The Biosynthetic Pathway of the Aminonucleoside Antibiotic Puromycin, as Deduced from the Molecular Analysis of the pur Cluster of Streptomyces alboniger*

(Received for publication, July 24, 1995, and in revised form, October 23, 1995)

José A. Tercero*, J. Carlos Espinosa, Rosa A. Lacalle*, and Antonio J. Imenez†

From the Centro de Biología Molecular “Severo Ochoa”, Consejo Superior de Investigaciones Científicas and Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain

The pur cluster which encodes the puromycin biosynthetic pathway from Streptomyces alboniger was subcloned as a 13-kilobase fragment in plasmid pJ1702 and expressed in an apparently regulated manner in the heterologous host Streptomyces lividans. The sequencing of a 9.1-kilobase DNA fragment completed the sequence of pur. This permitted identification of seven new open reading frames in the order: napH, pur7, pur10, pur6, pur4, pur5, and pur3. The latter is followed by the known pac, dmpM, and pur8 genes. Nine open reading frames are transcribed rightward as a unit in opposite direction to that of the pur8 gene, which is expressed as a monocistronic transcript from the rightmost end. napH encodes the known N-acetylpuromycin N-acetylhydrolase. The deduced products from other open reading frames present similarities to: NTP pyrophosphohydrolases (pur7), several oxidoreductases (pur10), the putative LmbC protein of the lincomycin biosynthetic pathway from Streptomyces lincolnensis (pur6), S-adenosylmethionine-dependent methyltransferases (pur5), a variety of presumed aminotransferases (pur4), and several monophosphatases (pur3). According to these similarities and to previous biochemical work, a puromycin biosynthetic pathway has been deduced. No cluster-associated regulatory gene was found. However, both pur10 and pur6 genes contain a TTA codon, which suggests that they are translationally controlled by the bidA gene product, a specific tRNA

Nucleoside antibiotics constitute an important group of microbial secondary metabolites some of which are effective agents against plant and human diseases. Examples are ribavirin (antiviral), polyoxins (herbicides), and milidomycin (plant antifungal antibiotic). Given the key role accomplished by nucleosides and nucleotides in biochemical processes, the nucleoside antibiotics have found a fundamental application as specific inhibitors of a high variety of biochemical reactions (for a review, see Ref. 1). These features rise up the question of how the producing organisms defend themselves against the toxic effects of their products and, in most cases, of the relevant biosynthetic precursors.

Puromycin is an aminocycl nucleoside antibiotic produced by Streptomyces alboniger. It is a broad spectrum secondary metabolite active against Gram-positive bacteria, protozoans, and mammalian cells, including tumor cells. It has been a key compound in various cell-free systems directed to elucidate the mechanism of protein synthesis and the mode of action of other inhibitors of this process (2, 3). Concerning the biosynthetic pathway of puromycin, some data are available. Adenosine is known to be a direct precursor for the 3'-amino-3'-deoxyadenosinemoiety of puromycin (4). Moreover, a commercial sample of puromycin was found to be contaminated by small amounts of N6,N1,O-tridemethylpuromycin, N6,O-didemethylpuromycin, and O-demethylpuromycin. This led to the proposal that puromycin biosynthesis probably proceeds through these intermediates and in that order (5). Three enzymes of the pathway, an O-demethylpuromycin O-methyltransferase (DmpM),1 a puromycin N-acetyltransferase (Pac), and an N-acetylpuromycin N-acetylhydrolase (NapH) have been characterized (6–8). Biochemical studies suggest that Pac inactivates the intermediate N6,N1,O-tridemethylpuromycin by acetylation, and that DmpM methylates N-acetyl-O-demethylpuromycin to form N-acetylpuromycin (7). The latter would be excluded from the cells and then hydrolyzed by the extracellular NapH activity (8) yielding the biologically active puromycin antibiotic. In addition to Pac, the putative transmembrane protein Pur8 was shown to confer resistance to puromycin in Streptomyces lividans and, consequently, in S. alboniger, possibly by promoting an active efflux energized by a proton-dependent electrochemical gradient (9). The complete set of genes (15 kb) encoding the puromycin biosynthetic pathway (pur cluster) from S. alboniger has been cloned in low-copy number cosmids and expressed in a regulated pattern in heterologous hosts, S. lividans and Streptomyces griseofuscus (10). Three genes of pur have been sequenced, pac, dmpM, and pur8, which encode Pac, DmpM, and Pur8, respectively (9, 11, 12). The three genes are contiguously located at the right end of the cluster and in this order. In addition, the napH gene, located at the left end of pur, was isolated and its product (NapH) characterized (8). Here, we report the completion of the nucleotide sequence of pur, an analysis of its transcriptional organization, and a novel proposal for the puromycin biosynthetic pathway.

1 The abbreviations used are: DmpM, O-demethylpuromycin O-methyltransferase; Pac, puromycin N-acetyltransferase; NapH, N-acetylpuromycin N-acetylhydrolase; kb, kilobase pair(s); ORF, open reading frame; AdoMet, S-adenosylmethionine; bp, base pair(s).
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**Fig. 1.** Restriction maps of the DNA inserts from several constructs, puromycin production phenotype of *S. lividans* transforms and organization of the ORFs of pur. A, the restriction maps of cosmids pPB5.13, pPB4.6, and pPB11.40 and plasmid pCSX were modified from (10). Plasmid pRPC11 was obtained by subcloning the 13-kb ClaI-EcoRI (labeled E*) in other restriction maps as well (data shown). From the resulting plasmid, this fragment was isolated as a KpnI-PstI piece, which was finally inserted in the KpnI-EcoRI replication fragment of plj 702, and + and – indicate production or no production of puromycin by the relevant *S. lividans* transforms, respectively. Enzymes: C, Clal; E, EcoRI; K, KpnI; N, NcoI; P, PstI; S, SphI; X, XhoI; XbaI, B, ORFs and transctional organization of the pur cluster. The incomplete orfA and orf1 are interrupted by the Clal and EcoRI sites, respectively. The gray region indicates the sequence obtained in this work. Only relevant restriction sites are indicated. Size of the ORFs is indicated by small arrows drawn immediately below. Lines a, b, c, and d indicate the DNA fragments used as probes in low resolution S1 protection assays. Above them, continuous arrows indicate the size and direction of clearly identified transcripts, whereas dotted arrows indicate possible transcripts.

**MATERIALS AND METHODS**

Strains, Plasmids, Media, and DNA Methodology—*S. alboniger* ATCC12461, the puromycin producer (13), *S. lividans* 66 (1326) (14), and *Escherichia coli* strains DH5 (15), TG1 (16), and GM119 (17), are described in the indicated references. Streptomycetes plasmid was plj 702 (18). *E. coli* vectors were “BlueScript” SK– (Stratagene), pUC19 (19), and M13 mp18/M13 mp19 (20). Plasmid DNA from Streptomycetes and *E. coli* was prepared as described (14). Growth of Streptomycetes on solid media was carried out on R5 (14). Liquid media for Streptomycetes were YEME containing 34% sucrose and 5 mM MgSO4, or puromycin-producing *E. coli* carrying pRCP11, puromycin production start at the middle of the log-phase, similarly to *S. lividans*. Puromycin was produced in the high-copy number plasmid plj 702. The resulting construct (pRPC11; Fig. 1) was introduced into *S. lividans*. Puromycin was present in the fermentation broth of a relevant transformant, as determined by TLC and Pac assays (not shown), which indicates that all the structural genes for puromycin production are present in the 13-kb ClaI-E fragment. Moreover, the amount of puromycin produced in *S. lividans*(pRPC11) is similar to that in a control *S. lividans*(pPB5.13) (Fig. 1 and data not shown). In transformants carrying pRPC11, puromycin production starts at the middle of the log-phase, similarly to *S. lividans*(pPB5.13) and *S. alboniger* (10). In the particular case of *S. lividans*(pRPC11) this cannot be attributed to a variation of plasmid copy number structure, since these apparently remained unchanged during all stages of the growth curve (not shown). Therefore, it appears that in *S. lividans* expression of the pur cluster from pRPC11 is regulated in a similar manner than in *S. alboniger* or *S. lividans*(pPB5.13) (10).

**Sequence of pur**—Starting at the ClaI site (Fig. 1), a total of 9.12 kb was sequenced (Fig. 2). The sequence covered the
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Fig. 2—continued
unknown pur region and overlapped with the known sequence of pac (Ref. 11; Fig. 1). It contained seven complete and one incomplete ORFs (Figs. 1 and 2). All shared a codon usage and a G+C content at the third position typical of Streptomyces (28), with an exception made of the 5′-end of the napH coding sequence (see below). From left to right these ORFs were named/identified as: orfA, napH, pur7, pur10, pur6, pur4 (previously named prg1; Ref. 10), pur5 and pur3. The incomplete orfA probably did not pertain to the cluster, because its incompleteness did not prevent puromycin production from pRCP11 (Fig. 1). Therefore, the left end of the cluster should reside between orfA and napH. Similarly, in plasmids pPB11.40 and pRCP11 (Fig. 1), 5′ to pur8 there is another incomplete ORF (orf1) (Ref. 9; Fig. 1), which indicates that the right end of pur lies between these two ORFs. Several characteristics of the intact ORFs are indicated in Table I.

Previously, it was shown that the napH gene is part of a 2.5-kb SphI-NcoI fragment from the left end of pur (8). The single complete ORF of 1458 nucleotides found in this fragment (Fig. 2) was, therefore, attributed to napH. Its deduced product has 485 amino acids (Table I). Both upstream and downstream of the chosen initiator codon there are other putative initiator codons in bold letters. An inverted repeat located 3′ to pur5, which could form a stem-loop for transcription termination is indicated by horizontal arrows. Putative –10 and –35 regions of napH are indicated. Possible transcription initiation and termination sites are indicated by bold triangles and an open triangle, respectively. Restriction sites with an asterisk are not unique in the sequence DNA; they are referred to in the text. The 5′ region of the previously reported pac sequence (11), which has been revised in this work, is indicated by small letters.

**Fig. 2. Nucleotide sequence of a 9.12-kb DNA fragment from the pur cluster.** The deduced gene products are indicated in the one-letter code under the DNA sequence. Possible ribosome binding sites (rbs) are indicated by dotted lines. Putative translation initiation codons are in bold letters. The start and direction of each of the ORFs are indicated by up arrows and named accordingly. A presumptive signal peptide of NapH is underlined and its putative cleavage site is indicated by a vertical arrow. A proposed motif for Pur10 and similar oxidoreductases is underlined. An inverted repeat located 3′ to pur5, which could form a stem-loop for transcription termination is indicated by horizontal arrows. Putative –10 and –35 regions of napH are indicated. Possible transcription initiation and termination sites are indicated by bold triangles and an open triangle, respectively. Restriction sites with an asterisk are not unique in the sequence DNA; they are referred to in the text. The 5′ region of the previously reported pac sequence (11), which has been revised in this work, is indicated by small letters.
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TABLE I
Relevant features of the ORFs described in this paper

| ORF | rbs | Start/stop codons | Amino acids/Mr | pI | Predicted/known functions |
|-----|-----|-------------------|---------------|----|--------------------------|
| napH | 711GGGGGA<sup>735</sup> | GTG<sup>749</sup>/752 TGA | 485/51,36 | 6.41 | Hydrolase |
| pur7 | 233GGAGGAGG<sup>757</sup> | ATG<sup>773</sup>/776 TGA | 152/16,82 | 5.35 | NTP-pyrophosphohydrolase |
| pur10 | 2699GGGGGA<sup>705</sup> | ATG<sup>737</sup>/740 TGA | 339/38,34 | 7.77 | Oxidoreductase |
| pur6 | 3752GGAGGAGG<sup>737</sup> | GTG<sup>756</sup>/760 TGA | 772/83,73 | 7.20 | Synthetase |
| pur4 | 6126GGAGGAAG<sup>2133</sup> | ATG<sup>2151</sup>/2154 TGA | 429/46,52 | 6.28 | Aminotransferase |
| pur5 | 7469GGGGGA<sup>475</sup> | ATG<sup>484</sup>/487 TGA | 228/24,90 | 4.86 | N-Methyltransferase |
| pur3 | 8227GGAGGAGG<sup>445</sup> | ATG<sup>825</sup>/828 TGA | 273/29,07 | 4.98 | Monophosphatase |

* rbs indicates ribosomal binding site.
with other proteins of the family. As indicated by Neuwald et al. (60), most proteins carrying these two domains could be phosphatases. This activity could, therefore, be attributed to Pur3.

Transcript Mapping—Previous work showed that the pac and dmpM genes were transcribed in a single RNA which terminated 3' to the second gene (12). The sequence reported here and elsewhere (11, 12) shows that nine ORFs of pur, closely linked to each other, share the same direction of transcription (Fig. 1). Moreover, an inverted repeat, which could form a strong stem-loop of $\Delta G = -35.2$ kcal/mol (66), is located 3' to pur5 (Fig. 2). It could act as a transcription termination structure similar to that found for other Streptomyces genes (67, 68). Other putative transcription termination structures were previously found 3' to dmpM and pur8 (9, 12). To determine the transcriptional organization of the pur gene cluster, total RNA was isolated from a mid-log phase culture of S. alboniger, when puromycin was actively synthesized. Initially, a broad localization of possible transcription initiation sites was carried out by low resolution S1 protection assays with probes indicated in Fig. 1. The results suggested that a transcript, which started 5' to naph and covered all nine ORFs with the same direction of transcription was present. In addition, transcription initiation between pur5 and pur3 was also detected (not shown). The clear lack of transcription initiation between pur3 and pac is in contrast to previous data, which indicated both promoter activity and transcription initiation 5' to pac (11). However, these data were obtained from S. lividans transfor- mants which contained the pac gene inserted in plasmid vectors and were grown in a different culture medium than the one used here. It is possible, therefore, that we have been unable to detect those events in S. alboniger.

A higher resolution study was also carried out using S1 protection experiments to confirm the existence of the transcription start points detected by low resolution nuclease-S1 assays. To locate the transcription initiation site 5' to naph, we used a probe (Fig. 6) covering its 5' coding region and the noncoding region between this gene and orfA. The nuclease assay showed a protected fragment of about 500 bp (Fig. 6), which indicated that naph transcription started around position 679, 41 bp upstream of the translational initiator GTG (Fig. 2). Possible 10- and 35 regions are indicated in Fig. 2. To identify a possible transcription initiation site 5' of pur3, the probe used (Fig. 6), covered the 5' end of this ORF and the region between pur5 and pur3. A protected fragment of approximately 290 bp (Fig. 6) suggested the presence of a transcription initiation signal around position 8210, 43-bp upstream of the pur3 initiator ATG (Fig. 2). No clear 10- and 35 regions were found. Transcription initiation upstream of pur8 was examined using a probe that covered the 5' end of pur8 and all the noncoding region up to orf1 (9). A protected fragment of 124
transcription initiation occasionally occurs at the region close to the 5'-end of the stem-loop. The protected band of approximately 512 bp may correspond to transcription termination close to the 5'-end of the 9

The stem-loop 3' to pur5 referred to above suggested that transcription termination could take place at this region. To examine this possibility, a high resolution S1 protection experiment was performed using a probe (Fig. 6) that comprised all the DNA between pur5 and pur3, including the stem-loop (Fig. 2), plus a Bluescript tail. The results indicated the presence of major protected fragments of approximately 840 and 512 bp, and minor protected ones of 535–545 and 1080 bp sizes (Fig. 6). The 1080-bp fragment corresponds to probe/probe reannealing. The 840-bp fragment corresponds to full protection of the probe minus the non-homologous plasmid sequence, thus confirming the existence of a transcript that extends through the stem-loop into pur3. The protected band of approximately 512 bp may correspond to transcription termination close to the 5'-end of the stem-loop. The minor protected bands suggest that transcription termination occasionally occurs at the region close to or covering the loop located within the inverted repeat 3' to pur5. However, since these protected fragments have not been found to the putative transcription terminator, it is questionable that these terminations really take place (see "Discussion"). Therefore, these studies and those reported elsewhere (12) show that the pur cluster is transcribed into at least two mRNA species: one of them is a polycistronic transcript that covers nine ORFs, and the other one is a monocistronic transcript. The expression of most ORFs of pur as a polycistronic transcript is in agreement with several related clusters for antibiotic biosynthesis (69–71). As indicated elsewhere (71), these polycistronic transcriptsshould facilitate the regulation of antibiotic biosynthesis improving its efficiency. In addition, two other transcripts, comprising napH through pur5 and pur3 through dmpM, respectively (Fig. 1).

DISCUSSION

Gene Organization of the Pur Cluster—This paper shows that the genes of the pur cluster, which determines the puromycin biosynthetic pathway of S. alboniger, are located in a single DNA fragment of approximately 13 kb. It comprises 10 ORFs, nine of which are contiguous and have the same direction of transcription and only one (pur8), located at the right end of the cluster, is transcribed in the opposite direction. The different enzymic steps of this pathway should be assigned to these ORFs. In fact, the biochemical studies of some of the expressed proteins (5, 7, 8), together with the similarities of the deduced gene products with known proteins, have allowed the proteins to attribute specific functions to all the genes of the cluster. As suggested by others, the possibility that some unlinked structural gene(s) could play a role in the biosynthesis of this antibiotic should not be excluded (47, 69). If so, such gene(s) should also be present in S. lividans, since puromycin production was achieved in this organism carrying the 13-kb fragment. The analysis of the biosynthetic gene cluster for puromycin is of special interest since it is the first one to be isolated for a nucleoside antibiotic. It should serve as a model system for related clusters.

Transcription of the Pur Cluster—The transcription analyses of the pur cluster described here and elsewhere (12) indicate the existence of a polycistrionic transcript which starts 5' to napH and terminates 3' to dmpM and comprises all ORFs of pur, except pur8, which is transcribed in the opposite direction as a monocistrionic transcript. The expression of most ORFs of pur as a polycistrionic transcript is in agreement with several other clusters for antibiotic biosynthesis (69–71). As indicated elsewhere (71), these polycistrionic transcripts should facilitate the regulation of antibiotic biosynthesis improving its efficiency. In addition, two other transcripts, comprising napH through pur5 and pur3 through dmpM, respectively, may be synthesized in the pur cluster. Nevertheless, as indicated above, the transcription terminator function of the stem-loop located between pur5 and pur3, which would explain the exist-
ence of these two transcripts, may be questioned. Therefore, transcription termination 3' to pur5 and transcription initiation 5' to pur3 may not occur in vivo. An alternative role for this stem-loop might be to confer variable stability to different segments of the polycistrionic transcript (71). Finally, additional promoters active under certain physiological conditions and that could contribute to the regulation of pur expression should not be discarded.

The Puromycin Biosynthetic Pathway—Sequencing of a gene cluster provides an analysis of its molecular organization and an insight, by sequence comparison with data banks, into the functions of many of its putative gene products. Moreover, if additional biochemical data are available, this insight can be achieved with a high degree of certainty. The rationale to propose the initial steps of the puromycin biosynthetic pathway from S. alboniger takes into account the finding that [U-14C]-adenosine is a direct precursor of the 3'-amino-3'-deoxyadenosine moiety of puromycin (4). The 3' addition of an amino group to the ribose moiety of adenosine should take place, similarly to other deoxysugars, through a 3'-keto intermediate (for a review, see Ref. 55). Therefore, in the puromycin pathway this intermediate should be 3'-keto-3'-deoxyadenosine, which by means of an aminotransferase would be converted to 3'-amino-3'-deoxyadenosine. However, in both Gram-positive and Gram-negative bacteria and in Ehrlich ascites tumor cells, 3'-amino-3'-deoxyadenosine is triphosphorylated by adenosine kinase, producing 3'-amino-3'-deoxy-ATP, a strong inhibitor of DNA-dependent RNA polymerase (72–74). Therefore, it seems likely that, if produced, 3'-keto-3'-deoxyadenosine would also be 5'-triphosphorylated. The resulting product, 3'-keto-3'-deoxy-ATP, could also be highly toxic. If so, S. alboniger should have a means to prevent the harmful effects of these putative intermediates. This could be either a resistant RNA polymerase or an adenosine kinase which does not recognize the 3'-keto-3'-deoxyadenosine, which, by means of an aminotransferase, would be converted to 3'-amino-3'-deoxyadenosine. Puromycin production would also be 5'-triphosphorylated. The resulting product, 3'-keto-3'-deoxy-ATP, could also be highly toxic. If so, S. alboniger should have a means to prevent the harmful effects of these putative intermediates. This could be either a resistant RNA polymerase or an adenosine kinase which does not recognize the 3'-keto-3'-deoxyadenosine.
The MutT protein from E. coli and humans hydrolyses 8-oxo-dGTP producing PP, and an inactive 8-oxo-dGMP (41, 75). Therefore, 3'-keto-3'-deoxy-AMP could be inactivated by Pur7 producing a supposedly nontoxic 3'-keto-3'-deoxy-AMP and PP. This proposal is also based on the finding that the mono- and diphosphorylated derivatives of 3'-amino-3'-deoxy-AMP lack biological activity (76, 77). Therefore, 3'-keto-3'-deoxy-AMP would then be the substrate for an aminotransferase to produce a non-toxic 3'-amino-3'-deoxy-AMP. In this way, the initial steps of the pathway could proceed through inactive intermediates. Because puromycin biosynthesis initiates when growth is still maintained (7), adenosine would preferentially be converted into ATP rather than into 3'-keto-3'-deoxyadenosine. Therefore, it seems plausible to propose that adenosine would enter the pathway via ATP (compound I; Fig. 7). If so, the latter would be converted by the putative oxidoreductase Pur10 which could be its cofactor; Ref. 55) into 3'-keto-3'-deoxy-ATP (compound II; Fig. 7), which would be hydrolyzed by Pur7 to yield 3'-keto-3'-deoxy-AMP (compound III; Fig. 7). This intermediate would then be modified by the presumptive aminotransferase Pur4 to produce 3'-amino-3'-deoxy-AMP (compound IV; Fig. 7). As described for aminotransferases which are implicated in amino sugar biosynthesis in Gram-negative bacteria (78), Pur4 would be a pyridoxal phosphate/L-glutamate-L-glutamine-dependent enzyme (54, 56). Assuming that the pathway proceeds in this manner, the 5'-phosphogroup should be eliminated at some step. This hydrolysis could be carried out by Pur3, which presents significant similarities to a variety of monophosphatases (Fig. 5). However, it should not be at this stage of the pathway because the resulting intermediate would be phosphorylated back to 3'-amino-3'-deoxy-ATP. Therefore, Pur3 should act on a subsequent intermediate.

It has been proposed that tyrosine would be attached by its COOH group to the 3'-NH2 group of 3'-amino-3'-deoxyadenosine to produce N6,N6,O-tridemethylpuromycin (5, 7). According to our proposal, formation of this bond should take place on the 5'-phosphoderviative (Fig. 7), thus removing it from the nucleotide pool to prevent any additional phosphorylation. This reaction would be equivalent to a peptide bond formation step, which would previously require an activation of tyrosine. Amino acid activation is performed by aminoacyl-tRNA synthetases, antibiotic peptide synthetases, the bacterial peptidoglycan precursor synthetases, and condensing enzymes of the β-lactams antibiotics (79–82). Assuming that the relevant gene(s) is(are) not on a separate locus(loci), the only putative enzyme, by elimination, of the puromycin biosynthetic pathway to which this function could be attributed is Pur6.

Although it presents low level similarities to these enzymes and apparently lacks any AMP binding domain, this might not be an impeding difficulty. Thus, LeuS, a leucyl-tRNA synthetase, has low similarities to members of the peptide antibiotic synthetases and lacks an apparent AMP binding site (83). The only ORF from data banks which presents a similarity to Pur6 is LmbC from the lincomycin biosynthetic gene cluster. This ORF appears to belong to the peptide synthetase family of proteins. It has been proposed to be an activating enzyme of either tyrosine, before conversion to L-dihydroxyphenylalanine, or propylproline (a tyrosine-derivative intermediate), prior to condensation at the -NH2 group of the sugar moiety of lincomycin (47). In our case, the activation of tyrosine could be performed by a different enzyme (i.e. a tyrosinyl-tRNA synthetase) and Pur6 might only catalyze its linkage to 3'-amino-3'-deoxy-AMP (compound V; Fig. 7). Given the rarity of the linkage attributed to Pur6, a sequence deviation from peptide bond forming enzymes should not be surprising. Indeed, Pur6 could be a member of a variety of enzymes which are implicated in the biosynthetic pathways of certain nucleoside antibiotics of bacterial and fungal origin, like chrisandin, A201A, and the agricultural fungicides polyoxins, where a variety of polycarbon chains are attached to the amino-ribofuranosyl moieties by forming a peptide bond-like linkage (1).

It has been proposed that N6,N6,O-tridemethylpuromycin is dimethylated at N6 (5). However, the resulting intermediate inhibits protein synthesis (84), and to prevent it, a Pac-dependent inactivation by N-acetylation of the -NH2 group of the tyrosinyl moiety of tridemethylpuromycin has been suggested (7). According to our proposal, this acetylation should take place on compound V (Fig. 7). The resulting intermediate (compound VI; Fig. 7) would be N6,N6,O-tridemethylpuromycin-5'-phosphate (compound V; Fig. 7). Given the rarity of the linkage attributed to Pur6, a sequence deviation from peptide bond forming enzymes should not be surprising. Indeed, Pur6 could be a member of a variety of enzymes which are implicated in the biosynthetic pathways of certain nucleoside antibiotics of bacterial and fungal origin, like chrisandin, A201A, and the agricultural fungicides polyoxins, where a variety of polycarbon chains are attached to the amino-ribofuranosyl moieties by forming a peptide bond-like linkage (1).

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compound VI (Fig. 7). The resulting N-acetyl-O-demethylpuromycin-5'-phosphate (compound VIII, via compound VII; Fig. 7) would be O-methylated by DmpM (5, 7). However, the affinity of DmpM for N-acetyl-O-demethylpuromycin (compound IX; Fig. 7) is very high ($K_m = 2.3 \mu M$), which suggests that this, instead of VIII, is the real substrate for this enzyme. Therefore, the 5'-phosphate group could be removed by Pur3 prior to O-methylation. If so, this removal could take place even at an earlier step (i.e. from compound V; Fig. 7). This sequence of reactions would produce N-acetylpuromycin (compound X; Fig. 7), which would be secreted to be N-hydrolyzed by NapH, the puromycin (compound XI; Fig. 7) activating enzyme (Ref. 8; Fig. 7).

Regulation of the Pur Cluster—It has been surprising not to find evidence for a pathway-specific regulatory gene associated to the pur cluster. While this is also the case for the ttn biosynthetic pathway isolated from the tetracyclomycin producer, Streptomyces glaucescens (69), many antibiotic biosynthetic gene clusters have a transcriptional activator associated with them (85). Given that puromycin production is clearly necessary and, indeed, may even be widespread among actinomycetes. The isolation and characterization of these genes may be of interest, since it suggests that both genes are controlled by the product of the bldA gene (43). Interestingly, according to our proposal for puromycin biosynthesis, Pur10 would catalyze the first step of the pathway, and it is tempting to speculate on the role of translational regulation in expression of the pur cluster.

Acknowledgments—We thank A. Martín for expert technical assistance and S. J. Lucania for the gift of thiostrepton.

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