Plasmid-directed Expression of Staphylococcus aureus $\beta$-Lactamase by Bacillus subtilis in Vitro*

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A plasmid carrying the Gram-positive Staphylococcus aureus PC1 $\beta$-lactamase gene is active in directing a cell-free transcription and translation system from Bacillus subtilis. The major protein synthesized has been identified as the S. aureus $\beta$-lactamase on the basis of peptide mapping. The protein is larger than the extracellular enzyme by about $M_r = 3100$. Significant in vitro translation of the $\beta$-lactamase mRNA occurs in the absence of the initiation factor fraction as is characteristic of translation of mRNAs of Gram-positive origin. The 1250 base transcript that encodes the $\beta$-lactamase and leader sequence has been mapped on the plasmid molecule.

Ribosomes from the Gram-positive bacterium, Bacillus subtilis, are extremely inefficient in translating mRNAs isolated from Escherichia coli and other Gram-negative bacteria (1-7). These ribosomes are as active as those from E. coli, however, in translating mRNAs of Gram-positive origin (1, 2). The lack of RNA phages with a Gram-positive host range (8) has hampered attempts to analyze specific Gram-positive mRNA-ribosome complexes. The development of an in vitro transcription system from B. subtilis (9) has enabled us to synthesize a defined population of mRNAs from the small bacillus phage $\phi 29$ DNA (10).

Utilizing the phage $\phi 29$ DNA-directed cell-free transcription and translation system, we were able to show that the B. subtilis translation system incorporates labeled amino acids into discrete protein products at an efficiency similar to the E. coli system. The most striking result of this study was that in both E. coli and B. subtilis translation systems, significant amounts (20-40%) of the same proteins are synthesized in the absence of the initiation factor fraction as are made in its presence (11). Previous studies indicated that this reduced dependence on the initiation factor fraction is not peculiar to $\phi 29$ mRNA but is a property of less well defined Gram-positive mRNA populations as well (3, 12). In marked contrast, translation of most mRNAs of Gram-negative origin by E. coli ribosomes is strictly dependent on the initiation factor fraction (3, 13-15). Thus, there is some feature of mRNAs derived from Gram-positive bacteria that facilitates protein synthesis in the absence of initiation factors and which may be, at least in part, responsible for the efficient mRNA recognition by ribosomes from Gram-positive bacteria.

This observation suggests that a structural difference between mRNAs isolated from Gram-positive and Gram-negative bacteria is involved in species specific translation. It seemed valuable, therefore, to characterize a specific mRNA from a Gram-positive source with regard to the initiation site for protein synthesis. Since none of the $\phi 29$ proteins has been identified by its NH$_2$-terminal amino acid sequence or by function, we chose instead to characterize the Gram-positive Staphylococcus aureus PC1 $\beta$-lactamase gene.

The S. aureus PC1 $\beta$-lactamase gene is carried on a variant of the naturally occurring Staphylococcus plasmid p1258 (pen I-443) and is expressed constitutively (16-18). An Eco RI fragment of this plasmid has been cloned in pSC101 and confers ampicillin resistance upon E. coli hosts (19). Strain PC1 was chosen for the main sequence studies because it produced the highest proportion of extracellular enzyme. Not only is the amino acid sequence of the secreted $\beta$-lactamase known (20), but the activity of the enzyme is easily assayed by its hydrolysis of the $\beta$-lactam bond of penicillin-like substrates (21, 22).

We report here the transfer of the staphylococcal fragment encoding ampicillin resistance to a pMB9 vector. The hybrid plasmid, designated pJM13, directs the in vitro synthesis of large amounts of S. aureus $\beta$-lactamase in a B. subtilis transcription and translation system. The $\beta$-lactamase formed in vitro has been identified by peptide mapping. It is larger than the extracellular enzyme by about $M_r = 3100$, suggesting the presence of a signal (leader) peptide sequence in the in vitro product. Furthermore, significant in vitro synthesis of the $\beta$-lactamase by both B. subtilis and E. coli systems occurs in the absence of the initiation factor fraction. The transcript encoding S. aureus $\beta$-lactamase has been identified and mapped on the plasmid molecule as have the other major transcripts specified by this plasmid. This specific mRNA, which is recognized efficiently by B. subtilis ribosomes, has enabled us to determine possible differences in initiation site structure that are important for recognition by Gram-positive ribosomes.

EXPERIMENTAL PROCEDURES

Materials—The sources of many materials have been cited previously (10). In addition, [a-$^{32}$P]ATP and l-[4,5-$^{3}$H]lysine were purchased from ICN. Ethidium bromide was purchased from Calbiochem. Cesium chloride (special biochemical grade) and sodium dodecyl sulfate were purchased from Gallard Schlesinger. Dowex AG 50W-X8 (200-400 mesh) and polyacrylamide gel reagents were from Bio-Rad. Ampicillin, tetracycline, and chloramphenicol were purchased from Sigma. TPCK-trypsin (223 units/mg) and DNase I (2029 units/mg) were obtained from Worthington. Nitrocefin (compound 87/312) was a gift of Glaxo-Research Ltd., Greenford, Middlesex, England. T. DNA ligase was purchased from Bethesda Research Labs.

Bacterial Strains and Plasmids—E. coli cell line SC489 containing pSC122 was obtained from Esther Lederberg, Dept. of Medical Microbiology, Stanford University Medical Center. pMB9 DNA was a gift of Alice Wong and Nancy Mazels, formerly of the Department of Biochemistry, University of California, Berkeley. E. coli RHI (22) was obtained from H. W. Boyer, Dept. of Biochemistry and Biophysics, University of California, San Francisco. Bacteria with antibiotic resistances were grown in L broth (10 g of Bactotryptone, 5 g of yeast

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extract, 10 g of NaCl, 1 NaOH pellet/liter in the presence of 10 µg/ml of tetracycline and/or 30 µg/ml of ampicillin. S. aureus PCI was obtained from the National Collection of Industrial Bacteria (No. 11195).

Nucleases—The sources of exoIII and S1 nuclease were previously cited (10). The restriction endonucleases Eco RI, Xba I, and Sma I were purchased from Bethesda Research Labs. HindIII was prepared by a published procedure (24) by Warren Gish working in this laboratory.

Purification of Plasmid DNA—Bacteria were grown to A660 = 0.5 in L broth and were treated 18-20 h with 25 µg/ml of chloramphenicol in order to amplify the plasmid DNA. Cleared lysates were prepared as described (25) and then precipitated with 0.6 volumes of isopropanol. Pellets were resuspended in 4.8 ml of 50 mM Na2-EDTA (pH 8.0). Solid CaCl₂ (4.75 g) and 0.2 ml of ethidium bromide (10 mg/ml) were added. The refractive index was generally 1.392. Samples were overlaid with paraffin oil (Fisher) and spun at 20 °C and 34,000 rpm for 40 h in a Spinco SW20 rotor. The plasmid band (visualized by UV illumination if necessary) was removed with a 1-ml disposable syringe and 18-gauge needle. Ethidium bromide was removed by passing the DNA over a 4-5 ml Dowex AG 50W-X8 column (1.1 x 45 cm) in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA buffer. The Dowex, 100 g, had been washed with water in a liter each of 20 mM Tris-HCl and 1 N HCl and autoclaving in H₂O at pH 6. Dowex stored in this manner was washed 3 times with 1 mM Tris-HCl (pH 8.0) prior to use in a column. CaCl₂ in the pooled DNA was removed by overnight dialysis at 4 °C against buffer containing 20 mM Tris-HCl (pH 8), 20 mM KC1, 1 mM EDTA.

Purification of DNA Fragments—Fifty µg of supercoiled pM13 was digested with Eco RI and Sma I and phenol extracted. The phenol-purified DNA was divided into 150-µl aliquots which were layered on each of two 10-ml gradients 10-30% sucrose gradients containing 20 mM Tris-HCl (pH 8.0), 12 mM MgCl₂, 10 mM EDTA, 150 mM KCl. Samples were spun at 5 °C and 25,000 rpm for 21 h in the Spinco SW20 rotor. Fractions were collected from the bottom of the tube and analyzed by agarose gel electrophoresis prior to pooling the largest fragment which was identified as the staphylococcal DNA.

Nucleic Acid Digestion—Cleavages of pM13 DNA with restricted endonucleases Eco RI, HindIII, Sma I, and Xba I were carried out at 37 °C according to the conditions described by Bethesda Research Labs. For use in transcription reactions, endonuclease-treated DNA was generally deproteinized by phenol extraction and dialyzed overnight at 4 °C against 10 mM Tris-HCl (pH 8), 1 mM EDTA.

Eco RI or HindIII digests of pM13 DNA were digested with exonIII as described (10) except that 50 µl aliquots of the reaction mixture were withdrawn and added to 7 µl of concentrated S1 nuclease containing buffer (2 µl NaCl, 5 µl MnCl₂, 300 µl NaAc (pH 4.7), 50 µg/ml of S1 nuclease) at 42 °C for 10 min. Aliquots were diluted 2-fold, phenol extracted and dialyzed against 4°C water and a buffer containing 20 mM Tris-HCl (pH 8), 20 mM KC1, 1 mM EDTA.

Plasmid Construction and Selection—One pg of Supercoiled pM13 and 1 µg of Eco RI-digested pSCE12 and 1 µg of Eco RI-digested pMB9 were ligated at 4 °C in ligase buffer (66 mM Tris-HCl (pH 7.5), 6.6 mM MgCl₂, 10 mM dithiothreitol, 0.4 mM ATP) with 0.8 unit of TI ligase. After this DNA was used to transform E. coli K12, plasmids were screened as described (26) except for decreasing volumes 10-fold so that 1.5 µl microfuge tubes could be used. Putative recombinant plasmids were treated with restriction endonucleases and analyzed on agarose gels.

In Vitro RNA Synthesis—RNA synthesis for gel analysis was performed as described (9). Reactions contained in a volume of 50 or 100 µl 100 mM Tris-HCl (pH 8), 10 mM MgCl₂, 0.1 mM EDTA, 0.2 mM dithiothreitol, 1.6 mM spermidine, and 0.4 µl T3, T7, and 0.2 µl [α-32P]ATP (500-1000 cpm/pmol) with KCl, DTT, and RNA polymerase concentrations as indicated. B. subtilis RNA polymerase was prepared as described (28). E. coli MRE 600 RNA polymerase was prepared according to Burgess and Jenndraak (27).

In Vitro DNA-directed Protein Synthesis—DNA-directed assays were performed in a 60-µl volume containing 3-5 µg of DNA 60–100

The abbreviations used are: exoIII, E. coli exonuclease III; S1 nuclease, S1 nuclease of Staphylococcus aureus S1; Sma I, sodium dimethyl sulfoxide; TPKC, L-t-lysyl-mono-2-phenylethylchloromethyl ketone.

1. D. Hanahan, personal communication.

2. C. L. Murray, B. L. Davison, and J. C. Rabinowitz, unpublished observations.
Cell-free Transcription and Translation of a Gram-positive Gene

pSC101 and a $4.6 \times 10^6$-dalton Eco RI restriction fragment of a derivative of a naturally occurring S. aureus plasmid p258 (17, 18). This staphylococcal plasmid DNA fragment carries genetic information for constitutive penicillin-ampicillin resistance and confers resistance on both E. coli and B. subtilis hosts in vivo (19, 40). In order to increase yields of plasmid DNA, it was desirable to transfer the staphylococcal ampicillin resistance fragment from the stringently controlled pSC101 replicon to the relaxed replicon, pMB9. Illustration of this recombination is shown in Fig. 1. Transformants resistant to both tetracycline and ampicillin were selected and characterized by restriction analysis. The $8.6 \times 10^6$-dalton plasmid pJM13 was further shown to consist of pMB9 ($3.5 \times 10^6$ daltons), the $4.6 \times 10^6$-dalton staphylococcal plasmid DNA, and an additional $0.5 \times 10^6$-dalton Eco RI fragment of unknown origin. E. coli (RRI) containing pJM13 are resistant to 50 μg/ml of tetracycline (minimum inhibitory concentration) and greater than 400 μg/ml of ampicillin.

In Vitro Translation Products Directed by pJM13 DNA—
Supercoiled pJM13 was purified as described under "Experimental Procedures." The relative activities of the cell-free coupled transcription and translation systems isolated from E. coli and B. subtilis as measured by incorporation of $[^3H]$lysine in response to supercoiled pJM13 DNA, phage ϕ29 DNA, and T7 DNA are shown in Table I. The B. subtilis system is about 50% as active as the E. coli system on pJM13 which suggests that the Gram-positive staphylococcal DNA is transcriptionally and translationally active since the Gram-negative pMB9 DNA directs little or no protein synthesis in the B. subtilis system (data not shown). Digestion of supercoiled pJM13 with endonucleases Eco RI, HindIII, or Xba I reduces amino acid incorporation in the E. coli system to 88, 84, and 65% of the value obtained with the supercoiled DNA, while digestion by Xba I lowers incorporation in the B. subtilis system further to 45% of the value obtained with supercoiled DNA.

The protein products of the pJM13 DNA-directed cell-free systems were analyzed by electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel (Fig. 2). There are no detectable products in the absence of added DNA (11). A major protein synthesized in pJM13 DNA-directed reactions by both the E. coli (lane a) and B. subtilis (lane c) systems is a product of $M_r = 32,000$. This protein is made very efficiently by both systems. In addition, significant amounts of this protein continue to be made by both the E. coli (lane b) and B. subtilis (lane d) systems when the salt wash fraction containing initiation factors is omitted from the assay. Although the amino acid incorporation in the absence of the initiation factor fraction in the experiment shown (Table I and Fig. 2) is 18% of the incorporation achieved in the presence of the initiation factor fraction, subsequent assays in the absence of salt wash have resulted in 28% of the incorporation observed in the complete system. We believe that this higher level is the more usual extent of factor-independent translation with pJM13 RNA. Based on the radioactivity in the 32-kilodalton band excised from a polyacrylamide gel relative to the remainder of labeled products in these later assays, 65% of the $[^3H]$lysine incorporated by the B. subtilis system in either the absence or presence of the initiation factor fraction is in the 32-kilodalton protein. These observations suggest that the 32-kilodalton protein is encoded in the Gram-positive portion of pJM13 since substantial initiation of protein synthesis in the absence of initiation factors is characteristic of messenger RNAs from ϕ29 and other Gram-positive sources (3, 12). No detectable 32-kilodalton protein is synthesized in reactions directed by Xba I-digested pJM13 in contrast to those directed
Cell-free Transcription and Translation of a Gram-positive Gene

**Fig. 2.** Electrophoretic analysis of proteins synthesized in cell-free pJM13 DNA-directed transcription/translation systems from S. aureus and E. coli. Assays were performed in the presence and absence of salt wash protein as described under "Experimental Procedures." All assays contained 0.35 pmol of pJM13 DNA and [^3H]lysine at 240 cpm/pmol. Lane a, E. coli system + salt wash; lane b, E. coli system - salt wash; lane c, B. subtilis system + salt wash; lane d, B. subtilis system - salt wash. Acid-insoluble [^3H]lysine applied the 15% polyacrylamide gel in lanes a-d was 221,760, 99,969, 113,040, and 20,160 cpm, respectively. Molecular weight markers included the 3 major p29 proteins formed in vitro of Mr = 10.5, 13.9, and 22.4 kilodaltons and T7 ligase of Mr = 41.7 kilodaltons. Exposure was for 8 days.

by Eco RI- or HindIII-digested pJM13 (data not shown). The Xba I site has been mapped in the vicinity of the staphylococcal β-lactamase gene (17).

**Peptide Mapping of the 32-kilodalton In Vitro Product—** Although the extracellular β-lactamase of S. aureus cultures has Mr = 28,823 (41), we recognized that the product formed from the same gene might be larger because of the presence of a leader amino acid sequence. There are 43 lysine residues and 4 arginine residues among the 257 amino acids of purified extracellular S. aureus β-lactamase (20). Lysine was therefore chosen to label the product formed in the *in vitro* synthesis to allow the detection of the maximum number of tryptic peptides if the 32-kilodalton protein were related to β-lactamase. The [^3H]lysine 32-kilodalton protein synthesized by the homologous B. subtilis transcription and translation system was eluted from a sodium dodecyl sulfate-polyacrylamide gel. Purified extracellular S. aureus β-lactamase from exponentially growing S. aureus cultures (30) was combined with the purified *in vitro* protein and digested with trypsin. The resulting peptides were chromatographed on the strongly acidic cation exchange resin Aminex A-5 (38). The column profile is shown in Fig. 3. The dotted line represents the radioactively labeled peptides of the *in vitro* 32-kilodalton protein. The solid line denotes peptides of S. aureus β-lactamase detected by ninhydrin assay (39). The large peak of ninhydrin reactivity at fractions 50-55 is due to free ammonia present in the sample applied to the column.

**Identification of 32-kilodalton Protein As β-lactamase—** The peptides formed by tryptic digestion of the 32-kilodalton protein formed in the *in vitro* system correspond very closely to those formed from a similar digest of purified S. aureus β-lactamase (Fig. 3). Every[^3H]lysine peptide of the *in vitro* product corresponds to a ninhydrin peptide of β-lactamase produced *in vivo* except for the three in the region where the ninhydrin peptides are obscured by ammonia. The peptides detected by ninhydrin that do not have corresponding[^3H] lysine peptides may result from any of the four unlabeled arginine peptides or from the unlabeled COOH-terminal peptide. The similarity of the tryptic peptide maps of the *in vitro* 32-kilodalton protein and S. aureus β-lactamase indicates that the major product synthesized by the B. subtilis coupled system is authentic S. aureus β-lactamase. The abundance of lysine residues in β-lactamase does not account for its appearance as the major *in vitro* product since incorporation of[^35S] methionine also results in β-lactamase as the major product (data not shown). The β-lactamase produced *in vitro* (e.g. lanes e and f) and *in vivo* (lane between e and f) is run on adjacent lanes of an SDS-polyacrylamide gel in Fig. 3. Radioactive ink marks the position of Coomassie brilliant blue-stained molecular weight markers (lane between e and f) including β-lactamase of Mr = 28,823 produced *in vivo* of β-Lactamase synthesized by the *in vitro* system is larger by about Mr = 3,100.

**Detection of β-Lactamase Activity in the B. subtilis Cell-free System**—The large amount of β-lactamase produced *in vitro* by B. subtilis prompted us to assay for β-lactamase activity after transcription and translation of pJM13 DNA. The chromogenic cephalosporin 87/312 (22) (Nitrocefin) was used as substrate in order to avoid interference by the high

![Fig. 3. Identification of the 32-kilodalton protein as β-lactamase.](image-url)
concentration of protein in the cell-free protein synthesis reaction. Mercaptoethanol, present at 10 mM in cell-free reactions, accounts for some yellow to red color production in the absence of pJM13 DNA corresponding to 26.5 milliunits (22). Reaction mixtures that contained pJM13 DNA resulted in the production of 11.8 milliunits of β-lactamase activity over this background. Similar levels of activity were consistently observed. The production of 3.2 milliunits of activity over background in assays directed by pJM13 DNA in the absence of the salt wash fraction (0.27) is consistent with the ratio of a result of in vitro β-lactamase synthesis. That is, the ratio of polyacrylamide gel. This represents about 17 times more.

The specific activity of purified S. aureus PC1 enzyme on Nitrocefin was found to be 66 ± 5 units/mg. An activity of 11.8 milliunits therefore corresponds to 10.8 ng or 0.34 pmol of active β-lactamase produced in a 60-μl reaction by the B. subtilis in vitro system. In this cell-free reaction, 5.8 pmol of β-lactamase protein were produced as determined by the [3H]lysine present in the 32-kilodalton band separated on an SDS-polyacrylamide gel. This represents about 17 times more protein than estimated on the basis of enzyme activity.

Transcription of pJM13 DNA in Vitro—Having demonstrated that the major protein synthesized in the B. subtilis cell-free system by pJM13 DNA-directed transcription and translation system in S. aureus β-lactamase, we chose to map the position for this gene on the plasmid molecule. The transcription products formed by both purified E. coli RNA polymerase (27) and B. subtilis RNA polymerase (9) directed by supercoiled pJM13 DNA at an enzyme/DNA ratio = 5 are shown in Fig. 4. Increasing the ionic strength from 2 mM (lanes c and e) to 160 mM (lanes d and f) not only stimulated synthesis (1.5-fold for B. subtilis RNA polymerase and 1.9-fold for E. coli RNA polymerase), but also enhanced the production of RNAs of discrete size classes as previously observed for transcription of φ29 DNA (9). As shown in lane d of Fig. 4, B. subtilis RNA polymerase produces products containing about 750, 1250, and a population ranging in size from roughly 3700 to 5900 bases. E. coli RNA polymerase (lane e, Fig. 4) also produces products containing 1250 and those ranging from 3700 to 5900 bases. E. coli polymerase produces an additional product of about 8000 bases (Fig. 4, lane f). The RNA marker of 1150 bases (Fig. 4, lane a) appears to co-migrate with a pJM13 transcript (Fig. 4, lanes d and f) that has been determined to be 1250 bases in length from a number of gels (such as that shown in Fig. 5) in which increased resolution was obtained (compare the separation of G5 and Ala markers in Figs. 4 and 5). Radioactivity is always seen at the top of the lanes when supercoiled DNA is used as a substrate. Treatment of completed transcription reaction mixtures with 0.02-0.2 unit of DNase I for 15 min at 25 °C, 6 units/μg of DNA of restriction endonuclease Eco RI for 10 min at 37 °C, heating at 60 °C for 2-10 min, heating at 90 °C for 1 min, or heating in 80% deionized formamide at 80 °C for 1–2 min was ineffective in redistributing the material at the top of the gel into discrete products.

Transcription of pJM13 DNA—Fig. 5 illustrates the RNA products formed by transcription of pJM13 (lane h), pJM13 restricted at a variety of sites (lanes b–g), and the purified Eco RI staphylococcal DNA fragment (lane i). Transcription of Eco RI-digested fragments of pJM13 (lane b) as well as purified staphylococcal DNA fragment (lane i) results in the synthesis of two major RNA species of 2000 and 1250 bases in length. Since the 1250-base RNA is also synthesized when supercoiled DNA is used as substrate, the sites for initiation and termination of the 1250-base RNA must lie entirely within the staphylococcal DNA fragment. By contrast, the 2000-base RNA is generated only in transcription of Eco RI-digested plasmid at the expense of the range of longer RNA species seen with the other substrates (lanes c–f and h, Fig. 5, and Fig. 4). Therefore, it is a run-off RNA-initiated 2000 base pairs from one of the Eco RI ends of the staphylococcal DNA. The 3700-base RNA (Fig. 5, lanes c–f and h) results from initiation at this same promoter and transcription across the Eco RI restriction site to a partially efficient terminator in the pMB9 portion of the plasmid. The 3700-base transcript can be placed as shown on Fig. 6, since HindIII-digested substrate is observed to leave its transcription unaltered (Fig. 5, lanes d and e). If this RNA were initiated at a promoter 2000 base pairs from the other end of the staphylococcal DNA, transcription of HindIII-digested pJM13 would result in an RNA of about 2330 bases. The 3700-base RNA was also found to hybridize to all three Eco RI fragments of pJM13 (data not shown), which lends further support to its position indicated in the map (Fig. 6).

A significant amount of RNA polymerase reads through the termination site for 3700-base RNA to produce an RNA species some 8000 bases in length. The exact size of this
11278  

Cell-free Transcription and Translation of a Gram-positive Gene

... the Xba I site. The location of this promoter relative to the Xba staphylococcal DNA is provided by transcription of Xba I-restricted volume of were: lane a, fragment, termini with exoIII and S1 nuclease for increasing time periods Consecutive digestion of DNA molecules with accessible 3'...
Cell-free Transcription and Translation of a Gram-positive Gene

**FIG. 7.** Transcription of exonucleolytically shortened pJM13 DNA. Eco RI- or HindIII-digested pJM13 DNA (40 µg) was digested with 20 µg/ml of exoIII in a 0.22-ml reaction containing 0.25 M Tris-HCl, pH 8.0, 0.02 M MgCl₂, 0.07 M β-mercaptoethanol. 50-µl aliquots were withdrawn from exoIII-treated Eco RI-digested pJM13 DNA after 1.5, 4, 6, and 8 min of digestion and from the exoIII HindIII-digested pJM13 DNA after 1 min and treated with S1 nuclease as described under “Experimental Procedures.” Time course samples were analyzed by electrophoresis on a 1% agarose gel (a) while the remainder of each sample was phenol extracted and dialyzed for subsequent transcription. Starting HindIII-digested pJM13 (a, lane d), exoIII-treated HindIII-digested pJM13 (lane e), starting A, B, and C fragments of Eco RI-digested pJM13 (lane f), and the 1.5-, 4-, 6-, and 8-min exoIII digests of Eco RI-digested pJM13 (lanes g-i) are visualized by ethidium bromide staining. The temporal decrease in size of the Eco RI A and B fragments as determined by standard log molecular weight versus migration plots (not shown) is plotted in b; solid circles represent the Eco RI A or staphylococcal DNA fragment bases, slightly shorter than the φ29 A1a transcript, which is probably unresolved at the bottom of the gel. Thus the 2000-base RNA is initiated at a promoter 2000 base pairs from the Eco RI site and results from RNA polymerase running off the end of Eco RI-digested DNA (Fig. 6).

**Translation Products Directed by exoIII-treated pJM13 DNAs—**Having mapped the two major transcripts directed by the staphylococcal portion of pJM13 DNA, we wanted to know which one encoded the β-lactamase protein. Gel electrophoretic analysis of the proteins synthesized in coupled transcription and translation assays directed by the same truncated DNAs used in Fig. 7 is shown in Fig. 8. β-Lactamase is produced in all reactions in which 1250-base RNA is present (Fig. 8, lanes a-h, i, l, and m), but no β-lactamase is made when the promoter for 1250-base RNA is removed as in the case of the exoIII-treated HindIII digest (Fig. 8, lane k). The small amount of β-lactamase which is apparently produced (Fig. 8, lane k) most likely results from 1250-base RNA transcribed from a fraction of DNA which is nuclease resistant (10). Furthermore, when only the 1250-base RNA and a 240-base RNA from the promoter for the 2000-base RNA are present (Fig. 7, lane g), β-lactamase is synthesized (Fig. 8, lane g). Thus, the 1250-base RNA is the mRNA encoding β-lactamase.

Several lower molecular weight products are also made when only the 1250-base RNA is present. The most major of
these products (Fig. 8, lane g) have approximate $M_n = 17,600 \pm 400, 14,300 \pm 400,$ and 11,400 \pm 400. Reduced amounts of products (but in a comparable ratio to $\beta$-lactamase) with similar molecular weights are formed in the presence of s aureus DNA. The genes for resistance to $\beta$-lactamase allowed a determination of the enzymatic activity of this region (see accompanying report) protein synthesis in the absence of the 1250-base RNA (fig. 8, lane g). Since the 1250-base RNA has only enough coding capacity for a protein of about $M_n = 12,000$ in addition to $\beta$-lactamase, the major products present in the cell's inner membrane. The observed activity could result from a low level of processing during the transcription and translation reaction. Second, the unprocessed form of the enzyme may be only about 6% as active as the processed form. We have not distinguished between these two alternatives. There is no evidence of inhibition of $\beta$-lactamase activity in the cell-free assay since addition of purified $\beta$-lactamase to a control cell-free assay yields the activity expected for purified enzyme alone. Although it has been reported that a cell-bound form of $B. licheniformis$ $\beta$-lactamase from which the extracellular enzyme is cleaved has enzymatic activity (45) and that the signal sequence need not inhibit activity, the $\beta$-lactamase from s aureus is related in any way to a stronger RNA-RNA complementarity for $S. aureus$ PC1 $\beta$-lactamase. The in vitro $\beta$-lactamase synthesized by both $B. subtilis$ and $E. coli$ systems, however, is larger than the extracellular enzyme on SDS-polyacrylamide gels by an apparent $M_n = 3100.$ Since $\beta$-lactamase is a secreted enzyme, the additional molecular weight of the in vitro product suggests the presence of a signal or leader peptide of 20 to 25 amino acids. Although the signal peptides of $\beta$-lactamases from $Bacillus licheniformis$ 749/C, $Bacillus cereus$ 569/H, and $E. coli$ pBR322 (R-TEM) have been studied (44), this is the first reported characterization of the $S. aureus$ signal peptide. Our determination of the nucleotide sequence of this region (see accompanying report) provides the cognate primary sequence of this leader peptide.

The capability of $B. subtilis$ ribosomes to efficiently and accurately synthesize a precursor form of $S. aureus$ $\beta$-lactamase allowed a determination of the enzymatic activity of this form. Although significant $\beta$-lactamase activity was detected over background, this activity was about 17-fold less than predicted from the amount of labeled protein synthesized in vitro. There are two possible interpretations of this observation. First, the precursor form of the enzyme may not be active because intracellular $\beta$-lactamase activity is unnecessary since penicillin and related substrates probably do not cross the cell’s inner membrane. The observed activity could result from a low level of processing during the transcription and translation reaction. Second, the unprocessed form of the enzyme may be only about 6% as active as the processed form. We have not distinguished between these two alternatives. There is no evidence of inhibition of $\beta$-lactamase activity in the in vitro assay since addition of purified $\beta$-lactamase to a control cell-free assay yields the activity expected for purified enzyme alone. Although it has been reported that a cell-bound form of $B. licheniformis$ $\beta$-lactamase from which the extracellular enzyme is cleaved has enzymatic activity (45) and that the signal sequence need not inhibit activity, this is apparently not the case with the precursor $\beta$-lactamase from $S. aureus$.

In addition to the efficient and productive recognition of the $S. aureus$ $\beta$-lactamase gene by $B. subtilis$ ribosomes, this gene has another feature commonly observed for mRNAs of Gram-positive origin (3, 11, 12). A significant amount of $\beta$-lactamase is synthesized in the absence of the initiation factor fraction. Since 28% of the $\beta$-lactamase synthesized in the presence of the salt wash fraction by the $B. subtilis$ system is produced in the absence of the salt wash fraction, $\beta$-lactamase synthesis is stimulated only 3.6-fold by the salt wash addition. Furthermore, assays in the absence of the salt wash fraction contained about 30% of the $\beta$-lactamase activity produced in the presence of the salt wash, indicating that the products of both assays have similar properties. This 3.6-fold stimulation is in marked contrast to the 50–60-fold stimulation of translation by salt wash addition of T7 and other Gram-negative mRNAs. An inverse correlation has been observed between the dependence upon initiation factors (salt wash components) and the degree of mRNA-16 S rRNA complementarity for stimulation of ribosome binding to initiator regions (14). Whether the feature of $S. aureus$ $\beta$-lactamase mRNA that facilitates protein synthesis in the absence of initiation factors is related in any way to a stronger RNA-RNA complementarity should be revealed upon analysis of the primary sequence.

**FIG. 8. Electrophoretic analysis of translation products directed by exoIII-treated pJM13 DNAs.** The exonucleolytically shortened pJM13 DNAs that served as templates for the transcription reactions shown in Fig. 7e were also used to direct protein synthesis in coupled reactions as described under “Experimental Procedures.” A homologous $E. coli$ system was used with [3H]lysine at 5,130 cpm/pmol. Templates and total [3H]lysine incorporated in the reactions analyzed on this 15% polyacrylamide gel include (a) supercoiled pJM13 DNA, 418 pmol; (b) exoIII-treated pJM13 that directs the transcription in lane a of Fig. 7c, 51 pmol; (c) supercoiled pJM13 DNA, 418 pmol; (d) 1.5-min exoIII-treated Eco RI-digested pJM13, 172 pmol; (e) 4-min exoIII-treated Eco RI-digested pJM13, 159 pmol; (f) 6-min exoIII-treated Eco RI-digested pJM13, 146 pmol; (g) 8-min exoIII-treated Eco RI-digested pJM13, 189 pmol; (h) Eco RI-digested pJM13, 351 pmol; (i) T7 DNA, 213 pmol; (j) HindIII-digested pJM13, 367 pmol; (k) 1-min exoIII-treated HindIII-digested pJM13, 136 pmol; (l) purified staphylococcal DNA fragment, 53 pmol; and (m) supercoiled pJM13, 418 pmol. Acid-insoluble radioactivity loaded on the gel for lanes a–m was 541,000, 175,340, 477,000, 264,800, 244,500, 225,370, 291,000, 539,600, 344,500, 421,200, 312,430, 120,700, and 329,230 cpm, respectively. Marker proteins are indicated with radioactive ink and included bovine serum albumin, purified extracellular $S. aureus$ $\beta$-lactamase, chymotrypsin, and cytchrome c.

**DISCUSSION**

$S. aureus$ $\beta$-lactamase is the major protein synthesized in a cell-free $B. subtilis$ transcription and translation system directed by pJM13 DNA. A similar amount of $\beta$-lactamase is produced by the analogous $E. coli$ system. The peptide map of the in vitro product correlates extremely well with that of purified extracellular $S. aureus$ PC1 $\beta$-lactamase. The in vitro $\beta$-lactamase synthesized by both $B. subtilis$ and $E. coli$ systems, however, is larger than the extracellular enzyme on SDS-polyacrylamide gels by an apparent $M_n = 3100.$ Since $\beta$-lactamase is a secreted enzyme, the additional molecular weight of the in vitro product suggests the presence of a signal or leader peptide of 20 to 25 amino acids. Although the signal peptides of $\beta$-lactamases from $Bacillus licheniformis$ 749/C, $Bacillus cereus$ 569/H, and $E. coli$ pBR322 (R-TEM) have been studied (44), this is the first reported characterization of the $S. aureus$ signal peptide. Our determination of the nucleotide sequence of this region (see accompanying report) provides the cognate primary sequence of this leader peptide.

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of the ribosome binding site region (see accompanying report).

In position to order the S. aureus β-lactamase gene on pJM13, a map of the major RNA transcripts produced in vitro was constructed. β-Lactamase is encoded on a 1,250-base RNA initiated at one of two major plasmid promoters. The promoter is mapped 90 ± 20 base pairs from the HindIII site in the staphylococcal DNA and is utilized by both E. coli and B. subtilis RNA polymerases. This 1250-base RNA has about 370 bases more coding capacity than required for β-lactamase (including 40 bases for ribosome binding). We have preliminary evidence to suggest that a protein of about \( M_r = 11,400 \) is also encoded in this RNA.

Although β-lactamase is produced in similar amounts by both E. coli and B. subtilis cell-free systems, it is a higher percentage of the total protein synthesized in the B. subtilis system (65%) than in the E. coli system (39%). That is, except for a product of about \( M_r = 14,000 \) (Fig. 2, lane c), the B. subtilis system yields lower amounts of most of the additional proteins synthesized by the E. coli system. The pattern of major translation products from the B. subtilis system directed by supercoiled (Fig. 2, lane a) Eco RI- or HindIII-cleaved (Fig. 8, lanes h and j) or truncated Eco RI-cleaved pJM13 (Fig. 8, lanes d and e) is quite similar. pMB9 DNA is reported to cause the synthesis in minicells of polypeptides of 34,000, 18,000, and approximately 14,000 daltons (46). Although two of the major additional products synthesized by the E. coli cell-free system are about \( M_r = 17,600 \) and 14,300, we do not believe these arise from pMB9 coding sequences for the following reasons. These proteins are synthesized when some 1,760 base pairs are removed from each end of Eco RI-digested pMB9 (Fig. 8, lane g) leaving about 1,930 base pairs of pMB9 DNA intact. Although this would contain ample coding capacity for these proteins if a promoter were present, it would not explain the disappearance of these products upon exonucleolytic removal of 220 base pairs from HindIII-cleaved plasmid (Fig. 8, lane h). Furthermore, small amounts of products with similar molecular weights are synthesized in the presence of the purified staphylococcal DNA fragment (Fig. 8, lane i). This analysis leads to the conclusion that many of the products including those of \( M_r = 17,600 \) and 14,300 synthesized by the E. coli system are related to β-lactamase since they are made in the presence of 1,250-base RNA which encodes β-lactamase and perhaps a protein of 12,000 daltons or less. The reason for the greater amino acid incorporation of the E. coli system relative to the B. subtilis system is not well understood. One contributing factor may be the synthesis of slightly more 1,250-base RNA by E. coli RNA polymerase than by B. subtilis RNA polymerase (Fig. 4).

The stronger of the two major promoters which has been mapped is located 2,000 base pairs from the end of the staphylococcal DNA. The absence of a termination site in staphylococcal DNA for this RNA is consistent with the finding that the cadmium resistance locus spans this Eco RI site in p258 from which this fragment was derived (17). Either the complete transcript encodes a protein larger than \( M_r = 73,000 \) (approximate coding capacity of a 2,000-base RNA) and is interrupted by the Eco RI site, or two or more genes for resistance to cadmium and possibly even to bismuth and lead which are mapped in this region (17) may be organized on an operon which contains an Eco RI site. The only major in vitro product which may be encoded in this RNA transcript is \( M_r = 10,500 \) since the other in vitro products in a supercoiled pJM13 DNA-directed assay are also present when all but 240 bases of the 2,000-base RNA are missing (Fig. 8, lane g). The identity of this protein is unknown.

A promoter for an operon containing the arsenate, arsenite, and antimony resistance genes is thought to be within 900 base pairs of the other end of the staphylococcal DNA fragment and transcribed in a clockwise direction (17). Since arsenate resistance is inducible, it is not surprising that a major transcript does not arise from this region. It is tempting to speculate that the minor 750-base transcript produced by B. subtilis RNA polymerase (Fig. 4, lane d) results from initiation at a promoter for these genes. E. coli RNA polymerase might not form this transcript due to interference from transcription of the opposite strand. Such transcription would occur during synthesis of the greater than 8,000-base read-through RNA and possibly utilizing a promoter near the Eco RI site (47).

Since pJM13 confers in vivo tetracycline resistance, there must be some transcription of pMB9 DNA in the direction opposing the synthesis of the 3700-base and its read-through RNA. The promoter responsible for this transcription is thought to be located at the HindIII site in pMB9 (48) and is salt sensitive (49). It is perhaps not unexpected that this promoter is much weaker than the staphylococcal promoters since the proteins encoded in pMB9 that are involved in tetracycline resistance act either intracellularly or at the membrane (46) and would not be expressed at the levels required for β-lactamase which is often dispersed extracellularly.

The S. aureus β-lactamase gene has been mapped on a plasmid which directs its efficient synthesis in both B. subtilis and E. coli. Cell-free transcription and translation systems are a good candidate for characterization of a ribosome binding site region since it has features typical of mRNAs from Gram-positive bacteria and this information will result in the elucidation of the signal peptide sequence of a Gram-positive protein.

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Cell-free Transcription and Translation of a Gram-positive Gene

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