0 (49.5)
pDAS121	Pro3		2,332.4 (54.6)	4,860.7 (149.8)
pDAS122	Pro3	ProA	2.83 (1.4)	1,181.2 (59.5)
pDAS123	Pro3	ProB	884.0 (145.4)	1,120.5 (29.1)
<i>S. aureus</i>				
pDAS100			<0.25	<0.25
pDAS101	Pro1		<0.25	<0.25
pDAS102	Pro1	ProA	ND	ND
pDAS103	Pro1	ProB	ND	ND
pDAS111	Pro2		76.1 (7.9)	183.4 (35.5)
pDAS112	Pro2	ProA	<0.25	<0.25
pDAS113	Pro2	ProB	<0.25	4.6 (0.74)
pDAS121	Pro3		129.5 (16.8)	257.8 (55.1)
pDAS122	Pro3	ProA	<0.25	26.4 (5.8)
pDAS123	Pro3	ProB	54.6 (3.8)	138.4 (9.1)

<sup>a</sup> Overnight cultures were diluted 1:100 and grown to an optical density at 600 nm ( $OD_{600}$ ) of approximately 0.1 at 31°C. The cultures were then divided equally and incubated at 31 or 42°C for 120 min (*E. faecium*), 95 min (*E. faecalis*), or 75 min (*S. aureus*) prior to being assayed for  $\beta$ -Gal activity ( $OD_{600}$  of approximately 0.6). The control strain carried a plasmid (pDAS100) containing a promoterless *lacZ* gene. Values are for multiple cultures ( $n = 3$ ) assayed in triplicate. ND, not determined.

Moreover, since the Pro3 promoter was generally stronger than the Pro2 promoter, the higher expression may also reflect the increased ability of RNA polymerase to compete with the repressor for binding to the unoccupied promoters. Nevertheless, low basal expression was still observed in *S. aureus* and *E. faecalis* when *c1* was expressed from the ProA promoter.

Under inducing conditions when either the Pro2 or Pro3 promoter was used, striking differences in the levels of induced expression were achieved depending on whether ProA or ProB was used to drive *c1* expression and which species was tested (Table 3). Induction was not observed when the Pro2 promoter in combination with the ProA promoter was used for any of the species (pDAS112). In contrast, high induced activity was obtained when Pro2 in combination with ProB was used to drive *c1* expression in *E. faecalis*, *E. faecium*, and, to a lesser extent, *S. aureus*. Using the Pro3 promoter in combination with ProA to drive *c1* expression (pDAS122) resulted in regulated expression in *E. faecalis* and *S. aureus* only, while using Pro3 in combination with ProB (pDAS123) did not result in regulated expression for any of the species due to the high basal expression. Therefore, use of only some of the constructs resulted in regulated expression. Moreover, the results suggested that dif-

ferences in C1 expression correlated with the ability to achieve derepression. Partial derepression when the repressor is in excess has been demonstrated previously for regulated promoter systems in gram-negative bacteria (33, 45). Indeed, ProA might be expected to be more active than ProB, resulting in higher levels of C1 expressed, since it contains more conserved nucleotides. However, low levels of basal activity and elevated induced expression were obtained in *S. aureus* and *E. faecalis* by using the Pro3 promoter in combination with ProA to drive *c1* expression. This suggests that induced expression depends on both the interaction between the repressor and operator site and the amount of repressor present. C1 has also been shown to be more thermally stable when tightly bound to DNA than it is in its unbound form, which can be dissociated only by further temperature increases (16). It should be noted that induced expression was achieved in *E. coli* with these constructs irrespective of the promoters utilized (Table 2). Nevertheless, these results demonstrated that a temperature-sensitive C1-regulated promoter can be effectively repressed to levels comparable to those for the control vectors yet yield high levels of induced expression. Induction/repression ratios for *E. faecium*, *E. faecalis*, and *S. aureus* were approximately 200, and

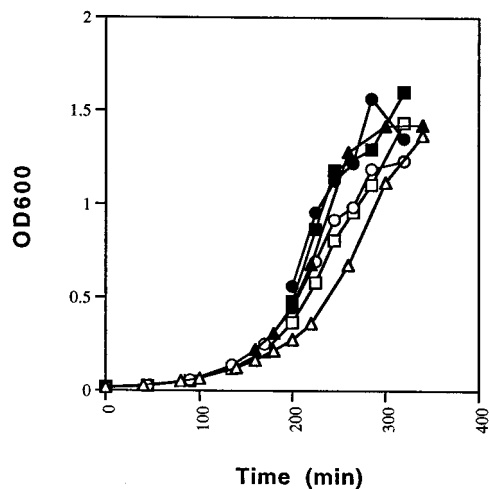


FIG. 2. Time course analysis of cell growth at 31 and 42°C. Overnight cultures were diluted 1:100 and grown at 31°C to early log phase. The cultures (*S. aureus* [squares], *E. faecalis* [triangles], and *E. faecium* [circles]) were then divided equally and incubated at 31°C (open symbols) or 42°C (solid symbols). Cell growth was monitored spectrophotometrically by measuring optical density at 600 nm ( $OD_{600}$ ).

at least 1,000, and 100, respectively. Consequently, these data represent the first heterologous regulated promoter system to be described for *E. faecium* and reflect a range of regulation in *E. faecalis* which is similar to those for the promoter systems described for *E. coli* (15). The level of regulation achieved for *S. aureus* is comparable, if not better, than those for previously described promoter systems (25, 56). In addition, since different combinations of promoters were evaluated, constructs can be selected depending on whether tight basal or highly induced expression is preferred.

However, note that the level of  $\beta$ -Gal activity detected varied by species and was highest in *E. coli*, followed by *E. faecium*, *E. faecalis*, and *S. aureus*. In particular,  $\beta$ -Gal activity was low in *S. aureus*, which may be due to weak promoter activity, inefficient translation of the *lacZ* message, or poor stability of the LacZ protein. Alternatively, it may reflect inefficient permeabilization of the *S. aureus* cells and hence a lower sensitivity of the  $\beta$ -Gal assay. However, since the promoters were designed on the basis of conserved elements from gram-positive species and since the relative *lacZ* message levels were similar, it is likely that the low expression levels were due to weak translation and/or detection rather than poor promoter activity.

To investigate the growth characteristics of *E. faecalis*, *E. faecium*, and *S. aureus* under the same conditions that were used for the promoter analysis, cell growth was monitored spectrophotometrically at 31 and 42°C (Fig. 2). When the culture temperature was changed from 31 to 42°C, cell growth rates increased. The increase in growth rate may result in differences in the levels of expression for the different constructs that are independent of the temperature-sensitive C1 repressor and hence the regulated promoter system. For example, in the absence of the repressor, activities from the promoter constructs (pDAS111 and pDAS121) would be expected to be similar at both temperatures. However, except for

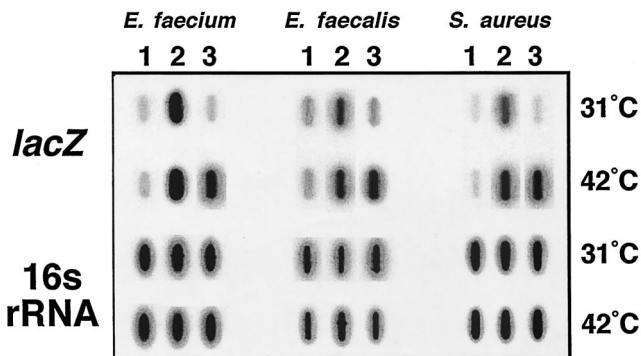


FIG. 3. Slot blot analysis of *lacZ* expression in *E. faecium*, *E. faecalis*, and *S. aureus*. Overnight cultures were diluted 1:100 and grown to an optical density at 600 nm of approximately 0.1. Cultures were then divided equally and incubated at 31 or 42°C for 120, 95, and 75 min, respectively. Cultures carried either a promoterless *lacZ* construct (pDAS100; lane 1); a reporter construct lacking the *c1* repressor (pDAS111 for *E. faecium* and *E. faecalis* and pDAS121 for *S. aureus*; lane 2), or a C1-regulated reporter construct (pDAS113 for *E. faecium* and *E. faecalis* and pDAS122 for *S. aureus*; lane 3). RNA was extracted from the cultures, blotted onto the membrane, and hybridized to either a *lacZ* or 16S rRNA complementary oligonucleotide probe.

pDAS121 in *E. faecium*, the activities were approximately two-fold higher at 42°C than at 31°C (Table 3). It is reasonable to speculate that these differences can be attributed to the increased growth rate at 42°C; although Miller units ( $\beta$ -Gal activity) account for the number of cells assayed, they do not take into consideration the growth rates of the different species. Therefore, the actual level of induced expression and hence the range of regulation may be slightly lower than the reported values.

**Transcriptional regulation of *lacZ* expression.** To analyze the regulation of *lacZ* expression at the transcriptional level, slot blot analysis was performed (32). Since promoters were located in both orientations in the plasmid, hybridizations were performed with an oligonucleotide complementary to *lacZ* as a probe. RNA was prepared from cultures carrying (i) promoterless *lacZ* control constructs, (ii) reporter constructs lacking the *c1* repressor, and (iii) reporter constructs under repressed and derepressed conditions (Fig. 3). The blots were also hybridized with a complementary oligonucleotide homologous to a conserved region of 16S rRNA to verify equal loading of the RNA. Levels of *lacZ* expression from the promoterless *lacZ* control constructs and the constructs lacking *c1* were low and high, respectively, as expected (Fig. 3). Furthermore, the levels of *lacZ* transcripts produced from the control vectors and reporter constructs under repressed conditions were similar, indicating that C1 can efficiently repress transcription. In contrast, at elevated temperatures, *lacZ* expression from the reporter constructs was significantly increased. The results are therefore in agreement with enzymatic assays and confirmed that the regulation of *lacZ* expression occurred primarily at the level of transcription.

**Modulation of  $\beta$ -Gal activity in *E. faecium*, *E. faecalis*, and *S. aureus*.** The ability to obtain different levels of expression by partial induction of the promoter is an important feature of a controlled expression system. Therefore, to assess the ability to modulate the temperature-sensitive C1-regulated promoter

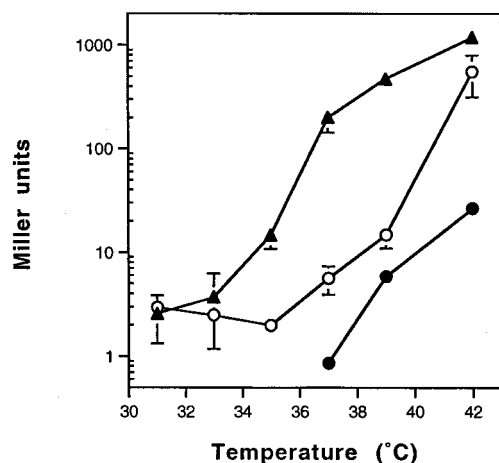


FIG. 4. Modulation of expression from the temperature-sensitive C1-regulated promoter in *S. aureus* (●), *E. faecium* (○), and *E. faecalis* (▲). Overnight cultures carrying the reporter construct were diluted 1:100 and grown at 31°C. The culture was then divided equally and incubated for 75 (*S. aureus*), 120 (*E. faecium*), or 95 min (*E. faecalis*) at the designated temperatures prior to assaying for  $\beta$ -Gal activity (optical density at 600 nm at the time of harvesting, approximately 0.6). Values ( $\pm$  standard deviations) are averages of triplicate cultures assayed in triplicate. The reporter constructs used for *S. aureus*, *E. faecalis*, and *E. faecium* were pDAS122, pDAS122, and pDAS113, respectively.

system in *E. faecium*, *E. faecalis*, and *S. aureus*,  $\beta$ -Gal activity was measured at different temperatures. The results indicated that, by varying temperature, it was possible to modulate expression (Fig. 4). However, the degree to which the promoter could be modulated was dependent on the host. For example, in *E. faecalis* there was a steady increase in  $\beta$ -Gal activity as the temperature increased. In contrast, the level of  $\beta$ -Gal expressed in *E. faecium* remained relatively unchanged until 39°C. For all three species, maximal induction was achieved at the highest temperature tested (42°C), which is in agreement with previous results indicating C1 instability at 42°C and above (16). Since enterococci can tolerate temperatures of 45°C (23), higher induced activities may be observed by a further increase in temperature.

To examine the kinetics of induction, the cultures were grown at low temperature and then induced at the elevated temperatures. At the times indicated in Fig. 5, cultures were harvested and  $\beta$ -Gal activity was measured. The kinetics of induction for *E. faecium*, *E. faecalis*, and *S. aureus* were similar and indicated that the temperature-sensitive C1-regulated promoter has a fast rate of induction. In addition, the results indicated that incubation under inducing conditions need only be maintained for 80 min to achieve maximal induction (Fig. 5).

In conclusion, we have demonstrated that the bacteriophage P1 temperature-sensitive C1 repressor can be used to control gene expression in clinically relevant gram-positive bacteria. For all three species investigated, the promoters were shown to be tightly repressed, an essential characteristic of a regulated promoter system. In *E. faecalis*, the level of regulation was 1,000-fold, bringing a level of efficiency comparable to those for promoter systems currently used in gram-negative bacteria.

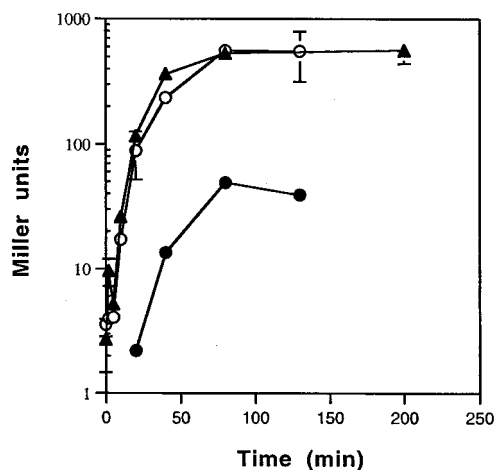


FIG. 5. Time course analysis of temperature induction of *lacZ* expression in *S. aureus* (●), *E. faecium* (○), and *E. faecalis* (▲). Overnight cultures carrying the reporter constructs were diluted 1:100 and grown at 31°C to early log phase. Aliquots of the culture were then incubated at 42°C for the indicated times prior to harvesting for  $\beta$ -Gal activity (optical density at 600 nm was approximately 0.6). Values reported ( $\pm$  standard deviations) are averages of duplicate cultures assayed in triplicate. The reporter constructs used for *S. aureus*, *E. faecalis*, and *E. faecium* were pDAS122, pDAS122, and pDAS113, respectively.

Furthermore, significant regulation was obtained in *E. faecium*, a species for which no heterologous regulated promoter systems have been described.

The C1-regulated promoters and promoters driving *c1* expression were designed based on conserved gram-positive species promoter elements and therefore should be active in a wide variety of bacteria. The vectors were also constructed in a broad-host-range vector capable of replication in gram-positive species as well as enteric gram-negative species. Tight basal expression and controlled induction with the same reporter plasmid were demonstrated in both *E. coli* and gram-positive species; these are features that may have many applications. Furthermore, as temperature is the inducer, the promoter system is not dependent on exogenously supplied inducers. For these reasons, we expect the temperature-sensitive regulated promoter system to be useful for genetic studies of both pathogenic gram-negative and gram-positive species.

#### ACKNOWLEDGMENTS

This work was supported by Hexal Gentech Forschungs GmbH. DNA sequencing data were obtained by the Biotechnology Resource Laboratory of the Medical University of South Carolina.

#### REFERENCES

- Bateman, B. T., N. P. Donegan, T. M. Jarry, M. Palma, and A. L. Cheung. 2001. Evaluation of a tetracycline-inducible promoter in *Staphylococcus aureus* in vitro and in vivo and its application in demonstrating the role of *sigB* in microcolony formation. *Infect. Immun.* **69**:7851–7857.
- Behnke, D., M. S. Gilmore, and J. Ferretti. 1982. pGB301 vector plasmid family and its use for molecular cloning in streptococci, p. 239–242. In D. Schlessinger (ed.), *Microbiology*. American Society for Microbiology, Washington, D.C.
- Citron, M., M. Velleman, and H. Schuster. 1989. Three additional operators, Op21, Op68, and Op88, of bacteriophage P1. Evidence for control of the P1 dam methylase by Op68. *J. Biol. Chem.* **264**:3611–3617.
- de Ruyter, P. G., O. P. Kuipers, and W. M. de Vos. 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl. Environ. Microbiol.* **62**:3662–3667.

5. Deuschle, U., R. Gentz, and H. Bujard. 1986. *lac* repressor blocks transcribing RNA polymerase and terminates transcription. Proc. Natl. Acad. Sci. USA **83**:4134–4137.
6. Eichenbaum, Z., M. J. Federle, D. Marra, W. M. de Vos, O. P. Kuipers, M. Kleerebezem, and J. R. Scott. 1998. Use of the lactococcal *nisA* promoter to regulate gene expression in gram-positive bacteria: comparison of induction level and promoter strength. Appl. Environ. Microbiol. **64**:2763–2769.
7. Eliason, J. L., and N. Sternberg. 1987. Characterization of the binding sites of *c1* repressor of bacteriophage P1. Evidence for multiple asymmetric sites. J. Mol. Biol. **198**:281–293.
8. Engel, H. W., N. Soedirman, J. A. Rost, W. J. van Leeuwen, and J. D. van Embden. 1980. Transferability of macrolide, lincomycin, and streptogramin resistances between group A, B, and D streptococci, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. J. Bacteriol. **142**:407–413.
9. Friesenegger, A., S. Fiedler, L. A. Devriese, and R. Wirth. 1991. Genetic transformation of various species of *Enterococcus* by electroporation. FEMS Microbiol. Lett. **63**:323–327.
10. Garvey, P., G. F. Fitzgerald, and C. Hill. 1995. Cloning and DNA sequence analysis of two abortive infection phage resistance determinants from the lactococcal plasmid pNP40. Appl. Environ. Microbiol. **61**:4321–4328.
11. Geissendorfer, M., and W. Hillen. 1990. Regulated expression of heterologous genes in *Bacillus subtilis* using the Tn10 encoded *tet* regulatory elements. Appl. Microbiol. Biotechnol. **33**:657–663.
12. Gibson, E. M., N. M. Chace, S. B. London, and J. London. 1979. Transfer of plasmid-mediated antibiotic resistance from streptococci to lactobacilli. J. Bacteriol. **137**:614–619.
13. Gonzalez, C. F., and B. S. Kunka. 1983. Plasmid transfer in *Pediococcus* spp.: intergeneric and intrageneric transfer of pIP501. Appl. Environ. Microbiol. **46**:81–89.
14. Graves, M. C., and J. C. Rabinowitz. 1986. *In vivo* and *in vitro* transcription of the *Clostridium pasteurianum* ferredoxin gene. Evidence for “extended” promoter elements in gram-positive organisms. J. Biol. Chem. **261**:11409–11415.
15. Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P<sub>BAD</sub> promoter. J. Bacteriol. **177**:4121–4130.
16. Heinrich, J., H. D. Riedel, B. R. Baumstark, M. Kimura, and H. Schuster. 1989. The *c1* repressor of bacteriophage P1 operator-repressor interaction of wild-type and mutant repressor proteins. Nucleic Acids Res. **17**:7681–7692.
17. Heinrich, J., M. Velleman, and H. Schuster. 1995. The tripartite immunity system of phages P1 and P7. FEMS Microbiol. Rev. **17**:121–126.
18. Heinzl, T., M. Velleman, and H. Schuster. 1989. *ban* operon of bacteriophage P1. Mutational analysis of the *c1* repressor-controlled operator. J. Mol. Biol. **205**:127–135.
19. Heinzl, T., M. Velleman, and H. Schuster. 1990. The *c1* repressor inactivates protein *coi* of bacteriophage P1. Cloning and expression of *coi* and its interference with *c1* repressor function. J. Biol. Chem. **265**:17928–17934.
20. Helmann, J. D. 1995. Compilation and analysis of *Bacillus subtilis*  $\sigma^A$ -dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. **23**:2351–2360.
21. Henkin, T. M., and A. L. Sonenshein. 1987. Mutations of the *Escherichia coli lacUV5* promoter resulting in increased expression in *Bacillus subtilis*. Mol. Gen. Genet. **209**:467–474.
22. Hiramatsu, K., H. Hanaki, T. Ino, K. Yabuta, T. Oguri, and F. C. Tenover. 1997. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J. Antimicrob. Chemother. **40**:135–136.
23. Huycke, M. M., D. F. Sahn, and M. S. Gilmore. 1998. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg. Infect. Dis. **4**:239–249.
24. Jensen, P. R., and K. Hammer. 1998. The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. Appl. Environ. Microbiol. **64**:82–87.
25. Ji, Y., A. Marra, M. Rosenberg, and G. Woodnutt. 1999. Regulated antisense RNA eliminates alpha-txin virulence in *Staphylococcus aureus* infection. J. Bacteriol. **181**:6585–6590.
26. Jones, R. N., S. A. Marshall, M. A. Pfaller, W. W. Wilke, R. J. Hollis, M. E. Erwin, M. B. Edmond, and R. P. Wenzel. 1997. Nosocomial enterococcal bloodstream infections in the SCOPE program: antimicrobial resistance, species occurrence, molecular testing results, and laboratory testing accuracy. SCOPE Hospital Study Group. Diagn. Microbiol. Infect. Dis. **29**:95–102.
27. Kim, L., A. Mogk, and W. Schumann. 1996. A xylose-inducible *Bacillus subtilis* integration vector and its application. Gene **181**:71–76.
28. Kreikemeyer, B., and P. G. Jerlstrom. 1999. An *Escherichia coli*-*Enterococcus faecalis* shuttle vector as a tool for the construction of a group B *Streptococcus* heterologous mutant expressing the beta antigen (Bac) of the C protein complex. FEMS Microbiol. Lett. **180**:255–262.
29. Lanzer, M., and H. Bujard. 1988. Promoters largely determine the efficiency of repressor action. Proc. Natl. Acad. Sci. USA **85**:8973–8977.
30. Lee, G., C. Talkington, and J. Pero. 1980. Nucleotide sequence of a promoter recognized by *Bacillus subtilis* RNA polymerase. Mol. Gen. Genet. **180**:57–65.
31. Lee, J. C. 1995. Electroporation protocols for microorganisms, p. 209–215. In J. A. Nickoloff (ed.), Methods in molecular biology, vol. 47. Humana Press, Inc., Totowa, N.J.
32. Leonhardt, H., and J. C. Alonso. 1988. Construction of a shuttle vector for inducible gene expression in *Escherichia coli* and *Bacillus subtilis*. J. Gen. Microbiol. **134**:605–609.
33. Lutz, R., and H. Bujard. 1997. Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I<sub>1</sub>-I<sub>2</sub> regulatory elements. Nucleic Acids Res. **25**:1203–1210.
34. McIntyre, D. A., and S. K. Harlander. 1989. Genetic transformation of intact *Lactococcus lactis* subsp. *lactis* by high-voltage electroporation. Appl. Environ. Microbiol. **55**:604–610.
35. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
36. Moran, C. P., Jr., N. Lang, S. F. LeGrice, G. Lee, M. Stephens, A. L. Sonenshein, J. Pero, and R. Losick. 1982. Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol. Gen. Genet. **186**:339–346.
37. Powell, I. B., M. G. Achen, A. J. Hillier, and B. E. Davidson. 1988. A simple and rapid method for genetic transformation of lactic streptococci by electroporation. Appl. Environ. Microbiol. **54**:655–660.
38. Poyart, C., and P. Trieu-Cuot. 1997. A broad-host-range mobilizable shuttle vector for the construction of transcriptional fusions to  $\beta$ -galactosidase in gram-positive bacteria. FEMS Microbiol. Lett. **156**:193–198.
39. Rehm, S. J. 2002. Two new treatment options for infections due to drug-resistant gram-positive cocci. Cleve. Clin. J. Med. **69**:397–401, 405–413.
40. Rosner, J. L. 1972. Formation, induction, and curing of bacteriophage P1 lysogens. Virology **48**:679–680.
41. Ruoff, K. L., L. de la Maza, M. J. Murtagh, J. D. Spargo, and M. J. Ferraro. 1990. Species identities of enterococci isolated from clinical specimens. J. Clin. Microbiol. **28**:435–437.
42. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. Schaefer, T. S., and J. B. Hays. 1991. Bacteriophage P1 Bof protein is an indirect positive effector of transcription of the phage *bac-1 ban* gene in some circumstances and a direct negative effector in other circumstances. J. Bacteriol. **173**:6469–6474.
44. Schofield, D. A., C. Westwater, J. W. Dolan, J. S. Norris, and M. G. Schmidt. 2002. Tight regulation and modulation via a C1-regulated promoter in *Escherichia coli* and *Pseudomonas aeruginosa*. Curr. Microbiol. **44**:425–430.
45. Schofield, D. A., C. Westwater, J. W. Dolan, M. G. Schmidt, and J. S. Norris. 2001. Controlled expression in *Klebsiella pneumoniae* and *Shigella flexneri* using a bacteriophage P1-derived C1-regulated promoter system. J. Bacteriol. **183**:6947–6950.
46. Stark, M. J. 1987. Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli*. Gene **51**:255–267.
47. Stieger, M., B. Wohlgensinger, M. Kamber, L. Rolf, and W. Keck. 1999. Integrational plasmids for the tetracycline-regulated expression of genes in *Streptococcus pneumoniae*. Gene **226**:243–251.
48. Velleman, M., T. Heinzl, and H. Schuster. 1992. The Bof protein of bacteriophage P1 exerts its modulating function by formation of a ternary complex with operator DNA and C1 repressor. J. Biol. Chem. **267**:12174–12181.
49. Velleman, M., M. Heirich, A. Gunther, and H. Schuster. 1990. A bacteriophage P1-encoded modulator protein affects the P1 *c1* repression system. J. Biol. Chem. **265**:18511–18517.
50. Voskuil, M. L., and G. H. Chambliss. 1998. The –16 region of *Bacillus subtilis* and other gram-positive bacterial promoters. Nucleic Acids Res. **26**:3584–3590.
51. Voskuil, M. L., K. Voepel, and G. H. Chambliss. 1995. The –16 region, a vital sequence for the utilization of a promoter in *Bacillus subtilis* and *Escherichia coli*. Mol. Microbiol. **17**:271–279.
52. Wieland, K. P., B. Wieland, and F. Gotz. 1995. A promoter-screening plasmid and xylose-inducible, glucose-repressible expression vectors for *Staphylococcus carnosus*. Gene **158**:91–96.
53. Wirth, R., F. Y. An, and D. B. Clewell. 1986. Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli*-*S. faecalis* shuttle vector. J. Bacteriol. **165**:831–836.
54. Wright, J. J., A. Kumar, and R. S. Hayward. 1992. Hypersymmetry in a transcriptional terminator of *Escherichia coli* confers increased efficiency as well as bidirectionality. EMBO J. **11**:1957–1964.
55. Yarmolinsky, M. B., and N. Sternberg. 1988. Bacteriophage P1, p. 291–438. In R. Calendar (ed.), The bacteriophages, vol. 1. Plenum Publishing Corp., New York, N.Y.
56. Zhang, L., F. Fan, L. M. Palmer, M. A. Lonetto, C. Petit, L. L. Voelker, A. St John, B. Bankosky, M. Rosenberg, and D. McDevitt. 2000. Regulated gene expression in *Staphylococcus aureus* for identifying conditional lethal phenotypes and antibiotic mode of action. Gene **255**:297–305.