Sensitivity of Translation by \textit{Brevibacterium lactofermentum} Ribosomes to Type 1 and Type 2 Ribosome-inactivating Proteins

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Received February 8, 1994

An active cell-free translation system was prepared from \textit{Brevibacterium lactofermentum}, a Gram-positive bacteria used in molecular cloning and protein expression. The system contained high speed postribosomal supernatant (S 370), purified ribosomes and a tRNA mixture from \textit{Escherichia coli}, and synthesized polyuridylic acid–directed polyphenylalanine once optimized for mono and divalent ions, time, and temperature. The translation system was evaluated for sensitivity to several translational inhibitors including several N-glycosidase ribosome-inactivating proteins (RIPs) isolated from plants. The pattern of inhibition by RIPs resembled that observed recently for Gram-negative bacteria such as \textit{Escherichia coli} and \textit{Agrobacterium tumefaciens} (Girbes et al., \textit{J. Bacteriol.}, 175, 6721–6724 (1993)). A typical inhibitory type 1 RIP such as crotin 2 promoted depurination of the tRNA, which upon treatment with acid aniline released a fragment of approximately 230 nucleotides. On these grounds, we propose that bacterial ribosome sensitivity to plant RIPs depends on the bacterial ribosome-specific presence of protein recognition domains in the RIP present only in some RIP but not in others.

Ribosome-inactivating proteins are plant toxins of unknown biological role\cite{1-5} that act as N-glycosidases of the larger tRNA of mammalian,\cite{6,7} yeast,\cite{8} plant,\textsuperscript{9,10} and bacterial ribosomes.\cite{11-15} RIPs are classified as follows:\cite{3} type 1, when they have only one polypeptide chain; type 2, when they are composed of two chains, the active one linked by a disulfide bridge to the other one, which is a lectin carrier able to translocate the dipeptide through the plasma membrane of animal cells\cite{2}; type 4 when they are formed of two dimers linked by non-covalent forces, each dimer being equivalent to a type 2 RIP. By contrast, type 2 and 4 RIPs may be classified as toxic, such as ricin, abrin, \textit{Viscum album} four-chain agglutinin, etc.,\cite{2,3} or non-toxic such as ebulin 1,\cite{16} nigrin b,\cite{17} \textit{Ricinus communis} four-chain agglutinin, etc.\cite{3} Analysis of the amino acid sequence of RIPs has shown that the active sites of some RIPs share common structural features.\cite{18}

Recent attempts to express genes coding for type 1 RIPs cloned in \textit{Escherichia coli} have faced insoluble problems arising from cell death promoted by inhibition of endogenous protein synthesis.\cite{19} This was promoted by a direct action of the expressed RIP on \textit{E. coli} ribosomes.\cite{20} Such inhibitory behavior of some type 1 RIPs disallows their overproduction using \textit{E. coli}.

In a recent study Hartley et al.\cite{11} speculated about the sensitivity of eubacterial ribosomes to plant RIPs. They suggested that, in contrast to type 2 RIPs such as ricin, which are inactive on \textit{E. coli} ribosomes, all type 1 RIPs may be active on all eubacterial ribosomes. This prompted us to investigate whether the inhibitory effect of these RIPs would be a general phenomenon or, instead, eubacterial ribosomes would display different and specific patterns of sensitivity towards RIPs. To answer this question we approached the issue of preparing highly purified eubacterial ribosomes, other than those of \textit{E. coli}, that are active in translation. Recent work has indicated that \textit{E. coli} and \textit{Agrobacterium tumefaciens} RIPs display nearly the same pattern of sensitivity to RIPs, both type 1 and type 2.\cite{21,22}

In this work we developed and optimized a cell-free translation system from \textit{Brevibacterium lactofermentum}. A survey of the literature in recent years on \textit{in vitro} translation by \textit{B. lactofermentum} found no references to this. Thus, to the best of our knowledge, this is the first optimized translation system prepared from this bacterium. It was found that not all RIPs were inhibitory.

Materials and Methods

\textbf{Materials.} The sources of the current chemicals and biochemicals were the same as indicated in previous works.\textsuperscript{5,21-23} L-[\textsuperscript{3}H]phenylalanine (sp. act. 117 Ci/mmol) was obtained from Amersham (Berica, Madrid, Spain). The ribosome-inactivating proteins assayed in this work were a generous gift from Prof. Fr. Strupe, University of Bologna, Italy.

\textbf{Preparation of supernatants and purified ribosomes.} Cells were grown at 30°C in 30 g/liter Difco TBS culture medium (tryptic soy broth; this medium contains 17 g/liter Bactotryptone, 3 g/liter Bactosoytone, 2.5 g/liter Bactodextrase, 5 g/liter NaCl, and 2.5 g/liter K\textsubscript{2}HPO\textsubscript{4}). The cells were harvested at an \textit{A}_{600} of 1.5–2.5 by centrifugation at 15,300 \texttimes g for 10 min. Pelleted cells were washed twice with buffer A (this buffer contained 10 mm Tris–HCl [pH 7.5], 10 mm magnesium acetate, 60 mm NH\textsubscript{4}Cl, and 10 mm 2-mercaptoethanol). From this point, all operations were done at 4°C. The cell pellets (a total amount of 8–10 g of cells) were placed in an unvitrified mortar precooled at −20°C, and ground with 1.5-fold their weight of alumina for 30 min. Extraction of the resulting cellular doughy fluid was done with two volumes of buffer A. The doughy fluid was placed in 30-ml centrifuge tubes and centrifuged at 20,300 \texttimes g for 15 min. Ten µg/ml of RNase-free DNase was added to the viscous supernatant and after 10 min at 0°C the viscosity due to undegraded DNA disappeared. The supernatant was then placed in clean 30-ml centrifuge tubes that were centrifuged at 30,100 \texttimes g for 10 min. This supernatant was designated S 30. The S 30 supernatant was centrifuged at 370,000 \texttimes g for 2.5 h. The

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\textit{Abbreviations.} ATA; aurin tricarboxylic acid; RIP; ribosome-inactivating protein; IC\textsubscript{50}; concentration of RIP that causes 50% protein synthesis inhibition.
resulting bright supernatant (called S 370) was processed as described below. The pellet contained ribosomes and was resuspended in buffer B (this buffer contained 10 mM Tris-HCl (pH 7.5), 1 mM NH₄Cl, 2 mM EDTA, and 2 mM 2-mercaptoethanol). These ribosomes were clarified by centrifugation at 30,100 x g for 10 min, sedimented by centrifugation at 184,000 x g for 2 h, resuspended in 100-200 µl of buffer B, and finally stored with 50% glycerol at -90 °C until use.

The S 370 supernatant was precipitated with ammonium sulfate (75% saturation) and centrifuged at 45,700 x g for 15 min. The sediment was resuspended in 500 µl of buffer A, dialyzed extensively against buffer A, and also stored with 50% glycerol at -90 °C until use.

Polyphenylalanine synthesis. Polyptide synthesis was measured as poly(U) directed poly(phe) synthesis. The standard reaction mixtures contained the following components in a final volume of 50 µl: 100 mM NH₄Cl, 10 mM magnesium acetate, 50 mM Tris-HCl (pH 7.8), 0.5 mM ATP, 30 µM GTP, 20 µM CTP, 1 mM diithiothreitol, 5 mM phosphenolpyruvate, 30 µg/ml pyruvate kinase; 200 µg S 370, 100 µg/ml RNA mixture from E. coli: 800 µg/ml polyuridylic acid; and 48 nm l-[¹H]phenylalanine (sp. act. 117 Ci/mmole). Some of these components were optimized previously by fixing the others under standard conditions, as described in the text. Unless otherwise indicated, incubation was done at 28 °C and lasted 15 min. Reactions were stopped and processed for radioactivity as indicated elsewhere.¹⁵

N-Glycosidase activity of ribosomes-inactivating proteins. The N-glycosidase activity of RIPs on bacterial RNA was followed by the release of the diagnostic fragment upon treatment of the rRNA from RIP treated ribosomes with acid aniline.¹¹,¹² Two hundred µg of ribosomes were incubated with 0.3 µg of cotin 2 for 1 min at 37 °C in a reaction mixture of 50 µl of buffer containing 40 mM Tris-HCl (pH 7.6), 125 mM NH₄Cl, 10 mM magnesium acetate, and 5 mM DTT. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA (pH 8.0) and 500 µl of 0.5% SDS containing 50 mM Tris-HCl (pH 7.6). Both control and cotin 2-treated RNAs were extracted by phenolization and ethanol precipitation as described elsewhere.²⁴ Aniline treatment of isolated RNAs was done as follows: 6 µg of RNA were dissolved in 10 µl of water and incubated in the darkness at 0 °C for 10 min with one volume of 2 M aniline (pH 4.5). The reaction was stopped by dilution with 200 µl of water, and the aniline was removed by two extractions with ether. Aniline-treated RNA was recovered by ethanol-precipitation. Electrophoresis of rRNA was done in 5% acrylamide gels at 21 mA for 40 min as described elsewhere.²⁵

Results and Discussion
Preparation of a cell-free poly(phe) synthesizing system from B. lactofermentum

To date, only two cell-free systems derived from Gram-negative bacteria have been used to study the effects of RIPs on bacterial protein synthesis, namely, E. coli¹¹,¹²,¹⁴,¹²,²⁰ and Agrobacterium tumefaciens.¹⁴,¹⁵,²⁶ Here we developed a cell-free system from a Gram-positive bacterium, B. lactofermentum, to investigate its sensitivity to RIPs.

The standard conditions for the preparation of the supernatants and purified ribosomes were as described in Materials and Methods and were those that gave a maximum of phenylalanine polymerization. Some points were found to be critical for obtaining a good rate of poly(phe) synthesis: 1) all operations should be done at 0-2 °C to prevent the activation of degradative enzymes and the deactivation of the ribosomes and enzymes required for translation, many of which are heat-sensitive; 2) the grinding should last not less than 30 min, adding the alumina in three portions; 3) treatment of the S 30 fraction with DNase should be done with an RNase-free DNase preparation; 4) the 37,000 x g supernatant should be dialyzed to remove low molecular weight compounds. Since some proteins belonging to the translational machinery are somewhat heat-sensitive, both supernatant and ribosome preparations were stored at -90 °C until use and were thawed only once.

Typical supernatants gave an A₂₈₀ of 50. The ribosome preparations gave typical A₂₆₀/A₂₈₀ ratios of 2. Current yields were 17 mg (in 0.3 ml) of supernatant per g of cells and 0.8 mg of ribosomes per g of cells.

Optimization of poly(U)-directed poly(phe) synthesis

Optimization of the ionic environment on poly(U)-directed poly(phe) synthesis was done by varying one parameter and fixing the others. As shown in Fig. 1, the optimum of magnesium ions for translation was between 9 and 12 mM. Below 6 mM magnesium acetate no appreciable degree of synthesis was observed. Monovalent ions affected translation much less than magnesium ions, as observed for other cell-free translation systems.¹⁵,²⁷ There was no effect at all on translation caused by the addition of a tRNA mixture from E. coli (data not shown), thus indicating that the high-speed supernatant contained enough endogenous tRNA to provide for pe-tRNA synthesis. Figure 1 also depicts the effects for variable S 370 and ribosome concentrations on translation. As regards S 370, saturation was reached at a protein concentration lower than 2 mg/ml. Ribosomes saturated the system at 4 mg/ml.

With respect to temperature optima and courses of poly(phe) synthesis, the best rates were obtained at 28 °C and saturation was reached in 20-30 min at 28 °C (data not shown). These requirements for poly(phe) synthesis are quite similar to those observed for E. coli²⁸ and A. tumefaciens,¹⁵ confirming that the reconstituted translation system is fairly stable, at least during translation.

To better define the reconstituted B. lactofermentum translation system we studied the sensitivity of poly(phe) synthesis to well-known antibiotics and ribosome-inactivating proteins. As shown in Fig. 2, ATA, and the antibiotic streptomycin, fusidic acid, chloramphenicol, and tetracycline effectively inhibited protein synthesis in the concentration range at which they inhibit protein synthesis in bacteria.²⁹

Sensitivity of poly(phe) synthesis to RIPs

We next studied the sensitivity of B. lactofermentum to a number of RIPs. As indicated in the Table, there are at least three groups of RIPs defined arbitrarily on the bases of their inhibitory powers. One category comprises all RIPs that were completely inactive on translation (saporin S6, gelonin and asparins) and those RIPs that only slightly inhibited translation (up to 10% close to 10,000 nm; dianthin 30, lycchnin, saporins L2 and R1, colocolis 1 and 3, morrococin S, trichokinin, curcins, manutin 1, PAP-C, and the type 2 RIPs ricin and volkensin). A second category comprises RIPs that inhibited translation between 10 and 50% at nearly 10,000 nm (saporins L1, R3 and S5, byrocin R, the barley inhibitor 1, and PAP-R). Finally, a third group is formed of those RIPs that inhibited translation by 50% at concentrations lower than 10,000 nm (dianthin 32, saporin R2, momordin I, Hura crepitans latex RIP 5, cotrins 2 and 3, and PAP-S).

It has been reported that up to 100 nicks in the rRNAs were compatible with translation.³⁰ However, the depurination of only one nucleotide at the highly conserved target rRNA loop irreversibly inactivates the ribosome.²¹
Fig. 1. Optimization of Key Requirements for Polyphenylalanine Synthesis by the Cell-free Translation System from B. lactofermentum.
Protein synthesis was done in reaction mixtures of 50 μl as described in Materials and Methods except that the corresponding participant in the reaction mixture was varied as indicated in the figure. Incubation was at 28°C for 15 min. The optimum of salt concentration found here was used for further experiments.

A highly inhibitory RIP such as crotin 2, despite the degradation state of the rRNA, modified B. lactofermentum ribosomes in such a way that the rRNA isolated upon treatment with acid aniline released an RNA fragment of 230 nucleotides (Fig. 3). This fragment was not released from untreated ribosomes, pointing to the specificity of the RIP action.

Concerning the question of whether the inhibitory effects of the RIPS are a general phenomenon or instead each eubacterial ribosome could display a specific pattern of sensitivity to RIPS, our results indicate that the patterns of sensitivity of B. lactofermentum, A. tumefaciens, and E. coli ribosomes to the large collection of RIPS tested in this work are nearly the same (Table, ref. 14). There is only one exception: dianthin 32 is somewhat more active on B. lactofermentum ribosomes than on the E. coli ones.

The key structure that governs the interaction of the elongation factor G (EF-G) with the E. coli ribosome is the highly conserved rRNA loop that contains the sequence 5′ ... AGUACGAGGGACC ... 3′. In fact, the interaction of EF-G with the ribosome prevents the interaction of the type 1 RIP crotin 2 with such an rRNA loop. Accordingly, the same pattern of inhibition of translation
Table  Effects of RIs on a Protein Synthesis System from *Brevibacterium lactofermentum* Translation

<table>
<thead>
<tr>
<th>FAMILY SPEcies</th>
<th>Part</th>
<th>RIp</th>
<th>IC50 (nm)</th>
<th>% of inhibition</th>
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<td>Lychnin</td>
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<td>Trichokrin</td>
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<td>Volkensin</td>
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<td>7.6</td>
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<tr>
<td>PASSIFLORACEAE</td>
<td>Root</td>
<td>Volkensin</td>
<td>&gt;3,300</td>
<td>7.6</td>
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</tbody>
</table>

C.c. cultured cells.

Barley inh. 1: barley inhibitor 1.

IC50 (nm) indicates the concentration of RIP that gives 50% inhibition of protein synthesis in the optimized conditions for translation.

% of inhibition means the degree of protein synthesis inhibition at the concentration of RIP indicated in the column of IC50. This value is given when the IC50 was not reached at the concentration tested.

Recent reports indicate that eukaryotic ribosomes display a sensitivity that is clearly dependent on the ribosomal conformation maintained by proper cation concentrations. In this sense, it has been described how rat liver ribosomes are more sensitive to ricin at high concentrations of monovalent cations. A similar observation has been described for magnesium ions and the inhibitory action of PAP, the RIP present in *Phytolacca americana*, on wheat germ and *Vicia sativa* ribosomes.

To ascertain whether bacterial ribosomes also display magnesium-maintained conformations sensitive to RIPs, we investigated the effects of magnesium concentrations on the extent of croton 2-triggered inhibition of protein
synthesis. As shown in Fig. 4, croton 2-treated ribosomes display maximum activity that peaks at 12–18 mm magnesium, in contrast to untreated ribosomes, which do so at 9–12 mm. The highest activity of croton 2 was observed at magnesium concentrations lower than the maximum.

Ribosomal structure conformates in a locked arrangement at high magnesium concentration. Therefore, the accessibility of the target rRNA loop would be hindered at high magnesium concentration. This is consistent with our results, since as the concentration of the cation decreases the effectiveness of croton 2 increases, reaching a plateau at 15 mm magnesium (see inset of Fig. 4).

In conclusion, we have prepared a very active cell-free translation system from B. lactfermentum that has allowed us to study the sensitivity of translation to RIPS. Further studies on this system will address the characterization of the ribosomes, specially in relation to RIP-ribosome interactions, to investigate in molecular terms why some RIPS are inhibitory and others are not.

Acknowledgments. This work was supported by grants from CICYT (BIO92-0231) and the Consejería de Educación de la Junta de Castilla y León (1992) to T. Girbés and University Grants-in-Aid to J. M. Ferreras and R. Iglesias. We are grateful to Mr. N. Skinner for correcting the English version of the manuscript and to J. E. Basterrechea for his technical assistance.

References