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amy as a Reporter Gene for Promoter Activity in *Nocardia lactamdurans*: Comparison of Promoters of the Cephamycin Cluster

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Promoter probe vectors containing the pA origin of replication and the *Streptomyces griseus* promoterless *amy* gene (encoding α-amylase) as reporter have been constructed to study transcription initiation regions in *Nocardia lactamdurans*. In some of the promoter probe vectors the phage fd terminator has been introduced to avoid readthrough expression from upstream sequences. By using these vectors, four different transcription initiation regions of the cephamycin gene cluster have been studied in *N. lactamdurans*. The *bla* gene encoding a β-lactamase has a relatively strong promoter. Two other separate promoters corresponding to the *lat* and *cefD* genes (encoding, respectively, lysine-6-aminotransferase and isopenicillin *N*-epimerase) showed weak transcription initiation ability. These two promoters are arranged in a bidirectional transcription initiation region located in the center of the cephamycin gene cluster. The *cmcH* gene (encoding 3-hydroxymethylcephem carbamoyltransferase) upstream region did not contain a functional promoter, suggesting that *cmcH* is transcribed as a part of a polycistronic mRNA. The native *amy* promoter is used very efficiently in *N. lactamdurans*, resulting in secretion of high levels of extracellular α-amylase.

Monitoring the expression of a promoterless reporter gene (encoding an easily assayable enzyme activity) fused to a promoter of interest offers a convenient method to analyze the function of promoters (20). This approach is particularly useful when specific mRNAs are difficult to isolate in an undegraded form for quantitative determination, as is the case with some actinomycetes, or when the gene products are difficult to assay. Several reporter genes have been used in gram-negative and some gram-positive bacteria (e.g., *lacZ*, encoding β-galactosidase; *cat*, encoding chloramphenicol acetyltransferase; *galK*, encoding galactokinase; *xylE*, encoding catechol 1,4-dioxygenase; and *luxAB*, encoding bacterial luciferase) (1, 3, 4, 6). Although each of them offers distinct advantages depending on the host system used, none of them is easily applicable to species of *Nocardia* and related gram-positive bacteria. Initial studies showed that catechol oxygenase (the product of the *xylE* reporter gene) was toxic when expressed from different cephamycin promoters in liquid cultures of *Nocardia lactamdurans* but not in cultures of *Streptomyces lividans*. However, *N. lactamdurans* transformants carrying *xylE* under weak promoters were able to grow.

During studies on protein secretion in *S. lividans* (10, 23) and corynebacteria (5) using the *amy* gene of *Streptomyces griseus*, we found that the amount of α-amylase secreted was proportional to the strength of the promoter used to express the *amy* gene since there is no bottleneck in amylase secretion up to very high levels of expression of the *amy* gene (24). Processing of the enzyme during secretion occurred by releasing the same leader peptide in both corynebacteria and *Streptomyces* species (5). Later, we observed that the *S. griseus amy* gene can be used as a marker for detection of *N. lactamdurans* transformants (15).

The α-amylase activity is easy to determine in clones growing on solid plates (as the zone of starch digestion) and in liquid cultures by spectrophotometric assays. Furthermore, quantification of extracellular amylase activity obviates the need to disrupt the cells as occurs with intracellular enzymes as reporters. Therefore, it was of interest to study the use of the *amy* gene as reporter in *N. lactamdurans.*

As described in this article, promoter probe vectors containing the promoterless *amy* gene as reporter have been constructed and used successfully to determine the activity of several promoters of the cephamycin gene cluster in *N. lactamdurans.*

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MATERIALS AND METHODS

Strains and culture conditions. The plasmids used in this study are listed in Table 1. Complex NYG medium (11) was used for growing wild-type and transformed cultures of *N. lactamdurans*. Flasks were inoculated with 5%, vol/vol, 48-h starter culture of *N. lactamdurans* LC411, or the different transformants, in NYG medium after the cells were washed with sterile saline solution (0.9% NaCl). NYGly medium, which has the same composition as NYG except that glucose (10 g per liter) was replaced by the same concentration of glycerol, and the defined Lechevalier medium (18) were used for α-amylase production in the *N. lactamdurans* transformants. Kanamycin (100-μg/ml final concentration) was added to the inoculum and production medium, when required to maintain the plasmids.

Amylase assay and polyacrylamide gel electrophoresis. Amylase was assayed by quantifying the release of maltose from soluble starch (Sigma) with 3,5-dinitrosalicylic acid (10). Polyacrylamide gel electrophoresis was performed as described previously (14), and the amylase activity was revealed in nondenaturing gels with starch and iodine as described by García-González et al. (10). One unit of amylase is defined as the amount of enzyme that forms 1 μmol of maltose per min. Specific activity is given as units per milligram of protein.

DNA procedures. Plasmid DNA of *S. lividans* was isolated as described by Hopwood et al. (12). Transformation of *N. lactamdurans* and isolation of plasmids from the transformants was as described previously (15). Digestions with restriction endonucleases, end filling with DNA polymerase (Klenow fragment), and resolution of DNA fragments by agarose gel electrophoresis were carried out by standard procedures (21).

RESULTS

Promoter-probe vectors containing the promoterless *amy* gene. Initial experiments revealed that *N. lactamdurans* has no

TABLE 1. Plasmids used in this study

Plasmid	Description	Reference(s)
pULVK2	Bifunctional *Nocardia-E. coli* plasmid; contains the origins of replication of an *Amycolatopsis* sp. and pBR322	15
pAM2	Derivative of pULVK2 containing the *amy* gene and its upstream promoter region	This work (Fig. 1)
pAM3	Same as pAM2, with the *amy* gene in the opposite orientation	This work (Fig. 1)
pAM3ΔP	Derivative of pAM3 by deletion of the 320-bp *Eco*RI fragment containing the *amy* promoter	This work (Fig. 1)
pULVK99	Bifunctional *E. coli-Streptomyces* positive selection vector containing the fd terminator; derivative of pIJ699	13, 13a
pAM3ΔPfd	Derivative of pAM3ΔP containing the fd transcriptional terminator subcloned from pULVK99	This work
pVK2ΔRI	Derivative of pULVK2 obtained by deleting one of the two *Eco*RI sites of pULVK2	This work
pAM2ΔRI	Derivative of pVK2ΔRI containing the *amy* gene and its upstream promoter region	This work
pAM2ΔPfd	Derivative of pAM2ΔRI in which the *amy* promoter region was replaced by the fd terminator	This work
pAM2PP	Derivative of pAM2ΔPfd with a synthetic polylinker upstream from the promoterless *amy* gene	This work
pAM2lat	Derivative of pAM2PP with the *lat* promoter expressing the *amy* gene	This work
pAM2cefD	Derivative of pAM2PP with the *cefD* promoter expressing the *amy* gene	This work
pAM2bla3	Derivative of pAM2PP with the *bla* promoter (three copies in tandem) expressing the *amy* gene	This work
pAM2bla1	Derivative of pAM2PP with the *bla* promoter (single copy) expressing the *amy* gene	This work
pULKS37	Plasmid containing the *cmcH* gene of *N. lactamdurans* in a 3.65-kb *Not*I fragment	9
pULJL1	pBluescript KS(+) derivative containing the *cmcH* gene in a 3.65-kb *Not*I fragment	This work
pULJL2	pBluescript KS(+) derivative containing the *cmcH* gene in a 2.63-kb *Pst*I fragment	This work
pULJL3	Derivative of pAM2PP with the *cmcH* upstream region expressing the *amy* gene	This work
pULJL4	Same as pULJL3, with the *cmcH* upstream region in the opposite orientation	This work

detectable extracellular amylase activity. Southern blot hybridizations with the *amy* gene of *S. griseus* as probe showed that no homologous gene was present in the DNA of *N. lactamdurans* (not shown).

The *S. griseus amy* gene (2.7-kb *Bgl*II fragment) contains its promoter region in a *Hin*dIII-*Eco*RI fragment that may be separated easily from the *amy* open reading frame (23). A 2.7-kb *Bgl*II fragment containing the *amy* gene (with its promoter region) was subcloned into the bifunctional *Nocardia-Escherichia coli* plasmid pULVK2 (15) in both orientations, yielding plasmids pAM2 (not shown) and pAM3 (Fig. 1). The 320-bp *Eco*RI fragment of pAM3 containing the *amy* promoter was removed to yield the promoter probe plasmid pAM3ΔP (Fig. 1). However, this plasmid still showed significant α-amylase activity (Fig. 2) due to readthrough expression from a promoter located in the pA-*rep* region required for replication of this plasmid (15). To prevent readthrough of the amylase gene from the pA-*rep* region, the fd transcriptional terminator of *E. coli* phage fd was subcloned from pULVK99 (Table 1) as a 1.1-kb *Eco*RI-*Eco*RV fragment in the *Eco*RI-*Eco*RV sites of pAM3ΔP to form plasmid pAM3ΔPfd. The construct pAM3ΔPfd was toxic when introduced in *N. lactamdurans*. The transformants obtained formed tiny colonies that did not grow to normal size.

As an alternative strategy to avoid readthrough from the promoter existing in the pA-*rep* region, the amylase gene was cloned in the opposite orientation in a modified pULVK2 named pVK2ΔR1. First, the *Eco*RI site of the polylinker of pULVK2 was removed (to leave a single *Eco*RI site in the plasmid), creating pVK2ΔR1, and then the complete *amy* gene of *S. griseus* (as a 2.7-kb *Bgl*II fragment) was cloned in the *Bam*HI site of pVK2ΔR1 and the correct orientation was selected, yielding pAM2ΔR1.

The *Dra*I-*Eco*RI fragment of pAM2ΔRI containing the *amy* promoter was removed and replaced by the 1.1-kb *Eco*RI-*Eco*RV fd terminator to create the promoter probe vector pAM2ΔPfd. An improved version of this promoter probe plasmid was obtained by inserting a synthetic polylinker containing five unique cloning sites (*Hin*dIII, *Bam*HI, *Sac*I, *Dra*I, and *Bgl*II) upstream of the promoterless *amy* gene and removing the *Hin*dIII fragment containing the fd terminator, originating the promoter probe vector pAM2PP (Fig. 1) (AM stands for amylase, and PP stands for promoter probe). Removal of the fd terminator greatly improved the stability of the new promoter probe vector pAM2PP. There was no significant background amylase activity in the promoter probe vectors pAM2ΔPfd and pAM2PP (with and without the fd terminator, respectively) either in solid or liquid medium (Fig. 2). Since the fd terminator was not required in these constructions, further studies were carried out with pAM2PP.

Comparison of the amylase levels synthesized by clones transformed with pAM3 (containing the entire *amy* gene with its native promoter) and pAM2PP (lacking the *amy* promoter) showed that the *amy* gene of *S. griseus* is very well expressed in *N. lactamdurans* from its native promoter (Fig. 2).

Characterization in *N. lactamdurans* of promoters of the cephamycin pathway using pAM2PP as promoter probe vector. The cephamycin biosynthetic genes (Fig. 3A) are transcribed in at least three transcriptional units (6a), but it was unclear whether the *cmcH* gene was expressed from a separate promoter. Several putative promoter regions of the cephamycin gene cluster were subcloned in the polylinker of pAM2PP, and the time course of amylase activity in cultures of transformants with each of the constructions was determined. All transcriptional fusions contained the ribosome binding site of the subcloned promoter region in addition to the ribosome binding site of the *amy* gene.

***lat* and *cefD* promoters.** The promoter regions of the *lat* and *cefD* genes of *N. lactamdurans*, which are located back-to-back, forming a bidirectional promoter region (6a, 8), were subcloned as a 700-bp *Nru*I-*Bst*EII fragment in pAM2PP, generating pAM2lat (with the *lat* promoter expressing the *amy* gene) and pAM2cefD (with the *cefD* promoter expressing the *amy* gene) (Fig. 3A). For these constructions the 700-bp *Nru*I-*Bst*EII fragment was first subcloned in pBluescript KS(+) and rescued with *Hin*dIII-*Bam*HI ends to insert it in pAM2PP.

***bla* promoter.** Similarly, the promoter of the *bla* gene (encoding the β-lactamase of the cephamycin cluster [7]) was subcloned as (i) a 510-bp *Eco*RI-*Xho*I segment in the *Dra*I site of pAM2PP after end filling and ligation, creating plasmid pAM2bla3, or (ii) a 310-bp *Eco*RI-*Pst*I fragment cloned in pBluescript KS(+), rescued as a *Hin*dIII-*Bam*HI fragment,

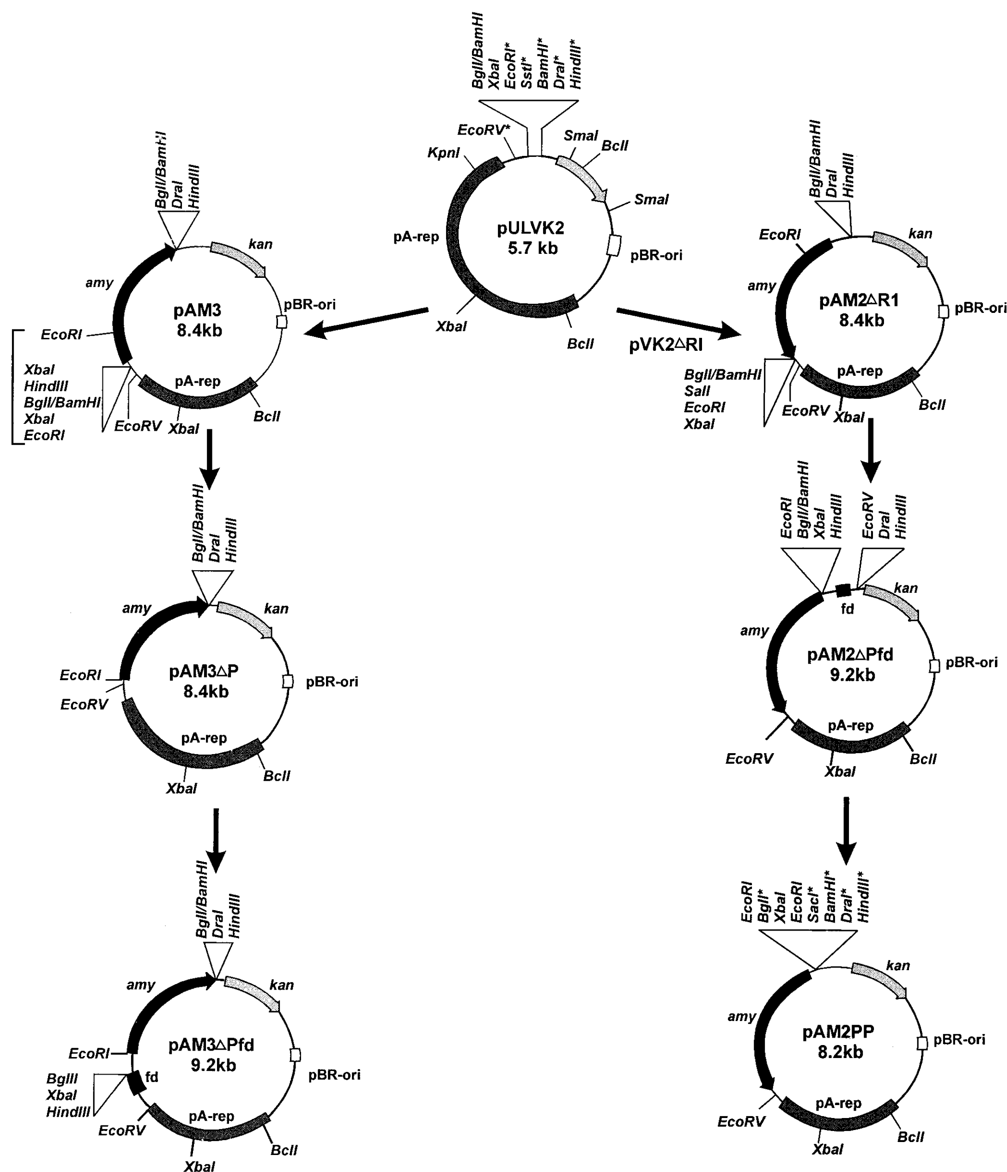

FIG. 1. Construction of different promoter probe plasmids using the promoterless *amy* gene as reporter. Plasmid pAM2 (not shown) is identical to pAM3 except that the *amy* gene is in the opposite orientation. Plasmids carrying the fd transcription terminator are designated with the abbreviation fd at the end of their names. ΔP indicates deletion of the promoter region of the *amy* gene. Note that pAM3ΔP was obtained by deleting the *Eco*RI fragment of pAM3 [bracket]. Asterisks in pULVK2 and pAM2PP, unique cloning sites; pA-rep, DNA fragment required for plasmid replication in *N. lactamdurans*; pBR-ori, origin of replication of pBR322 (see the text for details).

and subcloned in the *Hin*dIII-*Bam*HI sites of pAM2PP to create pAM2bla1 (Fig. 3A). After transformation of *N. lactamdurans* with the different constructs, amylase-producing transformants were selected. One of the clones from the first strategy selected at random was named pAM2bla3, and one clone from the second strategy was named pAM2bla1. *Mlu*I restriction fragment analysis and mapping of the inserts showed that pAM2bla3 contained three *bla* promoters in tandem whereas pAM2bla1 carried a single copy of the expected 310-bp fragment, expressing the *amy* gene.

Both the *lat* and the *cefD* promoter regions showed weak transcription initiation activity compared to the *bla* promoter in NYGly medium (Fig. 3B) and slightly better activity in defined Lechevalier medium (Fig. 3C). The *bla* promoter showed good transcription initiation ability in both Lechevalier and NYGly media. Plasmid pAM2bla3 showed clearly higher activity than pAM2bla1 in cultures at 24 h in NYGly medium, but activity was similar in Lechevalier medium. Since both plasmids contain the same *bla* promoter, it seems that the differences shown at 24 h in NYGly medium might be due to the three tandem copies of the promoter that exist in pAM2bla3. However, we cannot exclude the possibility that the different length of the upstream region may influence expression of the *amy* gene in pAM2bla1 and pAM2bla3.

All tested cephamycin promoters showed a clearly lower activity in *N. lactamdurans* than the amylase promoter of *S. griseus* (Fig. 3). Expression of the different cephamycin genes reached a peak at 24 h in complex NYGly medium, but the expression levels were maintained at 36 and 48 h in Lechevalier medium near peak levels. Expression of the *amy* native

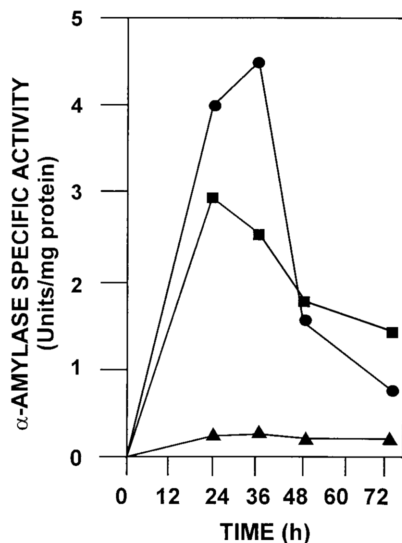

FIG. 2. α-Amylase activity in NYGly medium cultures of *N. lactamdurans* transformed with the promoter probe plasmids pAM3 (●), pAM3ΔP (■), and pAM2PP or pAM2ΔPfd (▲). Note that whereas pAM3ΔP transformants had a significant activity (due to readthrough from a promoter in the pA-rep region), no significant amylase activity was observed in transformants with the promoter probe plasmid pAM2PP. Data are averages of four determinations using duplicate flasks.

promoter reached a peak later than expression of the cephamycin promoters and remained at very high levels in Lechevalier medium up to 72 h.

***cmcH* promoter.** The putative *cmcH* promoter region was isolated from plasmid pULKS37, which carries a 3.65-kb *Not*I fragment of *N. lactamdurans* DNA containing the *cmcH* gene (7, 9), giving plasmid pULJ1. The *cmcH* gene was subcloned from pULJ1 as a 2.63-kb *Pst*I fragment in pBluescript KS(+), yielding pULJ2. The promoter region was rescued as an *Eco*RI fragment (1.37 kb) and digested with *Ava*I to obtain a 464-bp fragment that contained a 234-bp region upstream of the ATG translation initiation codon (Fig. 4). This region corresponds to the intergenic region between *cefF* and *cmcH* and also contains the 3′ end of the *cefF* gene (encoding deacetoxycephalosporin C hydroxylase) and an inverted repeat (IR in Fig. 4). The *Ava*I ends were filled with Klenow DNA polymerase and subcloned in both orientations in the *Dra*I site of the promoter probe vector pAM2PP, yielding pULJ3 and pULJ4.

Transformants with single and concatemeric copies of the promoter region in both orientations were used to transform *N. lactamdurans*. Amylase activity assays in both solid and liquid Lechevalier and NYGly media revealed that there was no promoter activity in the small intergenic region upstream of *cmcH*, since no significant amylase activity was observed in the transformants (see Discussion).

The *S. griseus* α-amylase is secreted and processed in *N. lactamdurans*. Studies of the expression in *N. lactamdurans* of the *S. griseus amy* gene with its native promoter (construct pAM2) showed that the amylase of *S. griseus* is very efficiently secreted in *N. lactamdurans*. As shown in Fig. 5, *N. lactamdurans* transformed with the *amy* gene secreted two enzymatically active forms. The proteins showed molecular masses of 57 and 50 kDa (by sodium dodecyl sulfate-polyacrylamide gel electrophoresis which were identical to those of the proteins formed in *S. griseus* and *S. lividans*. This result indicates that *N. lactamdurans* is able to secrete and to process the amylase, apparently by a processing mechanism identical to that in

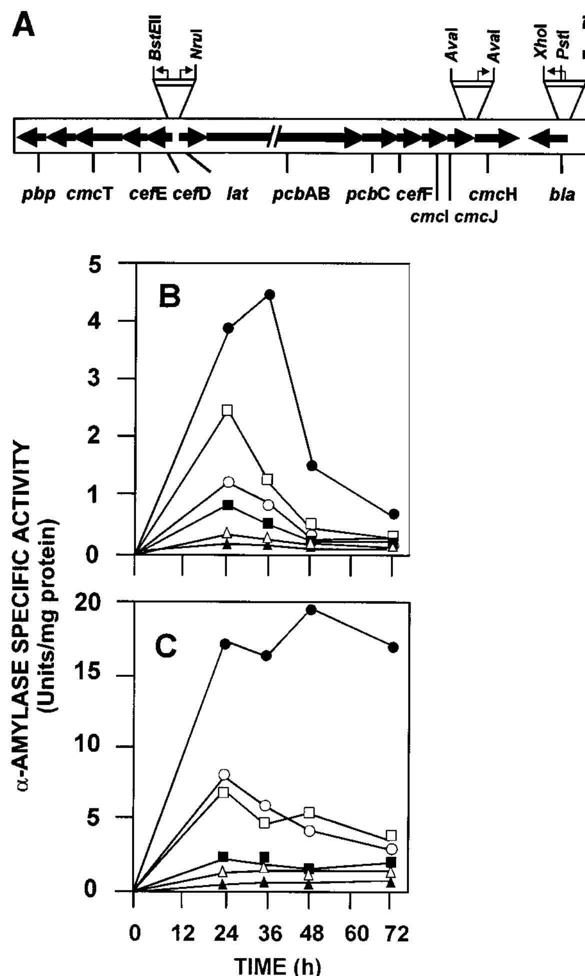

FIG. 3. (A) Restriction map of the 30-kb cephamycin gene cluster showing in the upper part the different promoter regions subcloned in pAM2PP. The orientation of the transcripts is indicated by bent arrows. The *pcbAB* gene (11.5 kb) is interrupted to save space. (B and C) α-Amylase activity in *N. lactamdurans* from different promoters of the cephamycin cluster in NYGly medium (B) and in defined Lechevalier medium (C): pAM2*cefD* (△), pAM2lat (■), pAM2bla1 (○), pAM2bla3 (□), pAM2 (native *amy* promoter) (●), pAM2PP (control without promoter), and pAM2cmcH (▲). Note the different scales of expression in panels B and C. The data are averages of four determinations using duplicate flasks.

Streptomyces species. The nontransformed *N. lactamdurans* lacked significant levels of endogenous amylase.

DISCUSSION

The efficient secretion of amylase and the formation of the 57- and 50-kDa forms from the preamylase in *N. lactamdurans*, as occurs also in *S. griseus* and *S. lividans*, suggests that all these actinomycetes are able to secrete the enzyme by a similar mechanism (2). In species of *Streptomyces* the processing of α-amylase is known to be mediated by intracellular specific proteolytic cleavage (10). The same proteolytic cleavage occurs in *N. lactamdurans* and also in corynebacteria (5). The amount of extracellular α-amylase is an indication of the level of expression of the reporter gene and can be used to compare different promoters.

The weak transcription initiation activity of the *lat* and *cefD* promoters using the reporter gene correlates well with their activity in the cell since the levels of the cephamycin biosyn-

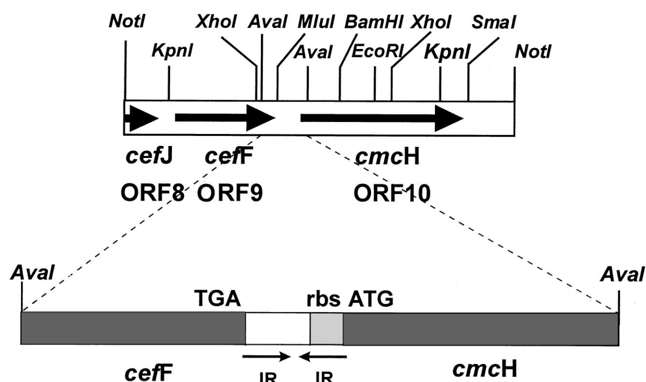

FIG. 4. Detailed map of the intergenic region between *cmcJ* and *cmcH*. Note the location of the inverted repeats (IR) between the *cefF* and *cmcH* genes and the ribosome binding site (rbs) immediately upstream of the ATG of the *cmcH* gene.

thetic enzymes encoded by these genes (lysine-6-aminotransferase and isopenicillin *N*-epimerase) are known to be very low in wild-type *N. lactamdurans* compared to another cephamycin-producing actinomycete, *Streptomyces clavuligerus* (17). The lack of expression of the *amy* reporter gene from the *cmcH* upstream region is in agreement with data about a polycistronic messenger in *N. lactamdurans* corresponding to several of the cephamycin genes (*lat*, *pcbAB*, *pcbC*, *cmcI*, *cmcJ*, *cefF*, and *cmcH*) (6a).

On the other hand, the *bla* promoter region which gives rise to a monocistronic mRNA for the *bla* gene has a stronger transcription initiation ability. It seems likely that cephamycin biosynthesis and β-lactamase production are strictly controlled to allow synthesis of the adequate levels of the antibiotic for survival or competition of the strains in nature. The *N. lactamdurans* β-lactamase was believed to play a role in resistance to β-lactam antibiotics in cephamycin-producing actinomycetes (7). It is involved in cell wall biosynthesis and morphogenesis in addition to its penicillin-inactivating activity, as shown in studies with β-lactamase-disrupted mutants (16). Expression of the cephamycin and β-lactamase genes may, therefore, be controlled to allow proper growth without damaging cell wall biosynthesis.

Promoters for cephamycin biosynthesis in *S. clavuligerus* (and probably also in *N. lactamdurans*) require a positively

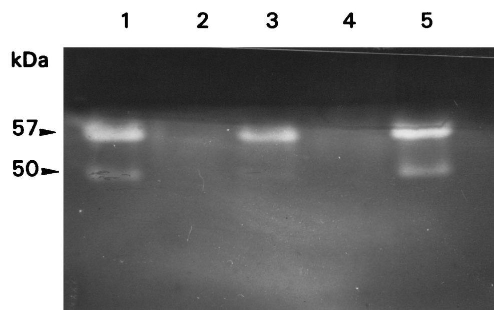

FIG. 5. Nondenaturing polyacrylamide gel electrophoresis showing the two active forms of α-amylase (57 and 50 kDa) encoded by the reporter *amy* gene after the gel was stained with starch and iodine. The stacking gel is stained in dark blue (upper part). Lanes: 1, enzymes secreted in *S. lividans*(pULTV1) (a transformant containing the *amy* gene with its native upstream region [23]); 2 and 4, control, *N. lactamdurans* LC411 (transformed with pULVK2 without an insert); 3 and 5, *N. lactamdurans* transformed with pAM3 (about 2.5 and 5 μg of protein, respectively). Sizes are indicated on the left.

acting regulatory protein, CcaR, that is limiting for cephamycin biosynthesis since amplification of the *ccaR* gene leads to cephamycin overproduction (19). Wild-type strains are likely to be limited in the availability of positively acting regulatory proteins. It is interesting that the promoter of the *amy* gene of *S. griseus* is used so efficiently in *N. lactamdurans*. The −10 and upstream regulatory regions of this gene are known (23–25) and are similar to those of primary metabolism genes (22), which explains its efficient recognition by the RNA polymerase of *N. lactamdurans*. These results suggest that cephamycin production may be improved by promoter replacement.

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REFERENCES

1. **Band, L., D. G. Yansura, and D. J. Henner.** 1983. Construction of a vector for cloning promoters in *Bacillus subtilis*. Gene **26:**313–315.
2. **Blanco, J., J. J. R. Coque, and J. F. Martín.** 1996. Characterization of the *sec*A gene of *Streptomyces lividans* encoding a protein translocase which complements an *Escherichia coli* mutant defective in the ATPase activity of SecA. Gene **176:**61–65.
3. **Boer, H. A.** 1984. A versatile plasmid system for the study of prokaryotic transcription signals in *Escherichia coli*. Gene **30:**251–255.
4. **Brosius, J.** 1984. Plasmid vectors for the selection of promoters. Gene **27:**151–160.
5. **Cadenas, R. F., J. A. Gil, and J. F. Martín.** 1992. Expression of *Streptomyces* genes encoding extracellular enzymes in *Brevibacterium lactofermentum*: secretion proceeds by removal of the same leader peptide as in *Streptomyces lividans*. Appl. Microbiol. Biotechnol. **38:**362–369.
6. **Clayton, T. M., and M. J. Bibb.** 1990. *Streptomyces* promoter-probe plasmids that utilize the *xyl*E gene of *Pseudomonas putida*. Nucleic Acids Res. **18:**1077.

6a. **Coque, J. J. R., F. J. Enquita, P. Liras, and J. F. Martín.** Unpublished data.

7. **Coque, J. J. R., P. Liras, and J. F. Martín.** 1993. Genes for a β-lactamase, a penicillin-binding protein and a transmembrane protein are clustered with the cephamycin biosynthetic genes in *Nocardia lactamdurans*. EMBO J. **12:**631–639.
8. **Coque, J. J. R., P. Liras, and J. F. Martín.** 1993. Characterization and expression in *Streptomyces lividans* of *cef*D and *cef*E genes from *Nocardia lactamdurans*: the organization of the cephamycin gene cluster differs from that in *Streptomyces clavuligerus*. Mol. Gen. Genet. **236:**453–458.
9. **Coque, J. J. R., F. J. Pérez-Llarena, F. J. Enguita, J. L. Fuente, J. F. Martín, and P. Liras.** 1995. Characterization of the *cmcH* genes of *Nocardia lactamdurans* and *Streptomyces clavuligerus* encoding a functional 3′-hydroxymethylcephem O-carbamoyltransferase for cephamycin biosynthesis. Gene **162:** 21–27.
10. **García-González, M., J. F. Martín, T. Vigal, and P. Liras.** 1991. Characterization, expression in *Streptomyces lividans*, and processing of the amylase of *Streptomyces griseus* IMRU 3570: two different amylases are derived from the same gene by an intracellular processing mechanism. J. Bacteriol. **173:**2451–2458.
11. **Ginther, C. L.** 1979. Sporulation and serine protease production by *Streptomyces lactamdurans*. Antimicrob. Agents Chemother. **15:**522–526.
12. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
13. **Kieser, D., and R. D. Melton.** 1988. Plasmid pIJ699, a multicopy positive selection vector for *Streptomyces*. Gene **65:**83–91.

13a. **Kumar, C. V., and J. F. Martín.** Unpublished data.

14. **Kumar, C. V., and J. F. Martín.** 1994. Thiostrepton induced proteins in *Streptomyces*, *Amycolatopsis* and *Nocardia* species. FEMS Microbiol. Lett. **118:**107–112.
15. **Kumar, C. V., J.-J. R. Coque, and J. F. Martín.** 1994. Efficient transformation of the cephamycin C producer *Nocardia lactamdurans* and development of shuttle and promoter-probe cloning vectors. Appl. Environ. Microbiol. **60:**4086–4093.
16. **Kumar, C. V., J. L. de la Fuente, A. L. Leitão, P. Liras, and J. F. Martín.** 1996. Effect of amplification or targeted disruption of the β-lactamase gene of *Nocardia lactamdurans* on cephamycin biosynthesis. Appl. Microbiol. Biotechnol. **45:**621–628.
17. **Martín, J. F., and P. Liras.** 1989. Enzymes involved in penicillin, cephalosporin and cephamycin biosynthesis. Adv. Biochem. Eng. Biotechnol. **39:** 153–187.

18. **Martín, J. F., and L. McDaniel.** 1976. Biosynthesis of candicidin by phosphate-limited resting cells of *Streptomyces griseus*. Eur. J. Appl. Microbiol. **3:**135–144.
19. **Pérez-Llarena, F. J., P. Liras, A. Rodríguez-García, and J. F. Martín.** 1997. A regulatory gene (*ccaR*) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: amplification results in overproduction of both β-lactam compounds. J. Bacteriol. **179:**2053–2059.
20. **Rosenberg, M., A. B. Chepelinsky, and K. McKenney.** 1983. Studying promoters and terminators by gene fusion. Science **222:**734–739.
21. **Sambrook, J., E. F. Fritsch, and J. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Strohl, W. R.** 1992. Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nucleic Acids Res. **20:**961–997.
23. **Vigal, T., J. A. Gil, A. Daza, M. D. García-González, and J. F. Martín.** 1991. Cloning, characterization and expression of an α-amylase gene from *Streptomyces griseus* IMRU 3570. Mol. Gen. Genet. **225:**278–288.
24. **Vigal, T., J. A. Gil, A. Daza, M. D. García-González, P. Villadas, and J. F. Martín.** 1991. Effects of replacement of promoters and modification of the leader peptide region of the *amy* gene of *Streptomyces griseus* on synthesis and secretion of α-amylase by *Streptomyces lividans*. Mol. Gen. Genet. **231:** 88–96.
25. **Virolle, M.-J., and J. Gagnat.** 1994. Sequences involved in growth phase-dependent expression and glucose repression of a *Streptomyces* α-amylase gene. Microbiology **140:**1059–1067.