[^{3}H]-*p*-Azidopuromycin Photoaffinity Labeling of *Escherichia coli* Ribosomes: Evidence for Site-Specific Interaction at U-2504 and G-2502 in Domain V of 23S Ribosomal RNA†

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Received October 14, 1987; Revised Manuscript Received January 22, 1988

ABSTRACT: Previously we (1) showed that [^{3}H]-*p*-azidopuromycin was a functional puromycin analogue that, on photolysis in the presence of 70S ribosomes from *Escherichia coli*, photoincorporated site specifically into proteins L23, L18/22, and L15 [Nicholson, A. W., Hall, C. C., Strycharz, W. A., & Cooperman, B. S. (1982) *Biochemistry 21*, 3809–3817] and (2) used immunoelectron microscopy to localize the principal sites of *p*-azidopuromycin photoincorporation within the 50S subunit [Olson, H. M., Nicholson, A. W., Cooperman, B. S., & Glitz, D. G. (1985) *J. Biol. Chem. 260*, 10326–10331]. These studies are here continued by identification of the principal sites of [^{3}H]-*p*-azidopuromycin photoincorporation into ribosomal RNA. The major portion of such photoincorporation, 72%, takes place into 23S rRNA. Analysis by hybridization of the photoaffinity-labeled rRNA to restriction enzyme fragments of plasmid pKK3535, which contains *rrn*B DNA, using a refinement of a recently developed methodology [Hall, C. C., Smith, J. E., & Cooperman, B. S. (1985) *Biochemistry 24*, 5702–5711], shows that the most prominent [^{3}H]-*p*-azidopuromycin photoincorporation occurs within bases 2445–2668 in domain V [Noller, H. F. (1984) *Annu. Rev. Biochem. 53*, 119–162] of 23S rRNA. Photoincorporation into this region is site specific, as demonstrated by the decrease in photoincorporation of radioactivity when unlabeled puromycin is included in the photolysis solution. Significant site-specific photoincorporation also occurs within bases 489–681 in domain II of 23S RNA. Further localization, by the method of reverse transcriptase primer extension [Barta, A., Steiner, G., Brosius, J., Noller, H. F., & Kuechler, E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3607–3611], provides evidence that U-2504 and G-2502 are the principal sites of *p*-azidopuromycin interaction. These results are considered along with the results of related studies in defining a ribonucleoprotein neighborhood at the peptidyl transferase center.

Puromycin, unique among ribosomal antibiotics, is a substrate for the peptidyl transferase activity of ribosomes. It acts as an aminoacyl-tRNA analogue in accepting the nascent peptidyl chain from peptidyl-tRNA bound in the donor site, thereby giving rise to premature peptide release from the ribosome. Photoaffinity labels based on puromycin are, therefore, prime candidates for labeling the peptidyl transferase center. In previous work from this laboratory, we have used both [^{3}H]puromycin and its photolabile derivative [^{3}H]-*p*-azidopuromycin [6-(dimethylamino)-9-[3′-deoxy-3′-(*p*-azido-L-phenylalanylamino)-β-D-ribofuranosyl]purine, Figure 1], which is also a substrate for ribosomal peptidyl transferase (Nicholson & Cooperman, 1978), as photoaffinity labels for the *Escherichia coli* ribosome. Protein L23 was the principal protein labeled by both compounds; in addition, proteins L18/22 and L15 were labeled to major extents by [^{3}H]-*p*-azidopuromycin. The photolabeling of these proteins was strongly inhibited by unlabeled puromycin analogues, in parallel with the inhibitory effects of these analogues on peptidyl transferase activity, thus providing evidence that L23, L18/22, and L15 are at or near the peptidyl transferase center (Jaynes et al., 1978; Nicholson et al., 1982a,b; Weitzmann & Cooperman, 1985). By contrast, the extents of photolabeling of other ribosomal proteins by [^{3}H]puromycin and/or [^{3}H]-*p*-azidopuromycin were less sensitive to the addition of unlabeled puromycin and puromycin analogues, indicating that such proteins are labeled either from sites of lower affinity or directly from solution.

We have also studied the photoincorporation of both puromycin and *p*-azidopuromycin into 50S subunits by immunoelectron microscopy, using antibody to N^6,N^6-dimethyladenosine to locate the sites of photoincorporation (Olson et al., 1982, 1985). In each case, the major site of antibody attachment was to a region of the 50S subunit, between the L1 projection and the central protruberance, that is believed (Prince et al., 1983; Stöffler & Stöffler-Meilicke, 1984; Olson et al., 1985) to contain the peptidyl transferase center.

In the present work we complete our localization studies of *p*-azidopuromycin photoincorporation by identifying its sites of rRNA labeling. Most of such labeling takes place within 23S rRNA, and within this chain we find significant site-specific labeling within bases 2445–2668 and, to a lesser extent, bases 489–681. Our results also indicate that the principal sites of interaction of *p*-azidopuromycin with rRNA are at bases U-2504 and G-2502.

Experimental Procedures

Materials

The following materials were purchased from the commercial sources indicated: [2-^{14}C]uracil (50 Ci/mol) and [α-^{32}P]dATP (400 Ci/mmol), Amersham; S1 nuclease and *Hpa*II restriction endonuclease, Boehringer-Mannheim; ribonuclease T1, Calbiochem; AMV reverse transcriptase (AMV-RT),[1] Seikagaku America; Na salts of dATP, dCTP,

† This work was supported by Research Grant AI-16806 from the National Institutes of Health.

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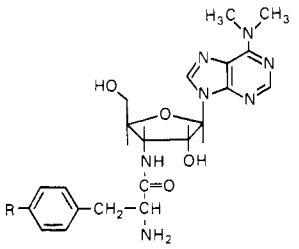

FIGURE 1: Structures of puromycin and *p*-azidopuromycin.

dGTP, and dTTP, Sigma; 2′,3′-dideoxy (dd) derivatives of ATP, CTP, GTP, and TTP, Pharmacia; NENSORB purification cartridges, Du Pont.

Deoxyoligonucleotide primers, complementary to 23S RNA residues 2741–2725 [primer 1, d(TTATCTCTTCCGCATTT)] and 2613–2597 [primer 2, d(AGGGACCGAACTGTCTC)], were provided by Dr. Mike Mitchell of the DNA synthesis Facility of the Cancer Center of the University of Pennsylvania.

All buffer pH values were measured at room temperature. Plasticware and glassware were pretreated as previously described (Hall et al., 1985).

Washed ribosomes were prepared from *E. coli* Q13 according to a modification of the Traub et al. (1971) procedure as previously described (Jaynes et al., 1978).

[^{3}H]-*p*-Azidopuromycin (5.76 Ci/mmol) was prepared by a modification of an earlier procedure (Nicholson & Cooperman, 1978; Nicholson et al., 1982a). RP-HPLC was used to purify both the intermediate [^{3}H]-t-BOC-*p*-azidopuromycin and the final product [^{3}H]-*p*-azidopuromycin, as described in Kerlavage et al. (1985).

Methods

[^{3}H]-p-Azidopuromycin Photoincorporation into Ribosomes. Photolyses were carried out on 0.4-mL solutions of *E. coli* ribosomes (100 A_{260} units/mL) and 0.13 mM [^{3}H]-*p*-azidopuromycin in buffer A (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM KCl) and 2 mM 2-mercaptoethanol for 10 min with Rayonet RPR-3500Å lamps at 4 °C. Procedure A, as described in Nicholson et al. (1982a), was used to prepare a washed ribosomal pellet free of contamination by noncovalently bound *p*-azidopuromycin and *p*-azidopuromycin photoproducts.

Radioactively Labeled rRNAs. [^{3}H]-*p*-Azidopuromycin-labeled rRNA was prepared from [^{3}H]-*p*-azidopuromycin-labeled ribosomes by phenol extraction of the washed ribosomal pellet from the photoincorporation experiment, in a manner paralleling that described earlier for 5-azido-2-(nitrobenzoyl)-[^{3}H]Phe-tRNA^{Phe}-labeled ribosomal RNA (Hall et al., 1985).

([^{14}C]Uracil + [^{14}C]cytosine)-labeled rRNA (0.0522 μCi/A_{260nm}) was prepared by phenol extraction of crude ribosomes (S-100 pellet) isolated from *E. coli* Q13 cells grown in the presence of [^{14}C]uracil (Moore & Boylen, 1955).

PAGE Analysis of Radioactively Labeled rRNA. [^{3}H]-*p*-Azidopuromycin-labeled rRNA was analyzed by electrophoresis in 2.85% acrylamide slab gels, ethidium stained, sliced, and counted as described in Hall et al. (1985). For double-labeling experiments, in which mixtures of [^{3}H]-*p*-azidopuromycin-labeled rRNA and ([^{14}C]uracil + [^{14}C]cytosine)-labeled rRNA were coelectrophoresed, peroxide solubilization of gel slices (Hall et al., 1985) was replaced by addition of 0.5 mL of H_2O–NCS solubilizer (Amersham) (1:9) to each slice followed by heating at 50 °C for 2 h and addition of 5 mL of 25% Triton–toluene-based counting medium (Jaynes et al., 1978).

Hybridization of [^{3}H]-p-Azidopuromycin-Labeled rRNA with pKK3535 HpaII Fragments, Nuclease Digestion, and PAGE Analysis. *E. coli* HB101 cells containing pKK3535, which includes the entire *rrn*B 23S, 16S, and 5S sequences (Brosius et al., 1981), was a gift of Dr. H. F. Noller. Cells were grown, the plasmid was isolated and digested into restriction enzyme fragments, and the fragments were hybridized to [^{3}H]-*p*-azidopuromycin-labeled rRNA alone, as described earlier for ribosomes labeled with a Phe-tRNA^{Phe} photoaffinity label (Hall et al., 1985), or to a combination of [^{3}H]-*p*-azidopuromycin-labeled rRNA and ([^{14}C]uracil + [^{14}C]cytosine)-labeled rRNA. The resulting mixtures, containing RNA·DNA hybrids as well as unhybridized rRNA and DNA, were next subjected to either S1 nuclease + ribonuclease T1 digestion or to ribonuclease T1 digestion alone.

S1 nuclease + ribonuclease T1 digestion was carried out essentially as described earlier (Hall et al., 1985), except that 3.2 rather than 6.4 units of S1 was used per microgram of total nucleic acids. The resulting RNA·DNA hybrid samples were analyzed by PAGE as before, with the same methods of gel staining and radioactivity determination, except that NCS solubilization (see above) replaced peroxide solubilization for double-labeled samples.

Ribonuclease T1 digestion alone was carried out by diluting the hybridization mixture with 9 volumes of T1 buffer (10 mM HEPES, pH 7.6, 0.2 M NaCl, 1 mM $ZnSO_4$) at 0 °C. The mixture was warmed to 25 °C, and digestion with ribonuclease T1 (70 units/mg of rRNA) was carried out for 30 min at this temperature. Digestion was terminated by addition of 0.5 volume of phenol. After phenol extraction, the nucleic acid was precipitated by addition of 2 volumes of ethanol and collected by centrifugation at 24000*g* for 1 h. The precipitate was resuspended in T1 buffer (1.1 mL/mg of original rRNA) and dialyzed for 4 h against 2 L of SET buffer (15 mM Tris-HCl, pH 7.6, 10 mM Na_2EDTA) at 4 °C. The dialyzed samples were analyzed by PAGE as described above.

Primer Extension. Hybridization of oligonucleotide primers to *p*-azidopuromycin-photolabeled rRNA, to control photolyzed rRNA (prepared from ribosomes photolyzed in the absence of *p*-azidopuromycin), and to rRNA (prepared directly from ribosomes and used for sequencing) and subsequent primer extensions by AMV-RT were performed essentially as described by Moazed et al. (1986).

rRNA Solutions. The 70S ribosomes were dissolved in SET$^+$ buffer (SET buffer plus 150 mM NaCl) and extracted 3 times with equal volumes of SET$^+$ buffer–equilibrated phenol and twice with equal volumes of chloroform. rRNA was ethanol precipitated and dissolved in H_2O at a final concentration of 1.2 mg/mL.

Primer Solutions. Deoxyoligonucleotide primers were dissolved in aqueous solution at a concentration of 2.3 μM.

Hybridization. The hybridization solution composed of 5 μL of rRNA solution, 1.5 μL of 5× hybridization buffer (25 mM potassium borate, 0.5 M KCl, 0.25 M K-HEPES, pH 7.0), and 1 μL of primer solution, was heated at 90 °C for 1

[1] Abbreviations: AMV-RT, avian myeloblastosis virus reverse transcriptase; AzPur, *p*-azidopuromycin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Na_2EDTA, disodium ethylenediaminetetraacetate; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reversed-phase high-performance liquid chomatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

min and slow cooled (about 20 min) to 42 °C.

Extension and Purification. Typically, 1 μL of hybrid solution was added to 5 μL of extension mix. The extension mix was made up in buffer (50 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM dithiothreitol, 10 mM $MgCl_2$) and contained the following components: 200 μM in each of dCTP, dGTP, and dTTP; 1 unit of AMV-RT, added from a solution [50% (v/v) glycerol, 50 mM Tris-HCl (pH 8.5), 2 mM DTT] containing 1 unit/μL; 10 μCi of [α-^{32}P]dATP. Sequencing reactions also contained the appropriate ddNTP (0.36 μM ddATP; 4.1 μM for the other ddNTPs).

Extensions on *p*-azidopuromycin-photolabeled rRNA or control photolyzed rRNA were carried out for 1 min at 42 °C. Chase mix (1 μL, containing 1 mM each of dATP, dCTP, dGTP, and dTTP in 125 mM Tris-HCl, pH 8.5, 125 mM KCl, 25 mM dithiothreitol, and 25 mM $MgCl_2$) was added, and incubation at 42 °C was continued for an additional minute. Reactions were stopped by addition of 5 μL of 5 mM EDTA, 3 μL of 0.3 M sodium acetate, and 90 μL of precooled 95% ethanol. Following cooling at −20 °C for 2 h, the precipitated nucleic acid was dried, redissolved in 200 μL of NEN reagent A [a mixture, adjusted to a final pH of 7.7 with concentrated HCl, of 7 parts of triethylamine to 5 parts of 0.1 M Tris-HCl–1 mM Na_2EDTA, pH 7.7 (v/v)], and applied to a NENSORB purification cartridge. After being washed with additional NEN reagent A and deionized water, the DNA/RNA hybrid was eluted with 1 mL of 50% HPLC-grade methanol (Fisher) in H_2O. This procedure removes excess nucleotide reagents, in particular [^{32}P]dATP. Samples were dried on a SpeedVac (Savant) and redissolved in 10 μL of electrophoresis loading solution (0.06% bromphenol blue–0.06% xylene cyanol in deionized formamide), and their radioactivities were determined. *p*-Azidopuromycin-photolabeled rRNA and control rRNA extension samples containing equal amounts of radioactivity were coanalyzed by PAGE and autoradiography.

Extensions for the sequencing reactions were performed similarly, except that initial reactions at 42 °C were for 30 min, incubations following the addition of chase mix were for 15 min, and NENSORB purification was not performed.

PAGE and Autoradiographic Analysis. Primer extension and sequencing reaction mixtures in loading buffer were applied to either 8% (w/v) or 12% (w/v) polyacrylamide–urea gels (33 cm wide × 40 cm long × 0.4 mm thick). The 8% gel was made up by combining 20 mL of 40% acrylamide stock [38 g of acrylamide plus 2 g of bis(acrylamide) in 100 mL of H_2O] with 50 g of urea, 10 mL of TBE buffer (100 mM Tris, pH 8.3, 100 mM boric acid, 2 mM Na_2EDTA) and 30 mL of H_2O. The 12% gel was made with 30 mL of 40% acrylamide stock and 20 mL of H_2O. Polymerization was induced by addition of 1 mL of 10% ammonium persulfate and 15 μL of *N,N,N′,N′*-tetramethylethylenediamine (Sigma). Electrophoretic runs were performed at a constant power of 40 W for 4 h. Following electrophoresis, autoradiography was carried out with preexposed X-ray film (Kodak) at −80 °C.

RESULTS

Photoincorporation of [^{3}H]-p-Azidopuromycin into rRNA. Photolysis of ribosomes with 0.13 mM [^{3}H]-*p*-azidopuromycin was carried out under conditions shown previously to maximize the fraction of observed labeling that was site specific (Nicholson et al., 1982a,b). The rRNA extracted from such ribosomes showed dominant (72%) incorporation of radioactivity into 23S rRNA, with 16S RNA (23%) and 5S RNA (5%, not shown) covalently labeled to much lower extents (Figure 2). The observed labeling corresponds to 0.032 *p*-azidopuromycin/23S RNA and 0.012 *p*-azidopuromycin/16S RNA. By comparison, under similar photolabeling conditions [and correcting for the difference in *p*-azidopuromycin concentration (0.05 mM) used earlier], the stoichiometries of *p*-azidopuromycin photoincorporation into the major labeled proteins can be estimated as follows: L23, 0.012; L18/22, 0.010; L15, 0.004 (Nicholson et al., 1982).

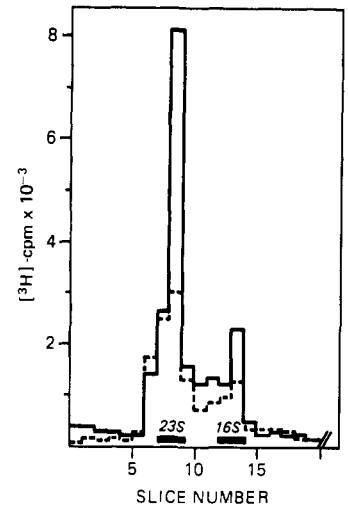

FIGURE 2: Radioactivity profile of PAGE analysis of [^{3}H]-*p*-azidopuromycin photoaffinity labeled rRNA. 70S ribosomes (100 A_{260}/mL) in buffer A were photolyzed for 10 min at 350 nm in the presence of 0.13 mM [^{3}H]-*p*-azidopuromycin and in the absence (solid line) or presence (dashed line) of 2 mM puromycin. A total of 79 μg of the extracted rRNA was subjected to PAGE as described under Methods. The 23S and 16S rRNA regions of the gel are indicated by bars.

RNA labeling by [^{3}H]-*p*-azidopuromycin was completely light dependent and was decreased in the presence of puromycin. Thus, as shown in Figure 2, addition of 2 mM unlabeled puromycin to the photolysis mixture led to decreased radioactive incorporation into both 23S rRNA (51% reduction) and 16S RNA (48% reduction).

Restriction Fragment Hybridization Analysis of [^{3}H]-p-Azidopuromycin Photoincorporation into rRNA. The distribution of [^{3}H]-*p*-azidopuromycin labeling in ribosomal RNA was further characterized by the restriction fragment hybridization analysis method described previously (Hall et al., 1985). Briefly, a threefold stoichiometric excess of total labeled rRNA is hybridized with restriction fragments of plasmid pKK3535, which includes all of *rrn*B DNA; S1 nuclease and ribonuclease T1 are added to digest single-stranded RNA and DNA; the surviving RNA·DNA hybrids are separated by PAGE, and the radioactivity in each hybrid is determined by gel slicing and counting. The known sequence of plasmid pKK3535 allows the identities of the hybrids to be identified by size.

Application of this method to the analysis of [^{3}H]-*p*-azidopuromycin photoaffinity labeled rRNA, using *Hpa*II digests of pKK3535, gave the results displayed in Figure 3. The hybrid segments are identified in Table I. Although the most prominently labeled region of the gel was consistently band 7 (corresponding to 23S bases 2445–2668), the overall recovery of radioactivity coelectrophoresing with the hybrids was extremely variable, ranging from a high of about 25% of expected, as is the case for the data in Figure 3, to a low of 1% of expected. Here, the expected value is determined by assuming a 100% yield of hybrids and estimating the extent of ribosomal labeling by the sum of the radioactivities coelectrophoresing with 23S, 16S, and 5S rRNA (as in Figure 2). Variability in the recovery of radioactivity was in marked

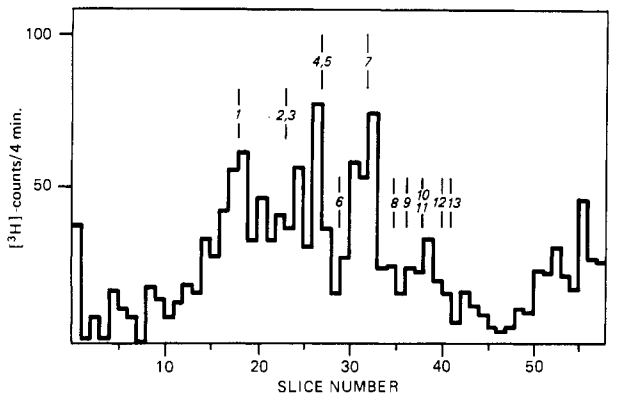

FIGURE 3: Radioactivity profile of PAGE analysis of [^{3}H]-*p*-azidopuromycin photoaffinity labeled rRNA hybridized to *Hpa*II-digested pKK3535 and digested with S1 nuclease and RNase T1. [^{3}H]-*p*-Azidopuromycin-photolabeled rRNA (342 μg) was hybridized to *Hpa*II restriction enzyme fragments of pKK3535 (600 μg), and the hybrids were digested with S1 nuclease and ribonuclease T1 as described under Methods. The ethidium-visualized positions of the *Hpa*II hybrid bands 1–13 are indicated (see Table I).

Table I: rRNA·DNA Hybrid Bands from *Hpa*II Fragments of pKK3535

band no.	no. of bases	rRNA	sequence of bases
1	505	16S	1–505
2	376	23S	924–1299
3	339	23S	1501–1839
4	320	23S	1897–2216
5	303	23S	186–488
6	280	16S	859–1138
7	224	23S	2445–2668
8	193	23S	489–681
9	180	23S	2217–2396
10	162	16S	1143–1304
11	157	16S	1386–1542
12	149	23S	1300–1448
13	137	16S	722–858
15	120	5S	1–120
18	107	23S	1–107
29	28	23S	2877–2904

contrast to the relative constancy in the intensity of hybrid bands on ethidium staining. Since the vast majority of rRNA present in the sample is not covalently labeled, these results led us to conclude that there is selective nuclease removal of sites of [^{3}H]-*p*-azidopuromycin photoincorporation from rRNA in the hybrids [see also Hall et al. (1985)].

On this basis we elected to eliminate the S1 digestion step, in order to recover as much as possible of the photoincorporated [^{3}H]-*p*-azidopuromycin with the hybrids. A schematic of the method using only digestion with ribonuclease T1 is shown in Figure 4. The majority of the hybrid segments generated are the same in the presence or absence of S1. However, hybrids formed from rRNA termini are expected to migrate with altered mobilities due to the attachment of unhydrolyzed single-stranded DNA (Figure 4). Thus, hybrid band 1 of Table I (505 bases) is expected to migrate with a mobility corresponding to 737 bases, the full-length restriction fragment overlapping this region of the plasmid pKK3535 sequence. Similarly, bands 11 (157 bases) and 18 (107 bases) are expected to migrate as a band of 704 bases, and band 29 (28 bases) and band 15 (120 bases) are expected to migrate as a band of 333 bases (see Figure 5).

Application of the method depicted in Figure 4 to the analysis of [^{3}H]-*p*-azidopuromycin photoaffinity labeled rRNA, using *Hpa*II digests of pKK3535, gave a labeling profile (Figure 5) similar to that seen in Figure 3, but with higher yields of recovered radioactivity and improved reproducibility. Further, replacement of ribonuclease T1 (Figure 4) with either S1 nuclease or ribonuclease A led to lower yields of recovered radioactivity (data not shown). Accordingly, for the remainder of the experiments reported in this section, RNA·DNA hybrid preparation proceeded as depicted in Figure 4. We note in passing that the lower yield of radioactivity obtained when ribonuclease A replaces ribonuclease T1 is consistent with results of Myers et al. (1985). They found that ribonuclease A, but not ribonuclease T1, generates nicks in the RNA of RNA·DNA hybrids at the sites of single-base mismatches, and it is possible that [^{3}H]-*p*-azidopuromycin covalent photoincorporation into rRNA may sometimes interfere with RNA·DNA hybrid base pairing.

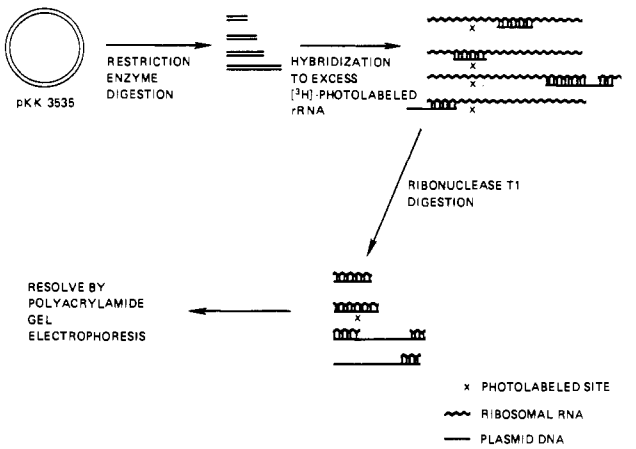

FIGURE 4: Schematic of method for localizing sites of labeling within rRNA solely by RNase T1 digestion.

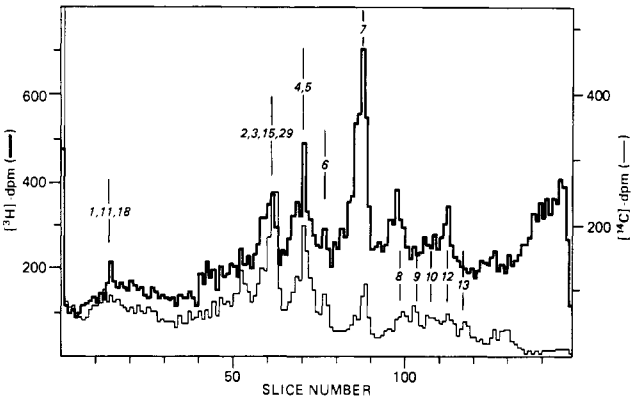

FIGURE 5: Radioactivity profile of PAGE analysis of a mixture of [^{3}H]-*p*-azidopuromycin photoaffinity labeled rRNA and ([^{14}C]uracil + [^{14}C]cytosine)-labeled rRNA hybridized to *Hpa*II-digested pKK3535 and digested solely with RNase T1. *Hpa*II restriction enzyme fragments of pKK3535 (700 μg) were hybridized to a mixture of 82 μg of ([^{14}C]uracil + [^{14}C]cytosine)-labeled rRNA (1937 dpm/μg) and 399 μg of [^{3}H]-*p*-azidopuromycin photoaffinity labeled rRNA (569 dpm/μg), and the hybrids were digested with ribonuclease T1 and electrophoresed as described under Methods. Note that the well-defined RNA·DNA hybrids (e.g., 4/5, 6, 7) give well-resolved peaks of ^{14}C radioactivity whereas hybrids that are expected to contain undigested single-stranded DNA (1, 11, 18) give a diffuse area of ^{14}C radioactivity.

Previously (Hall et al., 1985) we had shown that there is considerable variation in the yields of different RNA·DNA hybrids as measured following separation by PAGE. As a consequence, quantitative comparison of the extents of [^{3}H]-*p*-azidopuromycin photoincorporation into different regions of rRNA requires determination of the relative yields of each of the hybrids. In the present work we determined relative yields by adding ([^{14}C]uracil + [^{14}C]cytosine)-labeled rRNA to the hybridization reaction mixture (Figure 5, lower

Table II: rRNA·DNA Hybrid Bands from *Hpa*II Fragments of pKK3535: Measured Yields and Stoichiometry of [^{3}H]-*p*-Azidopuromycin (AzPur) Photoincorporation

band no.	hybrid yield (relative to band 7)[a]	mol of [^{3}H]AzPur photoincorporation/mol of hybrid ($\times 10^2$)[b]	[^{3}H]AzPur photoincorporation (+puro/−puro)[c]
1, 11, 18	0.73 ± 0.31 (3)	0.55	0.74
2, 3, 15, 29	0.845 ± 0.027 (3)	0.50	0.87
4, 5	0.77 ± 0.12 (3)	0.66	0.95
6	0.45 ± 0.05 (3)	0.27	0.52
7	1.00	0.95	0.53
8	0.62 ± 0.22 (3)	0.38	0.44
9	0.71 ± 0.26 (3)	0.13	0.63
10	0.73 ± 0.13 (3)	0.17	1.1
12	0.76 ± 0.05 (2)	0.23	1.0
13	0.62 ± 0.12 (2)	0.05	
		total: 3.89	

[a] Calculated from the [^{14}C]uracil + [^{14}C]cytosine labeling of gel regions. Number of determinations in parentheses. [b] Calculated from the [^{3}H]-*p*-azidopuromycin and [^{14}C]uracil + [^{14}C]cytosine labeling of gel regions. [c] Calculated by dividing the moles of [^{3}H]-*p*-azidopuromycin photoincorporation per mole hybrid in the presence of unlabeled 2 mM puromycin by the value obtained in the absence of puromycin. It should be emphasized that because of the background radioactivity present in the gel (due, in part, to hybrids formed with partially hydrolyzed rRNA), these ratios tend to underestimate the inhibitory effects of added unlabeled puromycin on [^{3}H]-*p*-azidopuromycin photoincorporation.

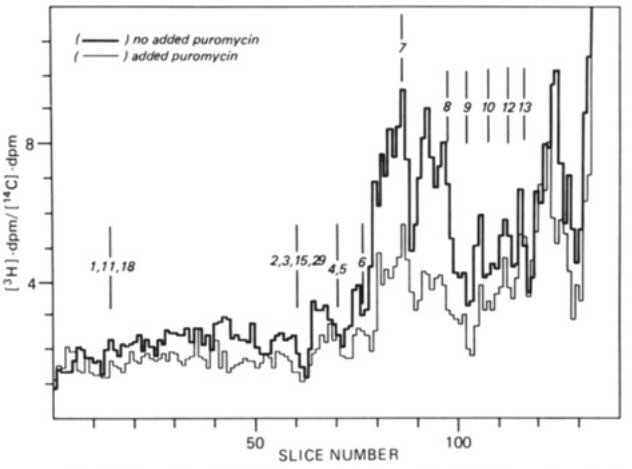

FIGURE 6: Effect of unlabeled puromycin added during photolysis on the ^{3}H/^{14}C ratio profile for double-labeled rRNA. *Hpa*II restriction enzyme fragments of pKK3535 were hybridized to mixtures of ([^{14}C]uridine + [^{14}C]cytidine)-labeled rRNA and rRNA extracted from ribosomes photoaffinity labeled with [^{3}H]-*p*-azidopuromycin in the absence (heavy line) and presence (fine line) of 2 mM unlabeled puromycin, as in Figure 5.

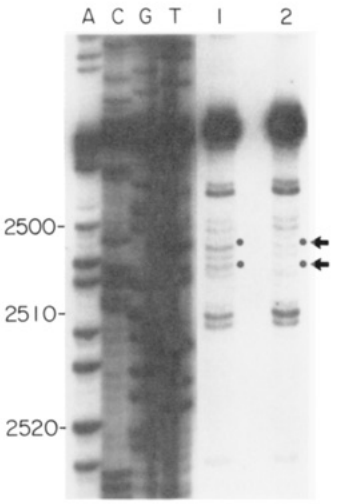

FIGURE 7: Reverse transcriptase extension of primer 2 on a *p*-azidopuromycin photoaffinity labeled rRNA template. Sequencing lanes A, C, G, and T are primer extensions with addition of corresponding dideoxynucleotides. Lane 1 is a primer extension on *p*-azidopuromycin photoaffinity labeled rRNA. Lane 2 is primer extension on rRNA extracted from ribosomes photolyzed in the absence of *p*-azidopuromycin. Note the much darker bands in lane 1 vs lane 2 at G-2505 and A-2503 (arrows).

trace). Simultaneous determinations of the ^{14}C and ^{3}H contents of a gel slice permitted calculation both of the yield of rRNA recovered from the gel, from the known cytosine + uracil content of the hybrid rRNA in the gel slice, and of the moles of [^{3}H]-*p*-azidopuromycin incorporation per mole of RNA·DNA hybrid. The results of these determinations are summarized in Table II for photolabeling experiments conducted in the absence and presence of 2 mM puromycin. Band 7 (bases 2445–2668) is labeled to the greatest extent. Furthermore, its labeling is markedly reduced in the presence of puromycin, providing strong evidence that labeling is site specific. *p*-Azidopuromycin photoincorporation into band 8 is only about 40% of its photoincorporation into band 7, but it is also strongly reduced in the presence of puromycin. The labeling of each of the other bands either proceeds to a still lower extent or is not strongly reduced in the presence of puromycin, or both.

A clear illustration of the site-specific labeling of bands 7 and 8 is provided by a plot of the ratio of ^{3}H/^{14}C counts in the absence and presence of puromycin (Figure 6). High values of this ratio are found for these bands, showing that they are labeled by [^{3}H]-*p*-azidopuromycin to higher extents than expected from their lengths alone, if labeling were occurring randomly into rRNA. In addition, these high values are markedly reduced in the presence of 2 mM puromycin.

The 50% reduction of [^{3}H]-*p*-azidopuromycin photoincorporation into band 7 in the presence of 2.0 mM puromycin is somewhat lower than the expected value of 75%, calculated on the basis of the previously determined K_D value of 0.7 ± 0.2 mM for puromycin binding to the site from which it photoincorporates into the 50S subunit. At least some of this difference is due to the presence of background radioactivity in the gel, arising from the formation of DNA hybrids with partially hydrolyzed rRNA, but a portion may also reflect photoincorporation into band 7 that is unrelated to a puromycin-specific site.

Adding up the values of [^{3}H]-*p*-azidopuromycin photoincorporation in all of the hybrids (Table II) gives a total of 0.039/rRNA. This agrees reasonably well with the value of 0.045/rRNA as measured by RNA electrophoresis gels (Figure 1), clearly showing that ribonuclease T1 digestion is not removing a major fraction of the photoincorporated [^{3}H]-*p*-azidopuromycin from the hybrids. Thus, the results summarized in Table II provide a faithful representation of the distribution of photoincorporated *p*-azidopuromycin within rRNA.

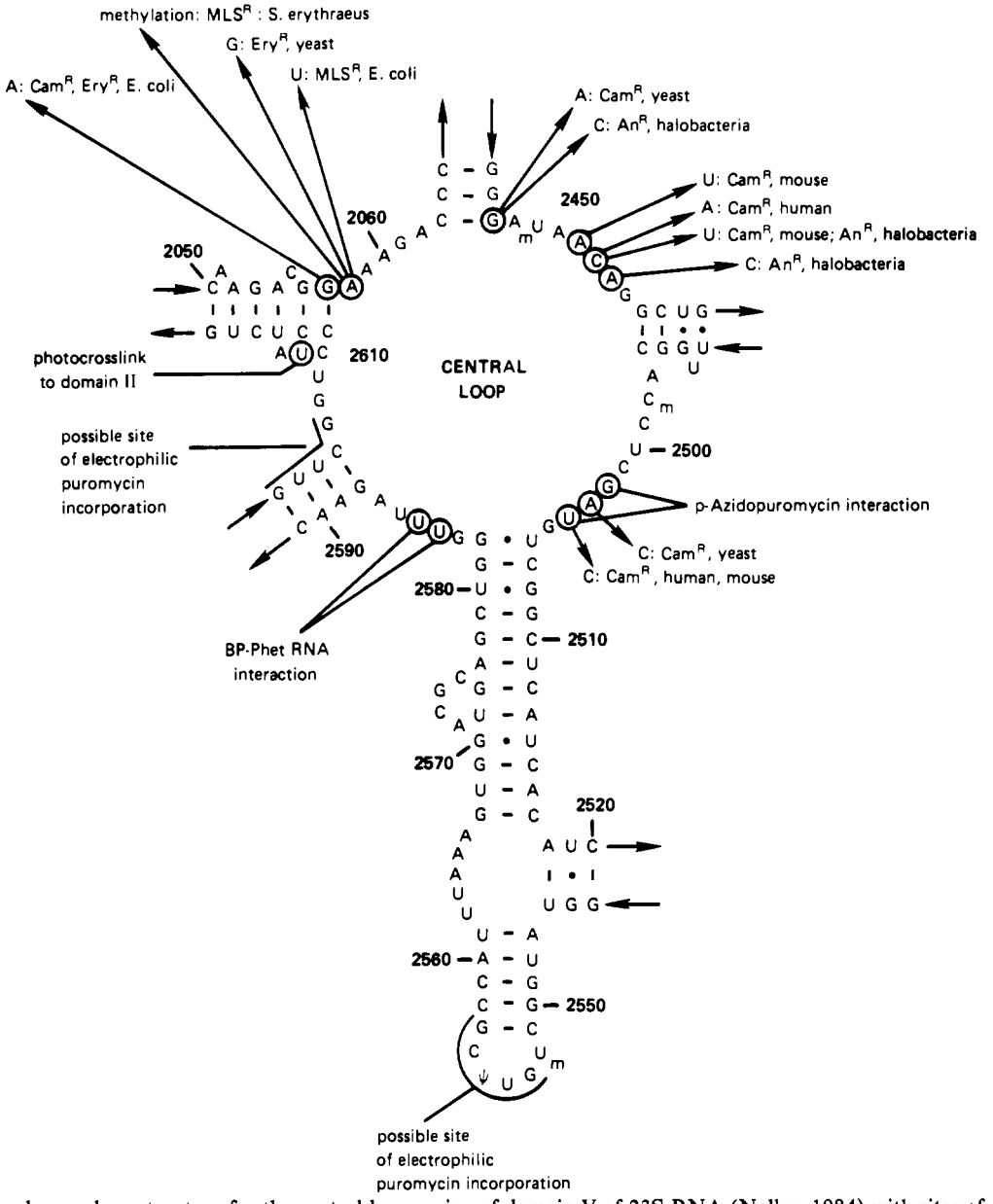

FIGURE 8: Proposed secondary structure for the central loop region of domain V of 23S RNA (Noller, 1984) with sites of affinity labeling, of intramolecular cross-linking, and of mutations leading to antibiotic-resistance indicated. This figure is adapted and updated from one presented in Ettayabi et al. (1985), based on the proposed 2° structure for 23S RNA of Noller (1984). BP-Phe-tRNA refers to the 3-(4-benzoylphenyl)propionyl-Phe-tRNA^{Phe} photoaffinity label of Barta et al. (1984). Two possible incorporation sites for the electrophilic bromoacetyl derivative of puromycin of Eckermann and Symons (1978) are indicated, as is a site of photo-cross-linking to domain II (Stiege et al., 1983). Sites of mutation leading to resistance to erythromycin (Ery), chloramphenicol (Cam), anisomycin (An), and collateral resistance to macrolides, lincomycin, and streptogramin B (MLS) are discussed in Ettayabi et al. (1985), Cundliffe (1986), and Hummel and Böck (1987).

Primer Extension in the Region of Band 7 Using [^{3}H]-p-Azidopuromycin-Labeled rRNA as Template. From the results presented above, band 7, corresponding to bases 2445–2668 in 23S RNA, clearly provides the major site of specific photoaffinity labeling of rRNA by *p*-azidopuromycin. The approach of Barta et al. (1984) was employed to localize the site of photoaffinity labeling to the individual base level. Both *p*-azidopuromycin-photolabeled rRNA and control rRNA prepared from ribosomes photolyzed in the absence of *p*-azidopuromycin were hybridized to single-stranded oligodeoxynucleotide probes that are complementary to rRNA either within 2445–2668 (no. 2) or to the 3′-side of this region (no. 1). The resulting heteroduplexes were examined as primer-templates for reverse transcriptase by PAGE and autoradiographic analysis. Here the underlying premise is that nucleotide modification caused by photoaffinity labeling could result in a halt or pause by reverse transcriptase at the position preceeding the site of labeling (Youvan & Hearst, 1979, 1981), resulting in a characteristic band darkening on autoradiographic analysis of the photoaffinity-labeled sample that would not be observed on analysis of a control rRNA sample. With the heteroduplex derived from probe 2, just such differences are seen reproducibly (five experiments) at positions 2505 and 2503 (Figure 7), providing strong evidence that U-2504 and G-2502 are major sites of photoincorporation of *p*-azidopuromycin within band 7 (see Figure 8). It should be noted that only weak stops are seen due to the low stoichiometry (~1%, Table II) of *p*-azidopuromycin photoincorporation into band 7.

No other such differentially intense bands were observed in the gel tracks corresponding to primer 2 extension, although clear extension bands were observed at least as far as nucleotide 2400, well past the 5′-terminus (nucleotide 2445) of band 7. Nor were darker bands observed for *p*-azidopuro-

mycin-photolabeled rRNA when the heteroduplex containing probe 1 was employed as substrate for reverse transcriptase. Extensions using this primer were clearly visible as far as nucleotide 2590.

Discussion

Coupling restriction fragment hybridization analysis (Hall et al., 1985) with double isotopic labeling, as illustrated by the results presented in Figure 5 and Table II, allows quantitative determination of the extent of incorporation of an affinity label, in this case [^{3}H]-*p*-azidopuromycin, into limited regions of rRNA. This approach thus provides a convenient method for determining both which regions are labeled to the greatest extents and, by extending such measurements to suitable control experiments, whether such labeling is site specific. As judged by both the magnitude of [^{3}H]-*p*-azidopuromycin incorporation and its sensitivity to added unlabeled puromycin, 23S rRNA regions 2445–2668 (band 7) and, to a lesser extent, 489–681 (band 8) show the greatest site-specific labeling. These results are in very good accord with those of other groups, as summarized in Noller et al. (1986), indicating that the peptidyl transferase center falls within domain V (bases 2043–2625; see below) of 23S RNA and that portions of domain V may interact closely with portions of domain II (bases 579–1261).

Utilization of the primer extension approach provides evidence that the principal sites labeled within bases 2445–2668 are U-2504 and G-2502. This elegant approach, first introduced by Barta et al. (1984) for mapping sites of rRNA affinity labeling, has, however, significant inherent ambiguities. Thus, it fails to distinguish between an interference with reverse transcriptase activity arising from photoincorporation as opposed to one arising from, for example, a photoaffinity label photosensitized change in rRNA structure. In addition, it cannot detect affinity label interaction at those sites that are preceded by a significant halt or pause in primer extension of control rRNA (e.g., base 2509, Figure 7) or that class of affinity label incorporation reactions that do not affect primer extension.

These ambiguities do not, however, call into question the evidence presented in Figure 7 for a strong interaction of *p*-azidopuromycin with rRNA in the vicinity of bases 2504 and 2502, and it is interesting to compare our present results with previous results bearing on the localization of the peptidyl transferase center within 23S RNA (Figure 8). The most pertinent earlier results are those of Barta et al. (1984) and ourselves (Hall et al., 1985) showing that two different photolabile N^{α} derivatives of Phe-tRNA^{Phe} have their major (if not exclusive) sites of rRNA photoincorporation within 2445–2668. In the Barta et al. (1984) work primer extension was used to provide evidence for photoincorporation into U-2584 and U-2585. The predicted 2° structure of 23S rRNA (Figure 8) places these two bases quite close to U-2504 and G-2502. Thus, two different classes of photoaffinity label that are nevertheless both directed toward the peptidyl transferase center, a tRNA derivative and an antibiotic derivative, appear to photoincorporate in close proximity to one another. It is tempting to speculate that the tRNA derivative, which is a functional analogue of peptidyl tRNA, photoincorporates into the P′ site (defined as the binding site for the 3′-terminus of peptidyl tRNA) whereas *p*-azidopuromycin, which is a functional analogue of aminoacyl tRNA, photoincorporates into the corresponding A′ site.

Mention should also be made of the work of Eckermann and Symons (1978) who labeled a 23S rRNA sequence identified as either GUUCG or GUCCG with an electrophilic derivative of puromycin. Of the four such oligonucleotides within 23S rRNA, two sequences, 2553–2557 and 2603–2607, occur within bases 2445–2668. Branlant et al. (1981) have suggested that bases 2553–2557 provide the most likely site of incorporation, since they fall within a predicted single-stranded region.

The strong evolutionary conservation of the central loop region of domain V (Noller, 1984; Noller et al., 1986), as well as the results of studies mapping sites of mutation leading to resistance to antibiotics that are either direct (chloramphenicol, anisomycin) or indirect (erythromycin) inhibitors of peptidyl transferase, provides additional evidence for the localization of the peptidyl transferase center within this central loop (Figure 8). Particularly striking for the current work is that mutations at U-2504 (human, mouse) and A-2503 (yeast), precisely where *p*-azidopuromycin interacts (and most likely photoincorporates), confer chloramphenicol resistance on mitochondrial ribosomes. In addition, several antibiotics have recently been shown to protect specific bases in the central loop from chemical modification, including chloramphenicol (A-2451 and G-2505), carbomycin (A-2058, A-2059, A-2062, A-2451, and G-2505), erythromycin (A-2058, A-2059, and G-2505), and vernamycin (A-2062 and G-2505) (Moazed & Noller, 1987).

With the completion of the current work, *p*-azidopuromycin becomes the first ribosome affinity label for which the interactions with both rRNA and ribosomal protein have been mapped in detail. Since [^{3}H]-*p*-azidopuromycin labeling of proteins L23, L18/22, and L15 occurs simultaneously with rRNA labeling and shows similar protective effects of added unlabeled puromycin (Nicholson et al., 1982b) to that demonstrated for bands 7 and 8, the two sets of results suggest that these proteins, along with the central loop of domain V and portions of domain II, form a ribonucleoprotein neighborhood within the 50S ribosome that is located at the peptidyl transferase center.

Acknowledgments

We acknowledge the assistance of Joseph Smith in providing ([^{14}C]uracil + [^{14}C]cytosine)-labeled rRNA, of Dr. Mike Mitchell of the DNA synthesis facility of the Cancer Center of the University of Pennsylvania for providing primers, of Nora Zuño in several technical aspects of this work, and of Danesh Moazed for helpful discussions of the primer extension procedure.

Registry No. AzPur, 67607-41-4; peptidyltransferase, 9059-29-4; uridine, 58-96-8; guanosine, 118-00-3.

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Cloning and Nucleotide Sequence of the 2,3-Dihydroxybiphenyl Dioxygenase Gene from the PCB-Degrading Strain of *Pseudomonas paucimobilis* Q1†

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Received October 29, 1987; Revised Manuscript Received January 20, 1988

ABSTRACT: The *bphC* gene encoding 2,3-dihydroxybiphenyl dioxygenase was cloned from biphenyl-degrading and chlorinated biphenyl-degrading *Pseudomonas paucimobilis* Q1, and its complete nucleotide sequence was determined. The DNA-derived protein sequence provides the primary structure of 298 amino acids. Polyclonal antibodies raised against this protein from *P. paucimobilis* Q1 failed to cross-react with the previously isolated 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 [Furukawa, K., & Arimura, N. (1987) *J. Bacteriol. 169*, 924-927. Furukawa, K., Arimura, N., & Miyazaki, T. (1987) *J. Bacteriol. 169*, 427-429], despite the close similarities of these proteins in terms of their native as well as subunit molecular weights, cofactor, and enzymatic activities. The sequence homology of the 2,3-dihydroxybiphenyl dioxygenase from the two different sources is examined.

2,3-Dihydroxybiphenyl dioxygenase (23OHBPO)[1] catalyzes the oxidative ring cleavage of 2,3-dihydroxybiphenyl to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (Figure 1). This is one of the key reactions in the metabolism of the widespread pollutant polychlorinated biphenyls (PCBs). Although the metabolic importance of PCB-degrading microorganisms has inspired extensive research on such organisms over the past decade, only quite recently have these systems become amenable to molecular studies (Furukawa, 1982, 1986).

We have recently cloned a gene cluster (*bphA*, *bphB*, *bphC*) encoding PCB-degrading enzymes from *Pseudomonas pseudoalcaligenes* KF707, and a major catabolic pathway of PCBs has been proposed as in Figure 1 (Furukawa & Miyazaki, 1986). In its first catabolic mechanistic stage a molecular oxygen is introduced at the 2,3-position of the nonchlorinated or less chlorinated ring to produce a dihydrodiol (compound II in Figure 1) by the action of a biphenyl dioxygenase

† This paper is dedicated to Dr. Akio Sato, the former Director General of FRI, for his 60th birthday as well as for his contribution to FRI until his retirement on Dec 1, 1987.

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[1] Abbreviations: 23OHBPO, 2,3-dihydroxybiphenyl dioxygenase; 34OHBP, 3,4-dihydroxybiphenyl; *bphC*, a gene that encodes 23OHBPO; Q1, *Pseudomonas paucimobilis* Q1; KF707, *Pseudomonas pseudoalcaligenes* KF707; 23OHBPO(Q1), 23OHBPO of Q1 origin; *bphC*-(KF707), *bphC* of KF707 origin; PCB, polychlorinated biphenyl; BP, biphenyl; kb, kilobase; SDS, sodium dodecyl sulfate; SD, Shine-Dalgarno sequence; Km, kanamycin; Sm, streptomycin; DEAE, diethylaminoethyl.

0006-2960/88/0427-3990$01.50/0