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Sequence analysis and in vitro maturation of five precursor 5S RNAs from Bacillus Q

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ABSTRACT

Bacillus Q, which is closely related to B.subtilis, contains at least six different precursors of 5S rRNA. The complete nucleotide sequences of four of these precursors, as well as the major part of the sequence of a fifth one, have been determined. They all contain the same 5'-terminal non-conserved segment which is to a large degree homologous with the corresponding segment of the B.subtilis p5S RNAs (Sogin, M.L., Pace, N.R., Rosenberg, M., Weissman, S.M. (1976) J. Biol. Chem. 251, 3480-3488). On the other hand the 3'-terminal non-conserved sequences of the various Bacillus Q precursors show considerable differences both in length and in nucleotide sequence, while there is also little or no homology with the 3'-terminal non-conserved sequence of the B.subtilis precursors. Bacillus Q p5S RNAs do not possess tetranucleotide repeats around the sites which are cleaved during maturation, as does B.subtilis p5S RNA. Like in B.subtilis, however, the cleavage sites are contained within a double-helical region of the precursor molecules. Crude RNAse M5 isolated from various Bacillus strains can maturate the Bacillus Q p5S RNAs with high efficiency. Despite considerable differences in primary structure between the precursors from the various strains, each RNAse M5 preparation can maturate all these precursors with about the same efficiency.

INTRODUCTION

The precursor 5S RNA molecules in Bacilli in contrast to those in E. coli (1-3) contain fairly long non-conserved sequences at both sides of the mature domain (4,5), which are split off in vivo by an endonuclease, called RNAse M5 (6). The maturation of these 5S RNA precursors provides an excellent model system for studying the processing of precursor RNA molecules in general, especially since Sogin and Pace (6,7) demonstrated that correct maturation of naked B.subtilis p5S RNA can be accomplished by RNAse M5.

The complete sequence of a p5S RNA from B.subtilis was reported by Sogin et al. (5) who noted several regularities in this sequence on which they based an elegant hypothesis to explain the specific interaction between p5S RNA and RNAse M5. These regularities were: tetranucleotide repeats around the two cleavage sites as well as two identical hexanucleotide sequences located in the same position relative to the 5'- and 3'-cleavage site

respectively. Further work by Meyhack et al. (8-10) on *in vitro* maturation of artificially constructed precursors, however, invalidated this hypothesis. These experiments led to the conclusion that RNAse M5 mainly interacts with the mature domain of p5S RNA and largely ignores the precursor-specific sequences. The only essential feature of these sequences as far as processing is concerned, appeared to be the presence of a G residue immediately adjacent to the 5'-terminus of the mature domain. This G can pair with the 3'-terminal C residue of the mature domain. This extends the double helical region known as the molecular stalk (11) by one base pair.

In this paper we report the nucleotide sequences of four of the at least six p5S RNAs occurring in *Bacillus Q*, which is closely related to *B.subtilis* (12). The sequences of the non-conserved parts of these precursors differ appreciably from those reported for B.subtilis p5S RNA, especially where the 3'-terminal non-conserved segment is concerned. Despite these differences *Bacillus Q* precursor 5S RNA is efficiently maturated by RNAse M5 from *B.subtilis* and other *Bacillus* strains. This confirms the relatively insignificant role of the precursor-specific segments in the interaction of p5S RNA with RNAse M5.

MATERIALS AND METHODS

Bacillus Q, a strain closely related to both *B.subtilis* and *B. licheniformis* was used throughout this study.

Isolation of precursor 5S RNA uniformly labeled with ^{32}P

Precursor 5S RNA was labeled uniformly with ^{32}P in the presence of chloramphenicol essentially as described by Stoof *et al.* (4). After the labeling period unlabeled phosphate was added to a final concentration of 30 mM and incubation of the culture was continued for 10 min. This procedure removes most of the label from unidentified material which causes a high heterodisperse background in the polyacrylamide gel used for final purification of the precursors.

Labeled cells were converted to spheroplasts by treatment with lysozyme (Sigma), and lysed by addition of Brij 58 (13). The lysate was incubated with DNAse (Sigma) and RNA was extracted with cold phenol/SDS (14). Low-molecular-weight RNA was isolated by sucrose gradient centrifugation (13). Finally precursor 5S RNA was purified by two-dimensional polyacrylamide gel electrophoresis. The first dimension was run on a 10% disc gel using the discontinuous buffer system of Richards *et al.* (15). The disc gel was then polymerized on top of a 12.5% slab gel (20x40x0.4 cm) in 50 mM Tris-borate buffer

(pH 8.3) containing 1 mM EDTA with the aid of some 5% acrylamide in 25 mM Tris-HCl (pH 6.3). After overnight electrophoresis at 4°C (10-20 V/cm) the precursors were located by autoradiography and subsequently eluted by shaking the crushed gel overnight at 40°C in 1.0 M NaCl containing 0.1% SDS. The eluate was freed from polyacrylamide by filtration through a glass-fiber filter, dialysed against distilled water and freeze-dried. The residue was redissolved in 0.3 M sodiumacetate and the RNA was precipitated twice with ethanol.

Digestion of RNA and analysis of oligonucleotides

Complete digestion of intact m5S RNA, p5S RNA or fragments with RNAse T_1 (Sankyo) or RNAse A (Boehringer) was carried out as described previously (13). The products were separated by high-voltage two-dimensional electrophoresis according to Sanger et al. (16). Oligonucleotides were analysed by standard methods using digestion with KOH, RNAse T_1, RNAse A, RNAse U_2, RNAse T_2 (17), T_3 endonuclease (18), P_1-nuclease (19), and spleen exonuclease (17,20). Products were separated by high-voltage electrophoresis and, where necessary, analysed further by tertiary digestion.

Partial digestion of precursor 5S RNA

Partial digestion of p5S RNA with RNAse T_1 was carried out as described previously (8) at an enzyme to substrate ratio of 1:3000 in the high-salt buffer described by Vigne and Jordan (21). Prior to digestion p5S RNA was renatured by the procedure of Meyhack et al. (8). Digestion was halted by adding a solution containing 0.1% SDS, 8 M urea and 0.5 M EDTA. The products were separated on 15% polyacrylamide slab gels (20x40x0.2 cm) in 50 mM Tris-borate buffer (pH 8.3) containing 1 mM EDTA and 8 M urea. The gels were autoradiographed and the products eluted and purified as described above.

Isolation of RNAse M5

Crude RNAse M5 was obtained from Bacillus Q, B.subtilis 168 and B. licheniformis (laboratory strain S244) cells essentially as described by Sogin et al. (7) by preparing a ribosomal wash. The cells were broken by grinding with Al_2O_3 powder (Sigma), however.

In vitro maturation of precursor 5S RNA

Precursor 5S RNA was incubated with crude RNAse M5 as described by Sogin and Pace (6) for 30 min. at 37°C. The reaction products were separated at room temperature on a 15% polyacrylamide slab gel (20x20x0.3 cm) in E-buffer (22) using a 6% stacking gel in ¼ E-buffer (8). The gels were autoradiographed and the appropriate products were eluted and purified as described above.

RESULTS

A. Isolation and characterization of precursor 5S RNA

The purification of p5S RNA by two-dimensional gel electrophoresis of low-molecular-weight RNA from Bacillus Q is shown in Fig. 1. Spots pA and pB represent precursor 5S RNA species as demonstrated by the fingerprint analysis shown in Fig. 2. The fingerprints of both pA and pB contain all oligonucleotides present in mature 5S RNA from Bacillus Q except for spot 18, which represents the 5'-terminal oligonucleotide pUUUG (12). The same result was obtained upon comparison of the RNAse A fingerprints of pA and pB to that of m5S RNA except that in this case the oligonucleotide $AAGC_{OH}$, the 3'-terminal sequence of m5S RNA (12), was absent from the pA and pB digests (data not shown). Therefore, Bacillus Q, like B.subtilis (3) and B. licheniformis (4), contains at least two types of p5S RNA, both of which have non-conserved sequences at the 5'- as well as the 3'-end. The lengths of these two types of

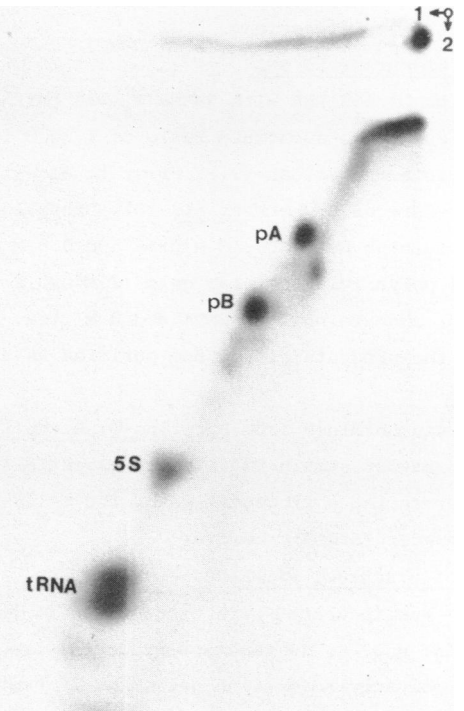

Fig. 1. Two-dimensional slab gel electrophoresis of ^{32}P-labeled low-molecular-weight RNA from Bacillus Q cells treated with chloramphenicol.

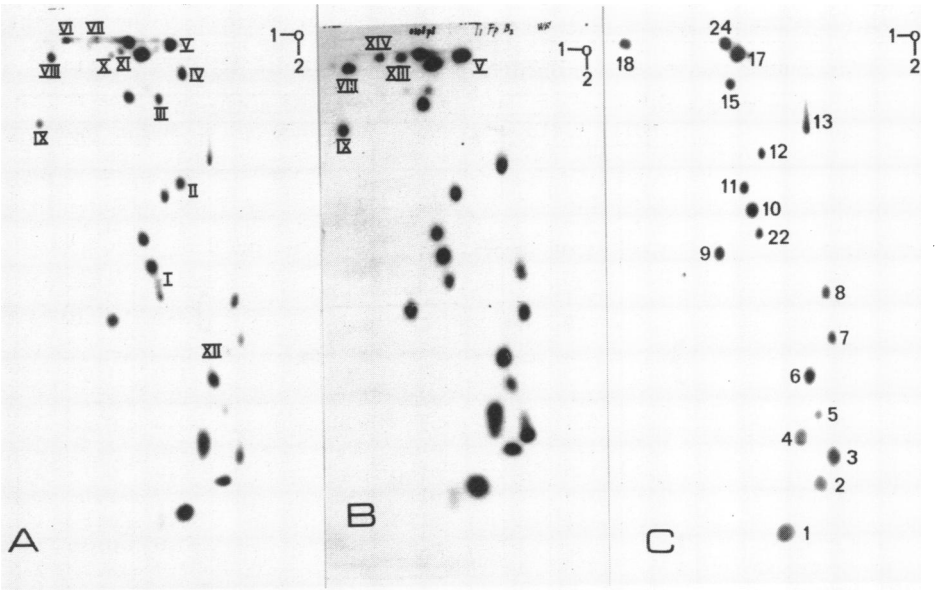

Fig. 2. Ribonuclease T_1 fingerprints of the material recovered from spots pA (panel A), pB (panel B) and m5S (panel C) in Fig. 1. The asterisk indicates the position of the xylene cyanol FF marker.

p5S RNA can be estimated from their electrophoretic mobility and sequence complexity (Fig. 1 and Table I) as being about 170 and 140 nucleotides respectively. These lengths are similar to the length of the two types of p5S RNA from B.subtilis (5), but quite a bit smaller than those of the B. licheniformis precursors (4).

B. Nucleotide sequence analysis of the Bacillus Q p5S RNAs

In Table I the nucleotide sequences and molar yields of the various oligonucleotides resulting from complete digestion of pA and pB with either RNAse T_1 or RNAse A are listed. The sequences of the oligonucleotides characteristic for m5S RNA have been determined previously (12,13). The sequences of the precursor-specific oligonucleotides were determined by secondary and, where necessary, tertiary digestion (see MATERIALS AND METHODS)*

*Since these data are of only limited interest to non-specialists they are not presented in full. The complete data are available, however, upon request.

Table 1 Sequences and molar yields of the oligonucleotides produced by complete digestion of pA and pB with either RNAse T_1 or RNAse A. Values are the mean of five independent determinations. The values for Bacillus Q m5S RNA, calculated from the known sequence(12), are included to facilitate comparison.

	RNAse Ti digest			
No	Sequence	Molar Yield		
		pA	pB	m5S
t1	G	12.5	11.4	10
t2	CG	3.3	2.8	3
t3	CCG	2.7	2.7	3
t4	AG	5.6	4.8	3
t5	ACG	0.9	0.8	1
t6	AAG	2.0	2.1	2
t7	CCAAG	0.9	0.8	1
t8a	AACACG	0.8	0.7	1
t8b	ACCCAG			-
t9	UG	2.1	2.0	2
t10	UAG	1.9	2.1	2
t11	AUG	1.7	1.2	1
t12	AUAG	1.0	1.0	1
t13	UCACACCCG	0.9	0.9	1
t15	UUAAG	1.0	1.2	1
t17a	CUCUUCAG	2.2	2.2	1
t17b	UUCCCAUG			1
t18	pUUUG	-	-	1
t21	UCG	0.8	0.9	1
t24	UUUCCCCCUG	1.3	1.2	1
tI	CUG	0.4	-	-
tIIa	CUAAAG	0.3	-	-
tIIb	UCAAAG	0.3	-	-
tIII	UCCCAUG	0.4	-	-
tIV	CAAUCAUACG	0.6	-	-
tV	AACACCUCUCAAUG	0.9	-	-
tVI	$CUUUUUUC_{OH}$	0.3	-	-
tVII	$CUUUUX_{OH}$	0.3	-	-
tVIII	UUUG	1.1	1.0	-
tIX	pUG	1.0	1.0	-
tX	UUCAUG	0.3	-	-
tXI	AUACUUG	0.3	-	-
tXII	ACAG	0.3	-	-
tXIII	$CCUUUUU_{OH}$	-	0.5	-
tXIV	$CUUUUU_{OH}$	-	0.5	-

	RNAse A digest			
No	Sequence	Molar Yield		
		pA	pB	m5S
p1	U	18	15	10
p2	C	24	20	18
p3	AC	4.4	4.1	3
p4	GC	4.2	3.8	4
p5	AU	2.4	1.2	1
p6a	AGC	2.8	2.0	2
p6b	GAC		-	-
p7	$AAGC_{OH}$	-	-	1
p8a	GAAC	1.0	1.0	1
p8b	AAGC	2.0	2.1	1
p9	GGC	0.8	0.7	1
p10	GU	2.6	2.3	2
p11	pUp	0.9	0.7	1
p12	GAU	2.7	2.1	2
p13	AGU	1.4	1.3	1
p14	AGGAC	0.8	0.8	1
p15	GGU	2.7	3.2	2
p16a	GGAAGU	2.1	2.1	1
p16b	GAGAGU			1
p17	GAAGAAGGU	1.0	1.0	1
p19	GGGGGU	0.7	0.9	1
pI	AAU	1.6	1.1	-
pII	AAAGAC	0.3	-	-
pIII	GAGC	0.7	-	-
pIV	AAAGGC	0.2	-	-
pV	AGAU	0.2	-	-
pVI	GAGAGAAC	0.7	0.8	-
pVII	GGGU	0.3	-	-

From Table I it is immediately clear that both pA and pB encompass more than one species of p5S RNA. The situation for pB is fairly simple: the RNAse T_1 digest contains only two (precursor-specific) oligonucleotides (tXIII and tXIV) in less than unimolar yield. Both were found to be 3'-terminal oligonucleotides by virtue of the fact that digestion with KOH did not produce Gp. Product tXIII is identical to tXIV except for an extra C residue at its 5'-end. Since both oligonucleotides occur in a molar yield of 0.5 (Table I) pB consists of two separate species which differ only by the insertion of a

single C residue close to the 3'-end. This difference does not show up clearly in the analysis of the RNAse A digest because the determination of free Cp and Up residues in these digests is relatively inaccurate.

Digestion of pA with RNAse T_1 or RNAse A produces several precursor-specific oligonucleotides in a molar yield of approximately 0.3 while others are found in a molar yield of about 0.7 (Table I). Considering the data in Table I it seems likely that pA comprises at least three different p5S RNA species. Unfortunately it is not possible to deduce the nature of the sequence differences directly from the data in Table I. It should be noted that neither pA nor pB gives rise to oligonucleotides characteristic of minor m5S RNA (12). Thus, there is likely to be a sixth precursor for minor m5S RNA which, probably due to its low amount, is not visible in Fig. 1.

In order to allocate the precursor-specific oligonucleotides to either the 5'- or the 3'-terminal non-conserved part both pA and pB were digested with RNAse T_1 under limiting conditions (see MATERIALS AND METHODS). We have previously established that under these conditions m5S RNA is cleaved predominantly at residue G39 only, producing "halves" of the m5S RNA molecule in high yield (13). Fig. 3a shows the products obtained after such a limited

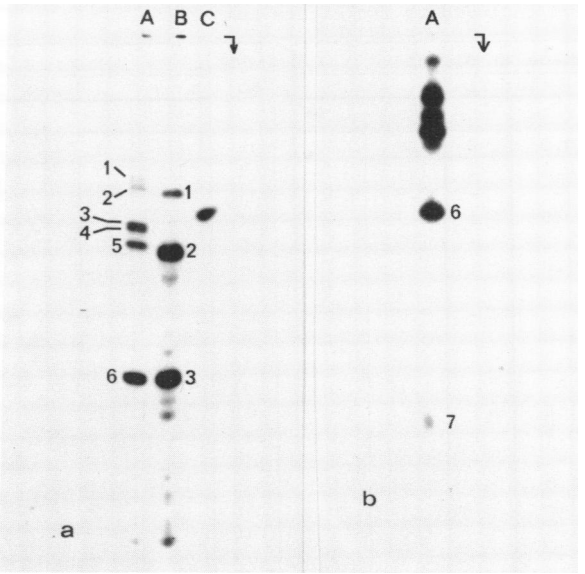

Fig. 3. Polyacrylamide gel electrophoresis of the products obtained by limited RNAse T_1 digestion of pA (lane A) and pB (lane B). Lane C shows intact m5S RNA. In Fig. 3b a similar separation of the products of pA is shown after a run for a shorter time.

RNAse T_1 digestion of pA and pB. Whereas pB gives rise to the expected three bands (band 1 represents remaining undigested material), digestion of pA produces a large number of bands indicating the presence of several additional G residues highly sensitive to RNAse T_1 which must be located in the precursor-specific parts of pA.

Table II lists the products obtained by complete digestion of bands pB-2 and pB-3 as well as band pA-6 with RNAse T_1 or RNAse A. When the known sequence of m5S RNA from Bacillus Q (12) is taken into account it is evident that band pB-3 represents the 5'-terminal "half" of pB. It contains the 5'-end group pUG of pB (cf. Table I) as well as oligonucleotides characteristic of the 5'-terminal part of m5S RNA. Band pB-2, by the same reasoning, is found to represent the 3'-terminal "half" of pB starting at residue C40 of the mature domain. Finally the digests of band pA-6 are identical to those of pB-3 and show no signs of sequence heterogeneity. No other fragments derived from the 5'-"half" of pA were detected. Therefore, all $p5S_A$ RNA species are

Table II. Molar yield of the oligonucleotides produced by complete digestion of the material from bands pB-2, pB-3 and pA-6 (Fig. 3) with either RNAse T_1 or RNAse A. Values are the mean of three independent experiments. The values for the 5'- and 3'-"halves" of m5S RNA (designated as 5'-m and 3'-m, respectively) have been included to facilitate comparison.

No	RNAse T_1 digest Molar Yield				
	pA-6	pB-3	5'-m	pB-2	3'-m
t1	4.0	4.0	3	7.0	7
t2	2.1	2.1	2	1.1	1
t3	-	-	-	2.8	3
t4	3.0	3.0	1	2.0	2
t5	-	-	-	1.0	1
t6	1.1	1.0	1	1.0	1
t7	-	-	-	1.0	1
t8	-	-	-	0.8	1
t9	1.0	1.0	1	0.9	1
t10	-	-	-	2.0	2
t11	-	-	-	1.1	1
t12	1.0	1.0	1	-	-
t13	0.7	0.7	1	-	-
t15	-	-	-	1.0	1
t17	1.0	1.0	1	1.0	1
t18	-	-	1	-	-
t22	-	-	-	1.0	1
t24	-	-	-	1.0	1
tV	0.9	0.9	-	-	-
tVIII	1.0	1.0	-	-	-
tIX	1.0	1.0	-	-	-
tXIII	-	-	-	0.5	-
tXIV	-	-	-	0.5	-

No	RNAse A digest Molar Yield				
	pA-6	pB-3	5'-m	pB-2	3'-m
p1	4.1	3.7	3	8.7	7
p2	8.2	7.6	6	9.6	13
p3	2.6	2.8	2	1.0	1
p4	-	-	-	2.6	3
p5	1.0	0.8	1	-	3
p6	0.9	0.9	1	1.1	1
p7	-	-	-	-	1
p8	-	-	-	2.9	2
p9	0.8	1.0	1	-	-
p10	1.0	1.0	1	1.0	1
p11	0.7	0.7	1	-	-
p12	1.0	1.1	1	0.9	1
p13	-	-	-	0.9	1
p14	-	-	-	1.1	1
p15	1.9	1.9	1	1.2	1
p16	-	-	-	2.0	2
p17	0.9	0.8	1	-	-
p19	-	-	-	0.9	1
pI	1.0	1.0	-	-	-
pVI	0.8	0.9	-	-	-

likely to have the same 5'-terminal precursor-specific sequence which, moreover, is identical to the corresponding sequence of the $p5S_B$ RNA species. This conclusion was confirmed by the sequence analysis of the 5'-terminal precursor-specific fragments obtained by in vitro maturation of pA and pB (see below). The sequence heterogeneity in pA, therefore, is restricted to the 3'-terminal non-conserved fragment.

The complete sequence of the 5'-terminal precursor-specific fragment of the p5S RNAs in Bacillus Q can be easily deduced from the data in Table II and is presented in Fig. 4. This fragment consists of 21 nucleotides and shows a large degree of homology with the corresponding sequence of B.subtilis p5S RNA.

Table II further shows that the 3'-terminal "half" of pB (band pB-2) gives rise to only two extra oligonucleotides compared to the 3'-"half" of m5S RNA. These extra products are the 3'-terminal oligonucleotides tXIII and tXIV discussed above. Consequently the 3'-terminal non-conserved sequences of the two $p5S_B$ RNA species are CU_4U_{OH} and U_4U_{OH} respectively (Fig. 4).

The large sequence heterogeneity of the 3'-terminal non-conserved segments of the various $p5S_A$ RNA species was a strong handicap in the analysis of these segments. Attempts to separate the different $p5S_A$ RNA species by various methods were in vain. Fortunately, however, the distribution of G residues highly sensitive to RNAse T_1 turned out to differ from one $p5S_A$ species to another. Consequently several partial RNAse T_1 digestion products could be obtained, that contained part of the 3'-terminal non-conserved sequence of

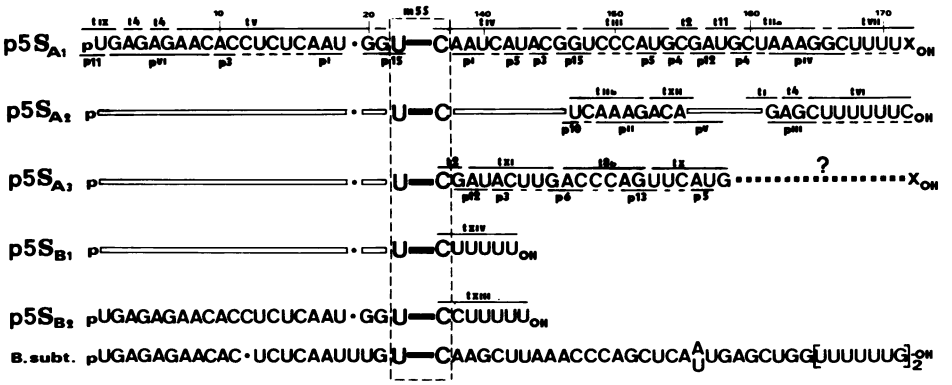

Fig. 4 Nucleotide sequences of the precursor-specific segments of the p5S RNAs from Bacillus Q. The positions of the RNAse T_1 and RNAse A digestion products are indicated. Open bars indicate sequence homology. The lowest line represents the sequence of B.subtilis $p5S_A$ RNA (5).

only one or at most two precursor species. Analysis of these products, combined with analysis of the mixture of intact 3'-terminal non-conserved fragments obtained by *in vitro* maturation of pA, allowed us to construct the complete 3'-terminal non-conserved sequence of two of the $p5S_A$ RNA species as well as the major part of this sequence of a third $p5S_A$ species. (*cf* Fig. 4).

Bands pA-3, pA-4 and pA-5 (Fig. 3a) are all derived from the 3'-terminal "half" of pA since they contain the complete set of oligonucleotides characteristic of the 3'-"half" of m5S RNA plus additional precursor-specific products. (Table III). The shortest of these fragments, pA-5, contains tIV (CAAUCAUACG) as the only extra RNAse T_1 oligonucleotide. Therefore tIV must span the junction between the mature and the 3'-terminal precursor-specific domain in one or more of the $p5S_A$ RNA species. The RNAse A digestion products of pA-5 are in agreement with the RNAse T_1 data (Table III). The extra copy of p8(AAGC) is equivalent to $p7(AAGC_{OH})$ of m5S RNA. The occurrence of product tIV in a molar yield of 0.6 in the RNAse T_1 digest of intact pA (Table I) implies that two of these three $p5S_A$ RNA species contain this oligonucleotide.

RNAse T_1 digestion of band pA-4 produces several precursor-specific oligonucleotides in a molar yield of about 0.5, indicating that pA-4 probab-

Table III Molar yields of the oligonucleotides produced by complete digestion of the material from bands pA-3, pA-4 and pA-5 (Fig. 3)with either RNAse T_1 or RNAse A. Values are the mean of three independent experiments. The values for the 3'-"half" of m5S RNA (3'-m) are included to facilitate comparison.

	RNAse T1 digest			
Spot	Molar Yield			
	pA-3	pA-4	pA-5	3'-m
t1	5.8	7.0	6.8	7
t2	1.9	1.2	1.1	1
t3	2.8	2.8	2.8	3
t4	2.0	1.9	2.0	2
t5	0.9	0.9	1.1	1
t6	1.0	1.1	1.0	1
t7	0.9	0.9	0.8	1
t8a,b	1.5	0.8	0.6	1
t9	0.9	0.8	1.1	1
t10	1.9	2.1	2.1	2
t11	1.0	1.1	1.1	1
t15	0.9	1.0	1.0	1
t17	1.0	1.1	1.2	1
t22	0.9	1.0	0.9	1
t24	0.8	1.0	1.1	1
tII	-	0.6	-	-
tIII	-	0.5	-	-
tIV	-	0.7	0.7	-
tX	0.8	-	-	-
tXI	0.9	-	-	-
tXII	-	0.6	-	-

	RNAse A digest			
Spot	Molar Yield			
	pA-3	pA-4	pA-5	3'-m
p1	8.7	5.8	5.6	7
p2	11.0	14.3	8.0	13
p3	1.9	1.9	1.8	1
p4	3.1	3.5	2.9	3
p5	1.1	1.5	1.2	-
p6	2.0	1.0	1.0	1
p7	-	-	-	1
p8	3.2	2.9	2.9	2
p10	0.9	1.4	0.9	1
p12	2.2	1.1	1.1	1
p13	2.1	1.2	1.2	1
p14	1.1	0.9	1.1	1
p15	0.9	1.6	1.1	1
p16	2.0	2.2	2.0	2
p19	0.8	1.0	0.8	1
pI	-	0.8	1.0	-
pII	-	0.5	-	-

ly is a 1:1 mixture of fragments derived from two different $p5S_A$ RNA species. Both species must contain product tIV connecting the mature and precursor-specific segments, since tIV is recovered in unimolar yield from pA-4. The presence of oligonucleotide pII(A_3GAC) in a molar yield of 0.5 in the RNAse A digest can only be explained by linking tII(UCA_3G) directly to tXII(ACAG) in one of the pA-4 fragments. On the basis of its electrophoretic mobility compared to pA-6 (60 nucl.), pB-2 (82/83 nucl.), pA-5 (86 nucl.) and pA-1 (142/143 nucl.) pA-4 should have a lenght of 94-98 nucleotides. Assuming one of the pA-4 fragments to have a precursor-specific sequence made up of tIV-tII-tXII in that order, a lenght of 96 nucleotides is obtained. The second pA-4 fragment should then contain tIV and tIII (UC_3AUG). This leaves products p15 (GGU), p4 (GC) and t2 (CG), all of which are present in a surplus molar yield of about 0.5 (Table III) still unaccounted for. In several experiments pA-4 was a much broader band than in Fig. 3. In these cases little or no surplus of t2 (CG) was present in the RNAse T1 digest of pA-4, indicating this sequence to be 3'-terminal. Even so the data do not allow a unique arrangement of the oligonucleotides into two sequences. However, the arrangement chosen in Fig. 4 accounts for the RNAse T1 sensitivity of the various G residues apparent from the partial digestion data (Fig. 3) which is likely to mirror base-paring within the p5S RNA molecules (see DISCUSSION). Also, this arrangement is the only one possible in which the two pA-4 fragments have exactly the same lenght.

The oligonucleotides produced by RNAse T1 and RNAse A digestion of band pA-3 (Fig. 3) are all recovered in approximately unimolar yield (Table III). The RNAse T_1 digest does not contain product tIV, however. Therefore pA-3 is part of a third $p5S_A$ RNA species differing in sequence from $p5S_{A1}$ and $p5S_{A2}$. Extra RNAse T_1 oligonucleotides derived from pA-3 are t2 (CG), t8b (ACCCAG), tX (UUCAUG) and tXI (AUACUUG). The presence of an extra copy of p13 (AGU) in the RNAse A digest of pA-3 shows that t8b is linked to tX. Furthermore, t2 (CG) must connect the mature and precursor-specific segments of pA-3 since it is the only extra RNAse T_1 product starting with a C residue. Finally the position of tX was deduced from the analysis of the RNAse A digest of the 3'-terminal precursor-specific fragment pf pA obtained by <u>in vitro</u> maturation. This digest contains the product pGAU (Fig. 6b) which places tX between t2 and t8b. The RNAse A digestion products of pA-3 are in agreement with this sequence (Table III and Fig. 4). Again the extra copy of p8a (AAGC) is the equivalent of p7 ($AAGC_{OH}$) produced by the 3'-"half" of m5S RNA. We have now determined the sequence of three $p5S_A$ RNA species up to residue 156-157. Since all three species have a length of approximately 170 nucleotides (see

above) this leaves 10-15 nucleotides at the 3'-terminus still to be sequenced. Band pA-2 (Fig. 3) showed too much sequence heterogeneity to be of any further help while band pA-1 contains remaining undigested material. However, by shorter electrophoresis of the partial RNAse T_1 digestion products of pA a fairly strong band (pA-7) in the lower part of the gel can be detected, having a length of about 15 nucleotides (Fig. 3b). RNAse T_1 digestion of this fragment (Table IV) produced the 3'-terminal oligonucleotides tVI (CU_6C_{OH}) and tVII (CU_4X_{OH}) each in half-molar yield. The molar yields of the other RNAse T_1 digestion products (Table IV) also show that pA-7 consists of two different fragments partly homologous in sequence.

The nucleotide sequences of the two pA-7 fragments are shown in Fig. 4 (residues 157-172 of $p5S_{A1}$ and $p5S_{A2}$). They were derived from the data in Table IV as well as those in Table I and analysis of the mixture of intact 3'-terminal non-conserved fragments obtained by in vitro maturation of pA with RNAse M5 from Bacillus Q (cf. Figs 5 and 6). An important additional consideration in this derivation was that the two different pA-7 fragments should have the same length. Neither these fragments nor the complete 3'-non-conserved segments of the various $p5S_A$ RNA species could be separated by gel electrophoresis. The direct linkage of one of the pA-7 fragments to the rest of the $p5S_{A1}$ RNA molecule was deduced from the presence of product p12 (GAU) - which can be clearly distinguished by its location from pGAU derived from the 3'-non-conserved part of $p5S_{A3}$ (cf. Fig. 6) - in a molar yield of 0.3 in the RNAse A digest of the intact 3'-non-conserved fragments of pA. The other pA-7 fragment is also directly linked to either $p5S_{A2}$ or $p5S_{A3}$ as could be concluded from the occurrence of product pV (AGAU) in a molar yield of 0.3 in the same digest. For reasons of homology (Fig. 4) and base-pairing (Fig. 7) this second pA-7 fragment is thought to be part of $p5S_{A2}$ and not of $p5S_{A3}$.

The complete analysis of the mixture of the 3' non-conserved segments ob-

Products	Sequence	Molar Yield pA-7
t1	G	0.5
t4	AG	0.4
t11	AUG	0.9
tI	CUG	0.4
tII	CUAAAG	0.4
tVI	$CUUUUUUC_{OH}$	0.4
tVII	$CUUUUX_{OH}$	0.5

Table IV. Molar yields of the oligonucleotides produced by complete digestion of the material from band pA-7 (Fig. 3b) with RNAse T_1.

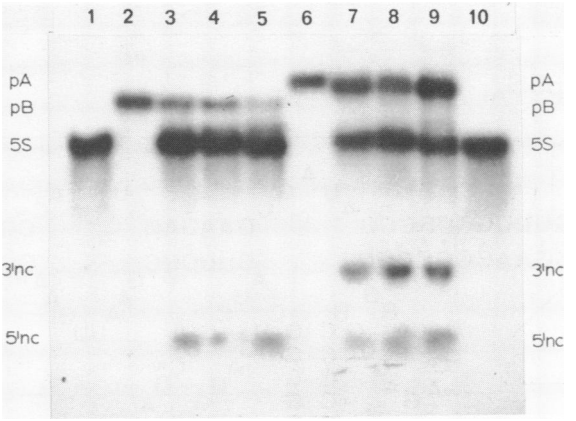

Fig. 5. In vitro maturation of Bacillus Q pA and pB with crude RNAse M5 from Bacillus Q (lanes 3 and 7), B. licheniformis (lanes 4 and 8), B. subtilis (lanes 5 and 9). Marker m5S RNA was run in lanes 1 and 10, pA in lane 6 and pB in lane 2.

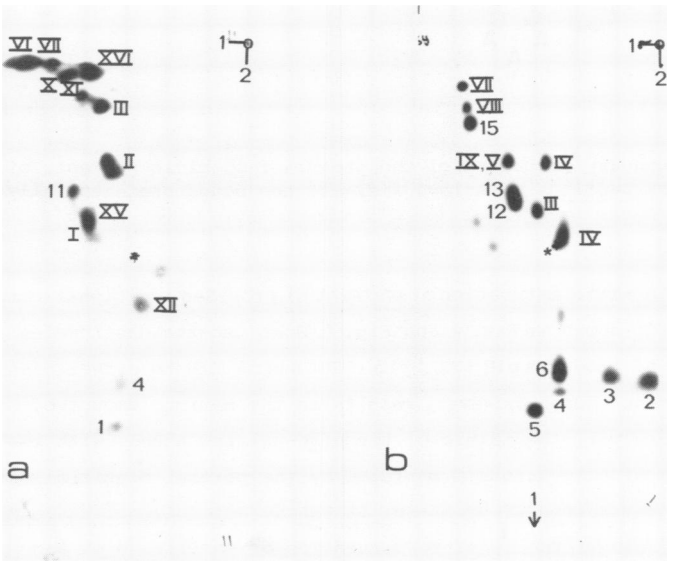

Fig. 6. RNAse T_1 (panel a) and RNAse A (panel b) fingerprints of the materials from the band indicated by 3'-n.c. produced by in vitro maturation of pA (Fig. 5). The products indicated by arrows were present in a molar yield of less than 0.2 and were not analysed further.

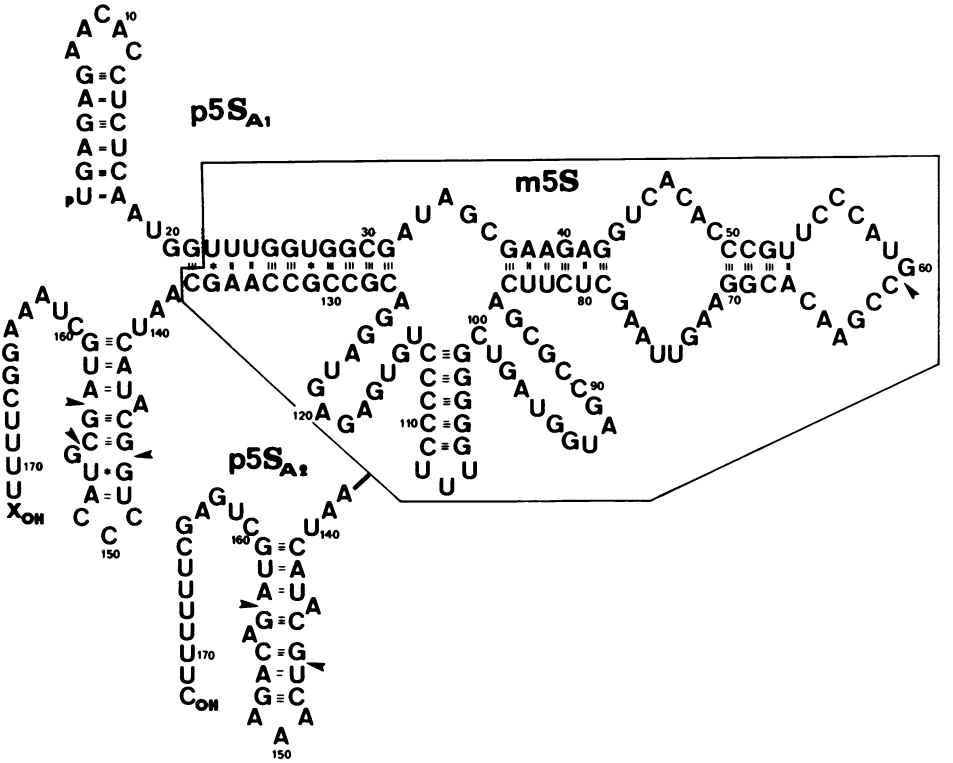

Fig. 7. Secondary structure of the precursor-specific segments of p5S RNA species from Bacillus Q. Arrows indicate the bonds preferentially cleaved by RNAse T_1.

tained by in vitro maturation of pA (Fig. 6) provided additional confirmation of the sequences shown in Fig. 4 and Fig. 7. Some uncertainty remains about the assignment of products p10 (GU), p15 (GGU) and t2 (CG) to either p5SA1 or p5S$_{A2}$. The assignment chosen, however, results in the maximum amount of base-pairing (Fig. 7). Moreover, the nature of the base-pairing agrees with the RNAse T_1 sensitivity of the various G residues by the partial digestion data (Fig. 7).

C. In vitro maturation of precursor 5S RNA by heterologous RNAse M5

Fig. 5 shows the in vitro maturation of Bacillus Q pA and pB with crude RNAse M5 from B.subtilis, Bacillus Q and B.licheniformis. In all cases it was found that the product comigrating with the marker m5S RNA gave rise to RNAse T_1 and RNAse A fingerprints identical to those of Bacillus Q m5S RNA (data not shown). In particular the 5'-terminal (pUUUG) and 3'-terminal (AAGC$_{OH}$) oligo-

nucleotides were recovered in unimolar yield. Clearly Bacillus Q p5S RNA is correctly maturated not only by RNAse M5 from the homologous strain but also by the corresponding enzyme from heterologous Bacillus strains. This conclusion was confirmed by the analysis of the other products of the in vitro maturation. The band indicated by 3'-n.c. in Fig. 5 was found to give rise to the products tXVI (pAAUCAUACG; molar yield 0.6), tXV (pGp; molar yield 0.3), pIX (pAAU; molar yield 0.6) and pVIII (pGAU; molar yield 0.3), (cf. Fig. 4) as well as the two 3'-terminal oligonucleotides tVI and tVII (Fig. 6). This band, therefore, represents the mixture of intact 3'-terminal non-conserved segments of the $p5S_A$ RNA species which bear the expected 5'-terminal sequences. The same conclusion could be drawn for the 5'-terminal non-conserved segments of both pA and pB which are present in the band indicated by 5'-n.c. in Fig. 5. The 3'-non-conserved segments of pB, because of their short lengths, have run off the gel.

In view of the lack of homology between the 3'-non-conserved sequences of the p5S RNA species of Bacillus Q and B.subtilis (Fig. 4) as well as B.licheniformis (manuscript in preparation) the highly efficient maturation by heterologous RNAse M5 preparations indicates that these non-conserved sequences play no role in the interaction between p5S RNA and the maturation enzyme.

DISCUSSION

A. Number of p5S RNA species in Bacillus Q

In Bacillus Q, like in B. subtilis (3) and B. licheniformis (4), two classes of p5S RNA accumulate upon treatment of growing cells with chloramphenicol which differ in length (Fig. 1). Class pA consists of at least three and class pB of two species which differ in sequence, but only as far as the 3'-terminal precursor-specific segment is concerned (Tables II and III). None of the $p5S_A$ and $p5S_B$ RNA species contain the sequences characteristic of minor Bacillus Q m5S RNA (12) as is evident from the digestion of these species with RNAse T_1 (Fig. 2) as well as the fingerprint analysis of the m5S RNA produced by in vitro maturation of pA and pB (data not shown). Bacillus Q minor m5S RNA, may thus be expected to have its own precursor different in length from pA and pB. Bacillus Q, thus, produces at least six different p5S RNA species with different genetic origins. Since $p5S_{A3}$ RNA probably consists of multiple species heterologous in the sequence of the 3'-terminal fifteen nucleotides a total of 7-8 different p5S RNAs is likely to be present in Bacillus Q. This would mean that each rRNA cistron in this strain produces a separate p5S RNA species. The same situation is

likely to prevail in B. licheniformis also (manuscript in preparation). In B. subtilis three different p5S RNA species have been detected (5) to which the precursor for minor 5S RNA probably has to be added.

B. Sequence of the p5S RNA species

All $p5S_A$ and $p5S_B$ RNA species in Bacillus Q have the same 5'-terminal precursor-specific fragment consisting of 21 nucleotides. This is concluded from the analysis of the partial RNAse T_1 digestion products pA-6 and pB-3 (Fig. 3 and Table II) as well as from fingerprint analysis of the 5'-non-conserved fragments produced by in vitro maturation of both pA and pB (Fig. 5). The sequence of this fragment could be unambiguously determined (Fig. 4). There is a large degree of homology between this sequence and the corresponding sequence of the B. subtilis p5S RNA (5) which becomes even more striking if one considers the lack of any significant homology between the 3'-terminal precursor-specific segments of p5S RNA in the two strains (Fig. 4). Moreover, even in the 5'-non-conserved segment the homology is limited to two features. Firstly, the G residue immediately adjacent to the 5'-terminus of the mature domain is conserved. As shown by Meyhack et al. (9) base-pairing between this G residue and the 3'-terminal C residue of the mature domain (cf. Fig. 7) is essential for cleavage of p5S RNA by RNAse M5. Our results confirm the importance of this base-pair for maturation. Secondly, the hairpin loop present at the 5'-end of B. subtilis p5S RNA (5) is also found in all Bacillus Q p5S RNAs (Figs 4 and 7), although in the latter strain it contains five instead of four residues in the loop. This hairpin is of no importance for cleavage of the precursor by RNAse M5 as is evident from the efficient in vitro maturation of both B. subtilis (23) and Bacillus Q (W.J. Stiekema, unpublished results) "p5S RNA" lacking this structure. The strong conservation of the hairpin, which also occurs at the 5'-end of the much longer 5'-precursor-specific segments of the B. licheniformis p5S RNAs (manuscript in preparation), nevertheless points to an important role for this double helical region. Conceivably it directs an RNAse E like enzyme (24) that cuts p5S RNA from an even longer precursor.

Finally our sequence data confirm that a tetranucleotide repeat around the 5'-terminal cleavage site, as present in B. subtilis p5S RNA (5), is unimportant in directing RNAse M5. This repeat is not conserved in the Bacillus Q precursors (Fig. 4). In fact, the only nucleotides of this repeat that are conserved are the G residue immediately preceding the mature sequence (discussed above) and the U residue at position 19 (Fig. 4). Nevertheless Bacillus Q p5S RNAs are efficiently maturated by B. subtilis RNAse M5

(Fig. 5). Residue U-19 may play a role in the interaction with RNAse M5 which was shown to have a certain specificity for the sequence immediately preceding the mature domain. An artificial precursor containing only C_6G at the 5'-end was maturated with only half the efficiency of one carrying either U_3G or the complete non-conserved sequence in this position (9).

The 3'-terminal precursor-specific sequences of the two $p5S_B$ RNA species could also be deduced quite simply from the analysis of the partial RNAse T_1 digests of pB (Table III). Both $p5S_B$ RNAs, like *B. subtilis* $p5S_A$ RNA (5), end in a stretch of U residues. Such a sequence is generally considered to constitute a stop signal for transcription (25). However, in *Bacillus Q* $p5S_B$ RNA the hairpin loop normally preceding such a set of U residues (25) is absent unless one considers the molecular stalk of the mature domain (extended by one or two G.C pairs respectively) to fulfil this role (Fig. 7). Possibly, however, transcription stops further downstream, like in some rRNA cistrons in *E. coli* (26), and $p5S_B$ has already lost the extra sequences by the action of an enzyme other than RNAse M5.

The $p5S_{A1}$ as well as the $p5S_{A2}$ species also end in a stretch of U residues (Figs 4 and 7). A hairpin, although not a perfect one, is likely to be present in both precursors as indicated by the results of partial RNAse T_1 digestion. This hairpin, however does not immediately precede the stretch of U residues. Thus, as in the case of $p5S_B$ RNA, there remains doubt whether the 3'-termini of $p5S_{A1}$ and $p5S_{A2}$ RNA corresponds to the termini of the respective rRNA cistrons or whether they arise by some previous processing event. The 3'-terminal sequence of $p5S_{A3}$ RNA could not be determined, probably due to further heterogeneity within this sequence.

A second feature of the 3'-terminal precursor-specific segments of the $p5S_A$ RNA species also deserves closer attention. Obviously an extension of the molecular stalk, formed by base-pairing between the 5'- and 3'-terminal sequences of the mature domain, by only a single base-pair suffices for correct cleavage at both the 5'- and 3'-terminal sites (Fig. 7). As far as the 5'-terminal site is concerned this confirms the results of Meyhack *et al.* (9) obtained with the use of artificial substrates in *in vitro* maturation. These authors could not determine, however, whether location of the 3'-terminal cleavage site *within* a double-helical region as occurring in *B. subtilis* p5S RNA (5), is a prerequisite for correct maturation. The result presented here clearly shows that such is not the case.

The almost complete absence of sequence homology between the 3'-terminal precursor-specific fragments of $p5S_A$ RNA from *B. subtilis* and *Bacillus Q* as

well as the large degree of sequence heterogeneity of these fragments between the individual Bacillus Q p5S RNA species contrasts strongly with the conservation of most of the 5'-non-conserved sequence (Fig. 4). Clearly the 3'-terminal sequence, apart from possibly being responsible for termination of transcription, is of little biological significance. Certainly it plays no role in maturation of p5S RNA.

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