Use of the Lactococcal *nisA* Promoter To Regulate Gene Expression in Gram-Positive Bacteria: Comparison of Induction Level and Promoter Strength

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We characterized the regulated activity of the lactococcal *nisA* promoter in strains of the gram-positive species *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, and *Bacillus subtilis*. *nisA* promoter activity was dependent on the proteins NisR and NisK, which constitute a two-component signal transduction system that responds to the extracellular inducer nisin. The nisin sensitivity and inducer concentration required for maximal induction varied among the strains. Significant induction of the *nisA* promoter (10- to 60-fold induction) was obtained in all of the species studied at a nisin concentration just below the concentration at which growth is inhibited. The efficiency of the *nisA* promoter was compared to the efficiencies of the *Spac*, *xylA*, and *lacA* promoters in *B. subtilis* and in *S. pyogenes*. Because *nisA* promoter-driven expression is regulated in many gram-positive bacteria, we expect it to be useful for genetic studies, especially studies with pathogenic streptococci in which no other regulated promoters have been described.

Many gram-positive cocci, such as *Streptococcus pyogenes* (group A streptococcus), *Streptococcus agalactiae* (group B streptococcus), *Streptococcus pneumoniae*, and some strains of *Enterococcus faecalis* (previously classified as group D streptococcus), are important pathogens that are major causes of a variety of infectious diseases in humans. The illnesses caused by these pathogenic streptococci range from local infections of moderate severity, such as impetigo, pharyngitis, sinusitis, and otitis media, to life-threatening invasive diseases, such as pneumonia, meningitis, endocarditis, bacteremia, myositis, necrotizing fasciitis, and streptococcal toxic shock syndrome. In addition, some types of streptococcal infections can result in severe complications, such as arthritis, rheumatic fever, and acute glomerulonephritis (2). Major progress has been made in the last decade in the genetic study of pathogenic streptococci due to the development of molecular tools such as vectors, reporter genes, transducing phages, and transposons that are functional in these bacteria (6, 13). However, the absence of other important tools, such as controlled gene expression systems, significantly limits the study of these gram-positive pathogens.

Bacterial pathogens often regulate the expression of virulence genes in a coordinated manner in response to changes in the environment. The ability to manipulate the expression of different genes is an important tool for understanding the regulatory cascade mechanisms and the importance of gene regulation in pathogenesis. In addition, to study the importance of the relative quantities of gene products on their interactions, it is essential to be able to control their expression independently. For these purposes, a regulated heterologous promoter system is essential.

In gram-negative bacteria, a variety of promoters (native or

chimeric) and vectors (ranging from low to high copy number) have been developed to allow quantitative modulation of gene expression over a range of levels. Unfortunately, many of these systems do not function in gram-positive organisms, probably because the requirements for promoter usage are more stringent in these bacteria than in *Escherichia coli* (34, 35). Only a few regulated promoters have been described for gram-positive bacteria, and some of these, such as the *Bacillus subtilis* sporulation promoters, require factors unique to the native species that are not present in heterologous systems, like specific sigma factors and their regulators (19). Some sugar-regulated promoters might not be functional in all hosts because they depend both on sugar entry and on some initial steps to convert the sugar into the inducer that relieves repression of the promoter involved. The appropriate enzymes to carry out these processes might not be present in all hosts. A considerable portion of the available information regarding gene expression in gram-positive bacteria was obtained from the extensively studied species *B. subtilis* and *Lactococcus lactis* (10, 11).

One well-characterized lactococcal gene expression system is based on the autoregulatory properties of the *L. lactis* nisin gene cluster. Two genes in the cluster, *nisA* and *nisF*, are induced by nisin via a two-component signal transduction pathway consisting of a histidine protein kinase, NisK, and a response regulator, NisR. Expression of both *nisR* and *nisK* is driven from the constitutive promoter of *nisR* (9, 29). Nisin acts as an inducer on the outside of the cell and is sensed by NisK. Recently, it has been reported that a two-plasmid system in which the *nisA* promoter and the regulatory genes *nisR* and *nisK* are used allows efficient control of gene expression by nisin in a variety of lactic acid bacteria (27, 30).

Another controllable expression system of *L. lactis* is based on the lactose-inducible transcription of the *lac* operon. Expression of this operon is repressed during growth on glucose and is regulated at the transcriptional level by the LacR repressor (40). LacR expression is repressed during growth on lactose, which allows expression from the *lacA* promoter. The

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TABLE 1. Plasmids used in this study

Plasmid	Characteristics	Reference
pDG1832	<i>xylR</i> , <i>xylA</i> promoter, pUC19 replicon	1
pDH88	<i>lacI^q</i> , <i>Spac</i> promoter, Amp	45
pEU308	<i>lacI^q</i> , <i>Spac</i> promoter, pSH71 replicon, Spec	This study
pEU327	<i>xylR</i> , <i>xylA</i> promoter, pSH71 replicon, Spec	This study
pEU352	pEU308 derivative carrying the <i>gusA</i> gene transcriptionally fused to the <i>Spac</i> promoter	This study
pEU356	pEU327 derivative carrying the <i>gusA</i> gene transcriptionally fused to the <i>xylA</i> promoter	This study
pLZ12Spec	pSH71, Spec	23
pMLK99	<i>gusA</i> gene, Bluescript-SK derivative, Amp	26
pMLK100 ^a	<i>gusA</i> gene, Bluescript-SK replicon, Amp	26
pNZ276	<i>lacR</i> , <i>gusA</i> gene transcriptionally fused to the <i>lacA</i> promoter, pSH71 replicon, Cm	37
pNZ8008	Promoterless <i>gusA</i> gene transcriptionally fused to the <i>nisA</i> promoter, pSH71 replicon, Cm	9
pNZ9520	<i>nisR</i> and <i>nisK</i> (both expressed from <i>rep</i> promoter), pAMβ1 replicon, Erm	27
pNZ9530	<i>nisR</i> and <i>nisK</i> (both expressed from <i>rep</i> promoter), derivative of pAMβ1 replicon with a deletion in <i>repF</i> repressor gene, Erm	27

^a Plasmid pMLK100 is identical to pMLK99 except that the *gusA* gene is in the opposite orientation.

lacA promoter and the LacR repressor have been used to express several heterologous genes in a lactose-glucose-dependent manner in *L. lactis* (40, 42).

In *B. subtilis*, expression of the xylose utilization operon is inducible via a repressor-mediated mechanism (18). The *xylA* promoter and the XylR repressor have been used to control expression of heterologous genes in bacillus species. In addition to such native promoters, a chimeric promoter, designated *Spac*, consisting of the *E. coli lac* operator fused to the SPO-1 phage promoter was constructed to regulate gene expression in bacillus (45). The promoter and the ribosome-binding site of the penicillinase gene of *Bacillus licheniformis* were linked to the *E. coli lacI* repressor gene to allow expression of *lacI* in bacillus. In this system, transcription from *Spac* is repressed by the LacI repressor and can be induced by lactose and the gratuitous inducer isopropyl-β-D-thiogalactoside (IPTG).

In pathogenic streptococci, no regulated foreign promoters have been identified. Furthermore, there is a paucity of information regarding comparative expression of specific promoters in different pathogenic streptococcal species. We demonstrate here that the *L. lactis nisA* promoter is active in all of the gram-positive species that we used, including the pathogenic streptococci. Furthermore, expression from the *nisA* promoter can be efficiently regulated experimentally in all of these organisms. When the same reporter gene was used, the *nisA* promoter was found to be stronger than the *lacA*, *xylA*, or *Spac* promoter in both *B. subtilis* and *S. pyogenes* and to show the greatest degree of induction in *S. pyogenes*. We expect the *nisA* promoter to allow manipulation of gene expression in pathogenic streptococci and to bring the level of sophistication of genetic studies of these gram-positive bacteria closer to that of gram-negative pathogens.

MATERIALS AND METHODS

Bacterial strains. Experiments were performed with the following gram-positive strains: *S. pyogenes* JRS4 (group A streptococcus) (15), *S. agalactiae* COH31 (group B streptococcus) (38), *S. pneumoniae* 1131 (47), *E. faecalis* OG1RF (12), and *B. subtilis* W168 (7). *L. lactis* FMCB1, derived from MG1363 (5), was used as a host for plasmids pNZ9520 and pNZ9530 (Table 1), and *E. coli* JM109 (44) and DH5α (20) were used as hosts for all of the other plasmids (Table 1).

Media. *S. pyogenes*, *S. agalactiae*, and *E. faecalis* were grown in Todd-Hewitt broth supplemented with 0.2% yeast extract (16). *S. pyogenes* was also grown on L3 medium (22). *S. pneumoniae* cells were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract. *B. subtilis* and *E. coli* cells were grown in Luria broth (39). *L. lactis* cells were grown in M17 broth (Becton-Dickinson) supplemented with 0.5% glucose. The following antibiotics were used: for *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae*, 0.5 μg of erythromycin per ml and 2 μg of chloramphenicol per ml; for *E. faecalis*, *B. subtilis*, and *L. lactis*, 5 μg of eryth-

romycin per ml and 10 μg of chloramphenicol per ml; and for all species, 100 μg of spectinomycin per ml.

Plasmid DNA preparation and transformation. Plasmid DNA was prepared from *L. lactis* by using glass beads for cell lysis (17) and from *E. coli* by the alkaline lysis method (32). Naturally competent *S. pneumoniae* and *B. subtilis* cells were transformed with plasmid DNA as previously described (43, 46). Electroporation was used as previously described to transform *S. pyogenes* (36), *S. agalactiae* (14), and *E. faecalis* (33).

Plasmid construction. The plasmids used in this study are described in Table 1. Plasmid pEU308 was obtained by ligating the 2.1-kb *StyI-EcoRI* fragment of plasmid pDH88 (45), containing the *Spac* promoter and the *lacI^q* repressor gene, to a 3.4-kb *HindIII-EcoRI* fragment of plasmid pLZ12Spec (23) carrying the pSH71 origin of replication and the spectinomycin resistance gene *add9*. The protruding ends of *StyI* and *HindIII* were filled in by using the Klenow fragment of DNA polymerase I prior to digestion with *EcoRI*. Plasmid pEU352 was generated by ligating the 1.85-kb *HindIII-BamHI* fragment of plasmid pMLK99 (26) carrying a promoterless *gusA* gene to plasmid pEU308 cut with *HindIII* and *BglII*, creating a transcriptional fusion of the *Spac* promoter to the *gusA* gene.

Plasmid pEU327 was constructed by ligating the 3.4-kb *HindIII-EcoRI* fragment of plasmid pLZ12Spec containing the pSH71 origin of replication and the *add9* spectinomycin resistance gene to the 1.5-kb *HindIII-EcoRI* fragment of plasmid pDG1832 (1) carrying the *xylR* repressor and the *xylA* promoter. Plasmid pEU356 was generated by ligating the 1.85-kb *NotI-HindIII* fragment of plasmid pMLK100 (26) carrying a promoterless *gusA* gene to plasmid pEU327 cut with *HincII* and *HindIII*, creating a transcriptional fusion of the *xylA* promoter and the *gusA* gene. The protruding ends of *NotI* were filled in with the Klenow fragment of DNA polymerase I prior to cutting with *HindIII*.

β-Glucuronidase activity. Nisin was obtained from Sigma as a 2.5% nisin solution in sodium chloride containing denatured milk solids. The nisin concentrations referred to below are in micrograms of total solids per milliliter. Stock solutions of nisin were made by suspending 100 mg of nisin per ml in 0.05% acetic acid and then diluting the preparations 10-fold with dimethyl sulfoxide and were stored at -20°C. Further dilutions were made with water and were used immediately.

Expression of the *gusA* gene was determined by assaying the rate of hydrolysis of the substrate *p*-nitrophenyl-β-D-glucuronide. Bacterial cultures were inoculated at concentrations of approximately 10⁶ CFU/ml into selective media along with various concentrations of nisin. Most cultures were grown overnight at 37°C statically; the only exception was *B. subtilis* cultures, which were grown with aeration. A 1.0-ml aliquot of each culture was pelleted, resuspended in 0.4 ml of lysis buffer [60 mM K₂HPO₄, 33 mM KH₂PO₄, 7.4 mM (NH₄)₂SO₄, 1.7 mM sodium citrate; pH 7.0], and lysed with a FastPrep cell disrupter (model FP120; Bio 101, Inc.) in tubes containing a glass bead matrix (Bio 101, Inc.) at a speed of 5 m/s for 30 s. A 0.1-ml aliquot of cell extract was added to 0.9 ml of reaction buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM MgSO₄ [pH 7.0], 20 mM dithiothreitol), and 0.2 ml of a 4-mg/ml solution of *p*-nitrophenyl-β-D-glucuronide in lysis buffer was then added. Samples were incubated at 30°C until yellow color development reached an optical density at 420 nm of approximately 0.2 to 0.6. Reactions were stopped by adding 0.5 ml of 1 M Na₂CO₃. Enzyme activity (units [U]) is given below as 1,000 times the increase in absorbance at 420 nm per minute per unit of optical density at 600 nm of the culture.

RESULTS AND DISCUSSION

Nisin gene expression system. To characterize regulated expression driven from the *L. lactis nisA* promoter, we used a

FIG. 1. Nisin sensitivity. Bacteria harboring both the reporter vector and one of the regulatory plasmids were inoculated into media containing different nisin concentrations. Cell growth is expressed as the culture optical density at 600 nm (O.D.600) following overnight incubation at 37°C. (A) *S. pyogenes* cells carrying pNZ9520 and pNZ8008. (B) Strains of the following species carrying pNZ8008 and pNZ9530: *S. agalactiae* (□), *S. pneumoniae* (●), *B. subtilis* (■), and *E. faecalis* (○).

plasmid-based system with *E. coli gusA* as a reporter gene. This system consists of a reporter plasmid and a regulatory plasmid, each derived from a broad-host-range replicon. The reporter plasmid, pNZ8008, is a pSH71 derivative that carries a transcriptional fusion of the *nisA* promoter to *gusA* (9) (Table 1). The *nisA* regulatory genes, *nisR* and *nisK*, were carried on either of two alternative plasmids, both of which are pAMβ1 replicons. One of the regulatory plasmids, pNZ9530, carries the native pAMβ1 origin of replication, and the other, pNZ9520, is a derivative that has a deletion in the replication repressor gene, *repF*, which results in increased plasmid copy number in *L. lactis* (27) (Table 1). Consistent with their copy numbers, plasmids pNZ9530 and pNZ9520 are expected to provide low and high gene dosages, respectively, of the regulatory genes *nisR* and *nisK*.

Nisin sensitivity among the species tested. Nisin has bactericidal activity against many gram-positive bacteria, including staphylococci, streptococci, bacilli, clostridia, and mycobacteria (24). However, nisin sensitivity has been reported to vary significantly, even between members of the same species (3, 4, 8). We found that there is considerable variation in nisin sensitivity among *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, *B. subtilis*, and *E. faecalis* (Fig. 1), which is consistent with observations made for other gram-positive bacteria (4, 8, 24, 41).

The *nisA* system is regulated by nisin in all strains tested. Modulation of the *nisA* promoter activity by nisin was charac-

TABLE 2. Regulated *nisA* promoter-driven expression

Organism ^a	Fold induction ^b	
	pNZ9520	pNZ9530
<i>S. pyogenes</i>	59	4.6
<i>S. agalactiae</i>	4	11
<i>S. pneumoniae</i>	1	10
<i>B. subtilis</i>	1	10
<i>E. faecalis</i>	3	20

^a Cells carried both the reporter vector, pNZ8008, and one of the regulatory plasmids (pNZ9520 or pNZ9530).

^b Ratio of the β-glucuronidase activity observed after maximal induction to the constitutive activity observed in the absence of nisin.

terized in *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, *B. subtilis*, and *E. faecalis*. Cells harboring both the reporter vector pNZ8008 and one of the regulatory plasmids were incubated in media containing different amounts of nisin. The β-glucuronidase activity (expressed from the *nisA* promoter-*gus* transcriptional fusion) was determined following overnight incubation. In all strains tested, cells harboring only the *gusA* reporter plasmid exhibited very low levels of β-glucuronidase activity. This indicates that *nisA* promoter function is dependent on its regulatory components, NisR and NisK, even in heterologous hosts. However, the plasmid supplying the regulatory proteins NisR and NisK for optimal induction differed in the different hosts (Table 2).

It was expected that pNZ9520 would have a higher copy number than pNZ9530 because of a deletion in the replication repressor gene (27). Although this was the case in *L. lactis* (27), we found that it was not true in all of the strains which we used. We compared the amount of linearized plasmids pNZ9520 and pNZ9530 with the amount of the coresident plasmid pNZ8008 by using ethidium bromide-stained agarose gel electrophoresis and/or Southern blot analysis (data not shown). In *B. subtilis*, pNZ9520 had a copy number similar to that of pNZ8008, while pNZ9530 had a lower copy number. However, in *S. pyogenes* and in *S. pneumoniae*, there was no detectable difference between the amounts of pNZ9520 and pNZ9530, and their copy numbers appeared to be much lower than the copy number of pNZ8008. Therefore, the copy numbers of these broad-host-range plasmids, which replicate by a rolling-circle mechanism, cannot be predicted in different species, and the amounts of NisR and NisK expressed from each plasmid cannot be predicted either.

In *S. pyogenes* cells harboring both the expression plasmid pNZ8008 and the regulatory plasmid pNZ9520, very low constitutive activity was observed in the absence of nisin, and nisin caused about 59-fold induction (Table 2 and Fig. 2A). In *S. pyogenes*, the regulatory plasmid pNZ9530 was less useful than pNZ9520 because the constitutive activity was high, and little induction was observed (Table 2). In contrast, in the *E. faecalis* strain carrying pNZ9520, although the constitutive β-glucuronidase activity was low, addition of nisin to the medium resulted in no significant induction (Table 2). In the other strains investigated, the *S. agalactiae*, *S. pneumoniae*, and *B. subtilis* strains, cells harboring pNZ9520 exhibited such high levels of constitutive β-glucuronidase activity that little induction above these levels was detected in the presence of nisin (Table 2).

In all of the strains tested in which pNZ9520 caused constitutive *nisA* promoter activity, the regulatory plasmid pNZ9530 resulted in controlled expression from the *nisA* promoter (Table 2 and Fig. 2). In response to nisin, 20-fold induction was observed in *E. faecalis* (Fig. 2B) and 10- to 11-fold induction of

FIG. 2. Dose response of the *nisA* promoter. Cells harboring both pNZ8008 and pNZ9520 (A) or pNZ9530 (B through E) were inoculated into media containing different nisin concentrations, and β -glucuronidase (GUS) activity was determined in cell extracts following overnight growth at 37°C. The enzyme activity is given as 1,000 times the increase in absorbance at 420 nm per minute per optical density unit of the culture. (A) *S. pyogenes*. (B) *E. faecalis*. (C) *S. agalactiae*. (D) *S. pneumoniae*. (E) *B. subtilis*.

β -glucuronidase activity was observed in *S. agalactiae*, *S. pneumoniae*, and *B. subtilis* (Fig. 2C through E).

The nisin concentration required for maximal induction of the *nisA* promoter was different for each of the strains. In general, the greatest induction was obtained with a concentration just below the inhibitory level (Fig. 1 and 2). A comparison of the growth curve obtained in the presence of this sublethal concentration of nisin to the growth curve obtained in the absence of nisin showed that the high nisin concentration had no effect on the growth rate (data not shown). The optimal nisin concentrations used for induction were 1 $\mu\text{g/ml}$ in *S. pyogenes*, 5 $\mu\text{g/ml}$ in *S. agalactiae*, 1 to 5 $\mu\text{g/ml}$ in *S. pneumoniae*, and 20 $\mu\text{g/ml}$ in *B. subtilis* and *E. faecalis* (Fig. 2). These concentrations are higher than those used to control the system in *L. lactis*, in which induction was observed with a nisin concentration far below the inhibitory concentration (27).

Characterization of *nisA* promoter strength. The level of β -glucuronidase measured with the induced *nisA* promoter varied considerably among strains, as has been observed previously with *Lactococcus*, *Leuconostoc*, and *Lactobacillus* species (27). The highest enzyme activity which we found, about 230 U, was observed following nisin induction in the strains of *S. pyogenes* (Fig. 2A) and *S. agalactiae* (Fig. 2C). In the inducible *S. pneumoniae* system, which required *nisR* and *nisK* on pNZ9530, the maximal β -glucuronidase activity induced was 55 U (Fig. 2D), which is fivefold lower than the constitutive level observed in the presence of pNZ9520 in this organism (data not shown). In the strains of *E. faecalis* and *B. subtilis* tested, the highest induced levels of β -glucuronidase activity were 72 and 18 U, respectively (Fig. 2B and E). These variations may reflect differences in the efficiencies of the transcription and translation machinery of each of the strains tested for recognizing the *nisA* promoter. Consistent with this idea, identical promoter sequences were found to have significantly different activities in *L. lactis* and *S. pneumoniae* or *B. subtilis* (28, 31). It has also been suggested that differences between the amino acid sequences of the RNA polymerase holoenzymes in different hosts may lead to different affinities for the same promoter sequence (25). In addition, differences in codon us-

age probably affect the amount of β -glucuronidase produced in the strains studied here.

Comparison of the strength of the *nisA* promoter to the strengths of the other regulated promoters in *B. subtilis*. To evaluate *nisA* promoter strength, we compared the activity of this promoter to the activities of other regulated promoters known to function in gram-positive bacteria. This comparative study was performed first with *B. subtilis*, because it is the gram-positive organism in which gene expression has been most extensively investigated. In this comparison, we used transcriptional fusions of different promoters to the *E. coli* *gusA* gene. To avoid potential differences due to gene dosage, all fusions were carried on the same type of replicon containing the pSH71 origin. The following promoters were studied: the *L. lactis* *nisA* promoter, which is regulated by nisin through NisR and NisK; the *L. lactis* *lacA* promoter, which is regulated by lactose through the LacR repressor; the *B. subtilis* *xylA* promoter, which is regulated by xylose through the XylR repressor; and the chimeric *Spac* promoter, which is regulated by IPTG through the LacI repressor. The following plasmids were used (Table 1): pNZ8008 and pNZ9530 (nisin system [see above]); pNZ276, carrying the *xylA* promoter-*gusA* transcriptional fusion and the repressor *lacR*; pEU356, carrying the *xylA* promoter-*gusA* transcriptional fusion and the repressor *xylR*; and pEU352, carrying the *Spac* promoter-*gusA* transcriptional fusion and the *lacI* repressor. To prevent catabolite repression of the promoters that are regulated by sugars (*lacA* and *xylA*), all assays were performed in Luria broth containing no sugar source other than that needed for induction. *B. subtilis* cells harboring the appropriate plasmids were grown under both inducing and non-inducing conditions, and the β -glucuronidase activity was determined following overnight growth (Fig. 3 and Table 3).

Sequence analysis of 236 promoters recognized by the *B. subtilis* sigma A subunit of RNA polymerase revealed an extended promoter structure (21). The most highly conserved bases include the -35 and -10 hexanucleotide core elements and a TG dinucleotide at positions -15, -14. In addition, several weakly conserved A and T residues are present upstream of the -35 region. All of these elements are found with dif-

FIG. 3. *nisA* promoter strength in *B. subtilis*. *B. subtilis* cells harboring the following plasmids were used: pNZ8008 and pNZ9530 (*nisA* promoter), pEU352 (*Spac* promoter), pEU356 (*xylA* promoter), and pNZ276 (*lacA* promoter). Cells were grown in Luria broth or in Luria broth supplemented with 20 μg of nisin per ml, 20 mM IPTG, 2% xylose, or 2% lactose. The β -glucuronidase (GUS) activity was determined following overnight incubation at 37°C.

TABLE 3. Induction of different regulated promoters in *B. subtilis* and *P. pyogenes*^a

Promoter	Inducer	Fold induction ^b	
		<i>B. subtilis</i>	<i>S. pyogenes</i>
<i>nisA</i> (pNZ8008) ^c	Nisin ^d	10	11
<i>Spac</i> (pEU352)	IPTG (20 mM)	16	4
<i>xylA</i> (pEU356)	Xylose (2%)	11	1
<i>lacA</i> (pNZ276)	Lactose (2%)	2	5

^a Cells were grown in Luria broth (*B. subtilis*) or L3 medium (*S. pyogenes*) with or without inducer overnight.

^b Ratio of the β -glucuronidase activity observed after maximal induction to the constitutive activity (no inducer). For the *lacA* promoter in *S. pyogenes*, the constitutive activity was determined with cells grown in L3 medium containing 2% glucose.

^c Cells carried both plasmid pNZ8008 and one of the regulatory plasmids, either pNZ9530 (*B. subtilis*) or pNZ9520 (*S. pyogenes*).

^d Nisin was used at a concentration of 20 μ g/ml with *B. subtilis* and at 1 μ g/ml with *S. pyogenes*.

ferent degrees of conservation in the four promoters which we tested (Table 4).

In *B. subtilis*, the *nisA*, *Spac*, and *xylA* promoters were all regulated to similar extents (about 10-fold induction), while expression from the *L. lactis lacA* promoter was too low to be detected even under inducing conditions (Fig. 3 and Table 3). The maximum induced expression was highest for the *nisA* promoter.

There are several possible explanations for the differences in the strengths of these promoters. It has been shown that the sequence between the -10 and -35 elements affects promoter strength several hundred-fold (25), probably by altering the context in which these elements are presented to the RNA polymerase holoenzyme. The sequences of the promoters which we compared differ in this spacer region, and this may also have some effect on the strengths of the promoters.

Comparison of the strength of the *nisA* promoter to the strengths of other regulated promoters in *S. pyogenes*. Each of the promoter systems mentioned above was also tested in *S. pyogenes* to learn whether it could be used to control gene expression in this organism. All assays were carried out in L3 medium with and without inducer. To study the effect of catabolic repression on the promoters that are regulated by sugars, expression from the *Spac*, *xylA*, and *lacA* promoters was also determined in L3 medium containing glucose (Fig. 4 and Table 3).

As in *B. subtilis*, in *S. pyogenes* the expression driven from the induced *nisA* promoter was higher than the induced activities of all of the other promoters (specific activity, about 67 enzyme

FIG. 4. *nisA* promoter strength in *S. pyogenes*. *S. pyogenes* cells harboring the following plasmids were used: pNZ8008 and pNZ9520 (*nisA* promoter), pEU352 (*Spac* promoter), pEU356 (*xylA* promoter), and pNZ276 (*lacA* promoter). Cells were grown in L3 medium or in L3 medium supplemented with 1 μ g of nisin per ml, 2% glucose, 2% lactose, 20 mM IPTG, or 2% xylose. The β -glucuronidase (GUS) activity was determined following overnight incubation at 37°C.

units). The *Spac* and *lacA* promoters had lower activities (specific activities, about 35 and 24 enzyme units, respectively), and no activity from the *xylA* promoter was detectable. *nisA* and *Spac* were the only promoters that showed increased activity in response to the inducers. *nisA* activity was induced 11-fold by nisin, while only 4-fold induction of the *Spac* promoter was obtained with IPTG. Induction of *Spac* by lactose was slightly lower than induction by IPTG (Fig. 4).

The levels of expression driven from the *lacA* promoter in L3 medium and in L3 medium containing lactose were similar; however, these levels of expression were about fivefold higher than the *lacA* activity in L3 medium supplemented with glucose (Fig. 4). This indicates that the differences in *lacA* promoter activity resulted from glucose repression rather than induction by lactose. Since lactose did not induce the *L. lactis lacA* promoter in either *B. subtilis* or *S. pyogenes*, it is possible that in these hosts lactose is not processed to the inducer form.

Because the constitutive expression of the *lacA* promoter was high in *S. pyogenes*, we investigated the effect of glucose on this system. There was a fivefold reduction in expression from the *lacA* promoter when *S. pyogenes* was grown in glucose compared to when it was grown either in the presence of lactose or with no added sugar (Fig. 4). Since the *lacA* promoter contains a catabolite repression element that is func-

TABLE 4. Comparison of the sequences of the regulated promoters used in this study^a

Source of sequence	Sequence in the following regions:						Space ^c	RBS sequence
	-45	-35	-15	-10	-5			
Gram-positive bacteria	Ta AAAAA	TTGAcA	a A a T TG	TATAAT	AatAt			AAGGAGG
<i>Spac</i>	TGCAAAAA GTG	TTGACT	TTATCTACAAGGTGTGG	CATAAT	GTGTGGA	26		AAGGAGG
<i>nisA</i> ^b	ATAATAAAC GGCT	CTGATT	AAATTCTGAAGTTTGTAGA	TACAAT	GATTTCG	26		AAGGAGG
<i>xylA</i>	AAAAACT AAAAA ATA	TTGAAA	ATACTGATGAGGTTATT	TAAGAT	TAAAATA	124		AAGGAGG
<i>lacA</i>	TAACAAAAA TAG	TTGCGT	TTGTTTGAATGTTGA	TATCAT	ATAAACA	77		TAGGAGG

^a Promoter sequences were aligned by using the -35 and Pribnow regions. The sequence derived from a compilation of 29 promoter sequences of gram-positive bacteria (18a) and the consensus sequence for the ribosome binding site (RBS) are shown. Bases in the consensus sequence that appear in more than 41% of the promoters tested are in lowercase letters, and bases that are present in more than 50% of the promoters tested are in uppercase letters. The bases in the regulated promoters that are the same as the bases in the consensus sequence are indicated by boldface type.

^b The *nisA* -35 and -10 regions are based on sequence homology data (9).

^c The space (in base pairs) between the transcription start site and the RBS is indicated.

tional in *L. lactis* (11), it is possible that glucose causes catabolite repression of this system in *S. pyogenes* as well.

Expression from the uninduced *xylA* promoter was undetectable in both *S. pyogenes* and *B. subtilis*. However, although this promoter was induced in the latter, it was not in the former. We believe that it is likely that xylose does not enter *S. pyogenes*, since we also found that xylose was not able to serve as a carbon source in this organism. An overnight culture in which glucose was added to L3 medium reached a higher optical density than a culture with no added sugar, while addition of xylose had no effect (data not shown).

Conclusions. In this study we demonstrated that the lactococcal *nisA* promoter is recognized in many different gram-positive species, including pathogenic streptococci. In *B. subtilis* and *S. pyogenes*, it was the most efficient of the regulatable promoters studied.

For efficient induction, the regulatory protein must be able to interact with the RNA polymerase, whose sequence differs in different bacteria. We have extended the list of species in which NisR is able to activate transcription to include *B. subtilis* and some of the pathogenic streptococci. We concluded that nisin interacts effectively with NisK in all of these strains since the *nisA* promoter was regulated by nisin in all species of AT-rich gram-positive bacteria which we studied.

The *nisA* gene expression system can be moved easily between strains of different species because it is contained on two broad-host-range replicons, and it should provide an alternative to the commonly used *Spac* promoter, which may not be active in all gram-positive organisms. Furthermore, in strains in which *Spac* is active, *nisA* should be compatible with the *Spac* system to allow independent regulation of different cloned genes. For all of these reasons and because it is the first heterologous regulatable promoter described for pathogenic streptococci, we expect the *nisA* gene expression system to be extremely useful in the future, especially for genetic studies of important gram-positive human pathogens.

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