Mass spectrometry-based detection of transfer RNAs by their signature endonuclease digestion products

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ABSTRACT

The separation of biologically active, pure, and specific tRNAs is difficult due to the overall similarity in secondary and tertiary structures of different tRNAs. Because prior methods do not facilitate high-resolution separations of the extremely complex mixture represented by a cellular tRNA population, global studies of tRNA identity and/or abundance are difficult. We have discovered that the enzymatic digestion of an individual tRNA by a ribonuclease (e.g., RNase T1) will generate digestion products unique to that particular tRNA, and we show that a comparison of an organism's complete complement of tRNA RNase digestion products yields a set of unique or "signature" digestion product(s) that ultimately enable the detection of individual tRNAs from a total tRNA pool. Detection is facilitated by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and proof-of-principle is demonstrated on the whole tRNA pool from *Escherichia coli*. This method will enable the individual identification of tRNA isoacceptors without requiring specific affinity purification or extensive chromatographic and/or electrophoretic purification. Further, experimental identifications of tRNAs or other RNAs will now be possible using this signature digestion product approach in a manner similar to peptide mass fingerprinting used in proteomics, allowing RNomic studies of RNA at the post-transcriptional level.

Keywords: TRNA; *E. coli*; mass spectrometry; RNase T1; MALDI-MS; signature digestion product; isoaccepting tRNA; isoacceptors

INTRODUCTION

Transfer ribonucleic acid (tRNA) plays a central role in gene expression as an adaptor molecule that translates the codons in messenger RNA (mRNA) to amino acids in a protein. tRNAs' crucial role in protein biosynthesis has been an active area of research since their discovery (Söll and Rajbhandary 1995). The identification and characterization of individual tRNAs and their post-transcriptional modifications are essential for fully understanding their cellular roles (Bjork et al. 2001). The separation of biologically active, pure, and specific tRNAs is difficult due to the overall similarity in secondary and tertiary structures of different tRNAs (Cayama et al. 2000), and numerous procedures for the fractionation of tRNAs have been reported (Holley and Merrill 1959; Cherayil and Bock 1965; Weiss et al. 1968; Rajbhandary and Ghosh 1969; Holmes et al. 1975; Garel et al. 1977; Kanduc 1994; Ribeiro

et al. 1995; Chinali 1997; Cayama et al. 2000). Because prior methods do not facilitate high-resolution separations of the complex mixture represented by a cellular tRNA population, global studies of tRNA identity and/or abundance are rare. We show that the enzymatic digestion of an individual tRNA by a ribonuclease (e.g., RNase T1) will generate digestion products unique to that tRNA and that a comparison of an organism's complete complement of tRNA digestion products yields signature mass values that enable the matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) detection of individual tRNAs from a cellular mixture.

Mass spectrometry (MS) offers a number of advantages for the characterization of nucleic acids arising from its ability to provide mass and sequence information (Meng and Limbach 2006). Characterization of RNAs by MS is commonly done at two levels: oligonucleotide analysis through the selective digestion of intact RNAs with endonucleases, and/or sequencing of oligonucleotides through the use of tandem mass spectrometry via collision-induced dissociation or post-source decay (Limbach 1996; Nordhoff et al. 1996). We and others have shown that RNAs can be characterized by mass spectrometry through the selective digestion of intact RNAs with endonucleases, which can be

analyzed using liquid chromatography electrospray ionization mass spectrometry (Kowalak et al. 1993, 2000; McCloskey et al. 2001) or MALDI-MS (Polo and Limbach 1998; Kirpekar et al. 2000; Berhane and Limbach 2003a,b; Hartmer et al. 2003; Meng and Limbach 2004). While these RNase mapping approaches have been used previously to identify post-transcriptionally modified RNAs, here we develop a more extensive RNA identification strategy based upon the generation of unique or signature RNase digestion products. This approach is demonstrated on the whole tRNA pool from *Escherichia coli*. With this approach, identification of tRNAs, even at the level of isoacceptors, is now feasible without requiring specific affinity purification, extensive chromatographic and/or electrophoretic separations, or post-isolation labeling. This approach is amenable to all tRNA families and is of appropriate specificity to identify nearly 90% of tRNA isoacceptors from an *E. coli* cell lysate.

RESULTS

Signature digestion products

The enzymatic digestion of an individual tRNA by an RNase (e.g., RNase T1) will generate a number of specific endonuclease digestion products. A comparison of an organism's complete complement of tRNA RNase digestion products yields a set of unique or "signature" digestion product(s) that ultimately enable the detection of individual tRNAs from a total tRNA pool. These signature products are the essence of this approach.

For example, the theoretical RNase T1, RNase U2, and RNase A digestion products of *E. coli* tRNA^{Cys} are listed in Table 1. From this list, nine mass values are identical or shared by the RNase T1 digestion products of other *E. coli* tRNAs, whereas four mass values are unique to *E. coli* tRNA^{Cys}. For RNase U2 digestion, nine mass values are shared with other *E. coli* tRNAs and three mass values are unique. Similarly, for RNase A, 12 mass values are shared and three mass values are unique. These unique mass values are the RNase signature digestion products of tRNA^{Cys}. The mass spectrometric detection of any one or more of these signature products from a complex mixture will confirm the presence of the corresponding tRNA.

Conducting a similar analysis on all of the tRNAs from *E. coli* results in the signature digestion products specified in Supplemental Table S1. The number of signature digestion products for all *E. coli* tRNAs, with the exception of tRNA^{Pro}, which is not found in the tRNA database, from RNases T1, A, and U2 are listed in Table 2. This analysis reveals the presence of three different types of signature digestion products: Type I—a signature product unique to one tRNA; Type II—a signature product specific to one tRNA family; and Type III—a signature product selective for two or more, but not all, of the members of one tRNA

TABLE 1. Endonuclease digestion products possible from *E. coli* tRNA^{Cys}

m/z	Sequence	Unique
RNase T1		
363.1	Gp	No
668.1	CGp	No
998.1	UAGp	No
1279.2	UCCGp	No
1294.2	TΨCGp	No
1304.2	AΨUGp	No
1326.2	AACGp	No
1609.2	UCUAGp	No
1614.2	DDAUGp	**Yes**
1793.3	CCUCCA	**Yes**
1913.3	ACUCCGp	No
2685.4	CAms^{2}i^{6}AAΨCCGp	**Yes**
2914.4	Us^{2}UAACAAAGp	**Yes**
RNase U2		
347.1	Ap	No
652.1	CAp	No
790.1	ms^{2}i^{6}AAp	No
1304.2	UGUAp	No
1609.2	ΨUGCAp	No
1687.2	GCGGAp	No
2258.3	CUCCGGAp	No
2303.3	GCGGDDAp	No
2525.3	ΨCCGUCUAp	No
3045.3	pGGCGCGUs^{2}UAp	**Yes**
3093.5	CGCGCCUCCA	**Yes**
3574.5	GUCCGGTΨCGAp	**Yes**
RNase A		
324.0	Up	No
323.1	Cp	No
669.1	GUp	No
668.1	GCp	No
997.2	GACp	No
997.2	AGCp	No
1028.1	GGTp	No
1093.1	pGGCp	No
1303.2	s^{2}UAACp	**Yes**
1655.3	AAAGCp	No
1649.2	GGAΨUp	**Yes**
1655.3	AAAGCp	No
1671.3	GGAACp	No
1730.3	Ams^{2}i^{6}AAΨCp	No
1959.3	GGDDAUp	**Yes**

family. For example, *E. coli* tRNAs Ini I and II yield no Type I RNase T1 signature digestion products and four Type II RNase T1 signature digestion products (*m/z* 748, 1888, 3501, and 3197) that identify the presence of an initiator tRNA within a mixture but cannot differentiate between the two isoacceptors. In *E. coli*, the only tRNAs that can have Type III signature products are Ala, Arg, Glu, Gly, Ile, Leu, Ser, and Val, with all but Gly having Type III RNase T1 signature products (Supplemental Table S1).

All tRNA families have multiple signature products, ranging from a low of six for tRNA^{Lys} to a high of 48 for tRNA^{Ser}. All tRNA isoacceptors have at least one signature

product with the exception of tRNA[Ala III], tRNA[Glu II], tRNA[Glu III], and tRNA[Tyr I]. Thus, the detection of all tRNA families except tRNA[Pro], and all but four tRNA isoacceptors

TABLE 2. Summary of theoretical signature digestion products for *E. coli* tRNAs

tRNA	RNase T1	RNase A	RNase U2	Total
Ala	7	5	10	22
Ala I	2	2	5	9
Ala II	2	1	2	5
Ala III	0	0	0	0
Arg	10	9	15	34
Arg I	0	2	1	3
Arg II	0	1	1	2
Arg III	5	2	3	10
Arg IV	1	1	2	4
Arg V	3	2	4	9
Asn	3	1	4	8
Asp	2	4	3	9
Cys	4	3	3	10
Gln	7	5	5	17
Gln I	2	1	1	4
Gln II	4	3	2	9
Glu	5	2	6	13
Glu I	0	1	1	2
Glu II	0	0	0	0
Glu III	0	0	0	0
Gly	7	3	12	22
Gly I	2	1	4	7
Gly II	2	1	3	6
Gly III	2	1	4	7
His	6	0	4	10
Ile	4	3	7	14
Ile I	0	1	1	2
Ile II	0	1	1	2
Ile III	2	1	3	6
Leu	7	5	14	26
Leu I	3	3	4	10
Leu II	1	0	5	6
Leu III	1	1	4	6
Lys	2	0	4	6
Met	4	4	4	12
Phe	3	4	2	9
Pro	N/A	N/A	N/A	N/A
Ser	9	16	23	48
Ser I	2	3	6	11
Ser II	2	4	4	10
Ser III	0	1	1	2
Ser IV	0	1	2	3
Ser V	2	6	5	13
Thr	4	2	5	11
Thr I	1	0	1	2
Thr II	2	0	3	5
Trp	6	3	4	13
Tyr	5	2	4	11
Tyr I	0	0	0	0
Tyr II	1	0	0	1
Val	4	4	9	17
Val I	0	1	2	3
Val II	1	0	2	3
Val III	2	2	4	8

(continued)

TABLE 2. *Continued*

tRNA	RNase T1	RNase A	RNase U2	Total
Sec6	1	4	6	11
Ini	4	3	6	13
Ini I	0	0	1	1
Ini II	0	0	1	1
5S rRNA	4	2	4	10

Specific *m/z* values of signature digestion products are listed in Supplemental Table S1. Type I signature products are listed for each tRNA isoacceptor. tRNA family signature product totals also include Type II and Type III signature products (see text for details).

from *E. coli*, is possible through the signature digestion approach.

Optimization of digestion conditions

Commercial tRNAs were used to optimize the RNase T1 and RNase A digestion conditions to ensure they were fully compatible with MALDI-MS analysis (data not presented). Complete digestion of tRNAs is obligatory for identifying specific digestion products from their respective *m/z* values. RNase digestion occurs via a 2′, 3′-cyclic phosphate intermediate, and the presence of this intermediate can be avoided by using an increased enzyme/substrate ratio or by lengthening the digestion time (Kirpekar et al. 2000). Although formation of alkali salts can be a concern in MALDI-MS of nucleic acids (Nordhoff et al. 1996), the presence of ammonium acetate in the RNase digestion solution reduced the formation of alkali-adduct digestion products, and no further purification prior to MS analysis was required after digestion. The absence of cyclic phosphate intermediates and alkali-adducts simplifies spectral interpretation and digestion product identification.

Analysis of simple mixtures

To initially confirm that MALDI-MS analysis of RNase digests of tRNAs will yield sufficient data for the identification of tRNAs through their signature digestion products, four samples, each containing three *E. coli* tRNA isoacceptors, were generated. Mixture A1 contained tRNA[Tyr I], tRNA[Val III], and tRNA[Glu II]. Mixture A2 contained tRNA[Phe], tRNA[Val III], and tRNA[Glu II]. Mixture A3 contained tRNA[Tyr I], tRNA[Phe], and tRNA[Glu II], and mixture A4 contained tRNA[Tyr I], tRNA[Val III], and tRNA[Phe]. Each mixture was digested with RNase T1, and those digestion products were analyzed by MALDI-MS (Fig. 1). A similar set of mixtures, with the exception that tRNA[Tyr I] was replaced by tRNA[Tyr II], was prepared for RNase A digestion and analysis (data not shown).

For both endonucleases, the presence of specific signature digestion product peak(s) in the MALDI spectrum confirmed the presence of the constituent tRNAs in each
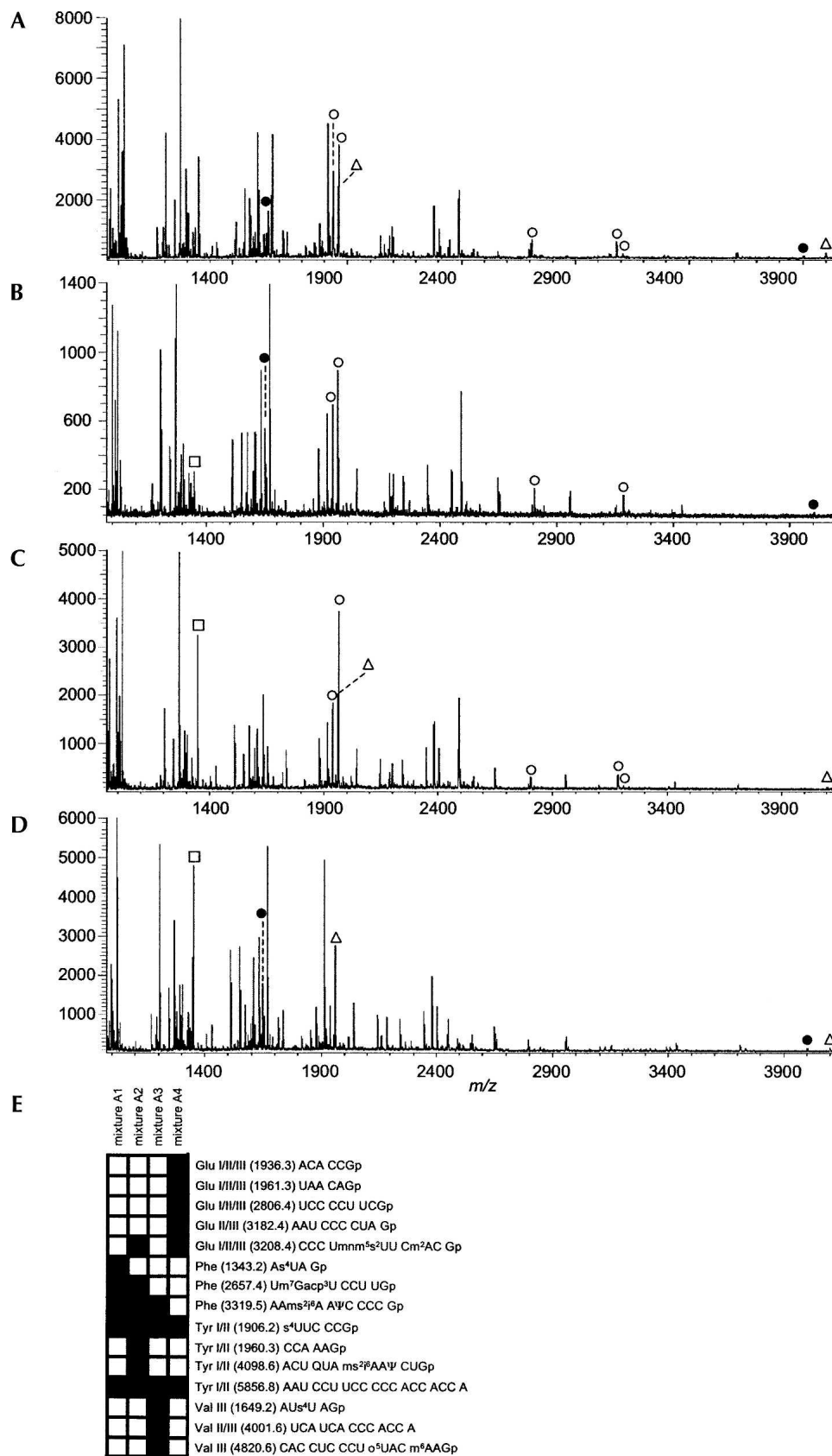
A 8000 6000 4000 2000

B 1400 1000 600 200

C 5000 4000 3000 2000 1000

D 6000 5000 4000 3000 2000 1000

1400 1900 2400 2900 3400 3900

m/z

E

	mixture A1	mixture A2	mixture A3	mixture A4	
					Glu I/II/III (1936.3) ACA CCGp
					Glu I/II/III (1961.3) UAA CAGp
					Glu I/II/III (2806.4) UCC CCU UCGp
					Glu II/III (3182.4) AAU CCC CUA Gp
					Glu I/II/III (3208.4) CCC Umnm^{5}s^{2}UU Cm^{2}AC Gp
					Phe (1343.2) As^{4}UA Gp
					Phe (2657.4) Um^{7}Gacp^{3}U CCU UGp
					Phe (3319.5) AAms^{2}i^{6}A AΨC CCC Gp
					Tyr I/II (1906.2) s^{4}UUC CCGp
					Tyr I/II (1960.3) CCA AAGp
					Tyr I/II (4098.6) ACU QUA ms^{2}i^{6}AAΨ CUGp
					Tyr I/II (5856.8) AAU CCU UCC CCC ACC ACC A
					Val III (1649.2) AUs^{4}U AGp
					Val II/III (4001.6) UCA UCA CCC ACC A
					Val III (4820.6) CAC CUC CCU o^{5}UAC m^{6}AAGp

FIGURE 1. MALDI mass spectra obtained from the RNase T1 digestion of mixtures of *E. coli* tRNAs. (*A*) tRNAs Tyr I, Val III, and Glu II. (*B*) tRNAs Phe, Val III, and Glu II. (*C*) tRNAs Tyr I, Phe, and Glu II. (*D*) tRNAs Tyr I, Phe, and Val III. RNase T1 signature digestion products for each tRNA are labeled: (Δ) Tyr I; (□) Phe; (●) Val III; and (○) Glu II. (*E*) Heat map representation of this data, along with *m/z* and sequences of signature digestion products. In all analyses, reproducible detection of the signature digestion products of the expected tRNAs is demonstrated.

mixture. As seen in Figure 1, for these smaller mixtures nearly all of the theoretical signature products were detected reproducibly. Further examination of the data did not reveal any *m/z* values indicative of signature products from other (absent) tRNA isoacceptors; thus, no false positives were noted in these analyses.

Analysis of *E. coli* cell lysate

After verifying the applicability of this method for simple mixtures of tRNAs, we next expanded into the analysis of tRNAs purified from *E. coli* cell lysates. After purification and digestion as described in the Materials and Methods section, the RNase digest was analyzed by MALDI-MS. There are 19 tRNA families that are identified from *E. coli* by RNase T1 (Fig. 2A,B) with their specific signature products noted in Table 3 and 13 tRNA families identified from *E. coli* by RNase A (Fig. 2C) with their specific signature products noted in Table 4. tRNA families identified by both endonucleases include Ala, Arg, Asp, Gln, Gly, Ini, Phe, Ser, Trp, Tyr, and Val with signature product coverage ranging from 10% to 100% (Fig. 3).

A number of 5S RNA signature digestion peaks (3135.4, 2829.4, and 1655.3 for RNase T1 and 2345.3 for RNase A) were also detected, which is to be expected given the small RNA isolation protocol used in this work. Importantly, the presence of other small RNAs, such as 5S rRNA, does not complicate or interfere with the detection of tRNA signature digestion products, and a single MALDI analysis is sufficient to identify these tRNAs. The ribonuclease-digested transfer RNAs were also labeled with ^{18}O and analyzed separately by MALDI-MS (Meng and Limbach 2004) to confirm the expected peaks (data not shown). All of the major peaks in the mass spectra are easily assignable to expected digestion products, and all expected products generate distinct signals. No prior fractionation is needed for this analysis.

Sample requirements and method reproducibility

The amount of sample required for this approach was determined. At the lower end, ~50 ng of RNA sample on the MALDI target plate is sufficient to detect tRNA sig-
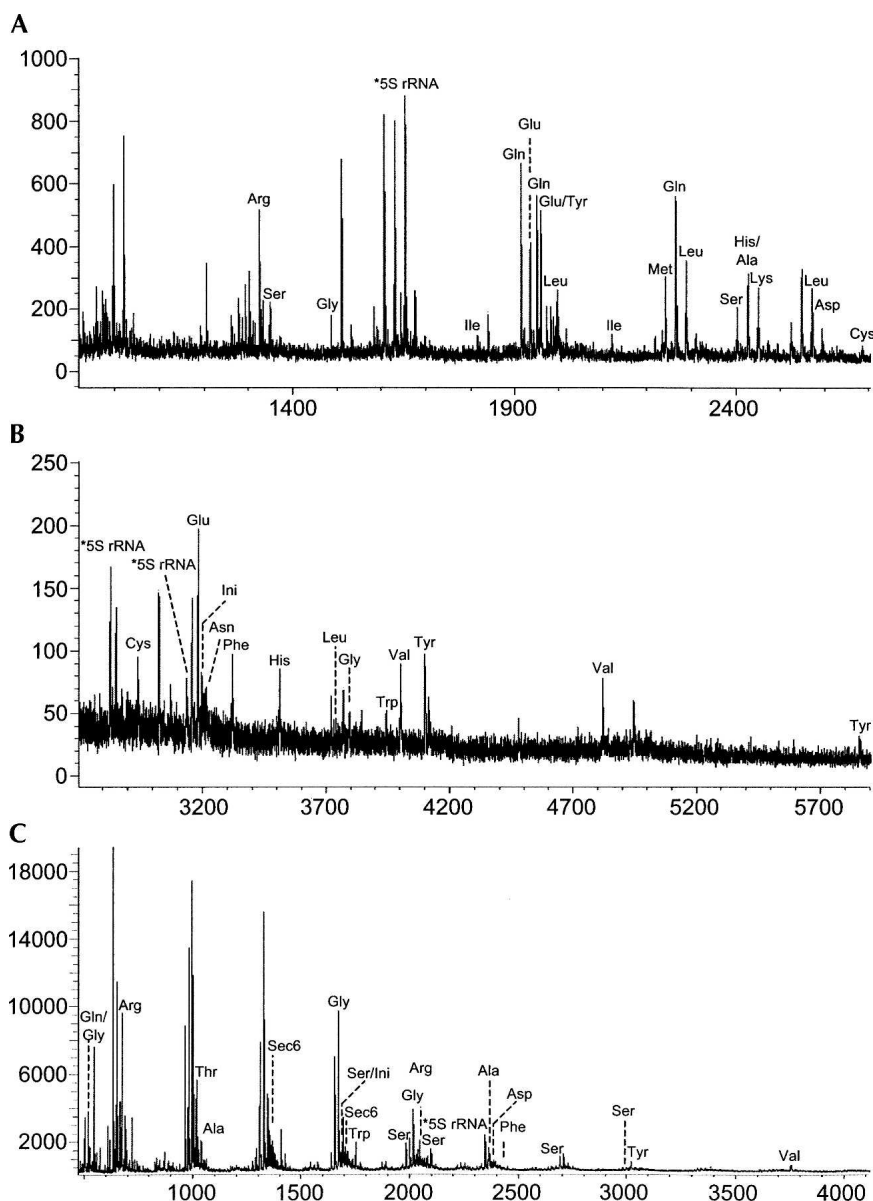

FIGURE 2. MALDI mass spectra obtained from the RNase T1 digestion of *E. coli* tRNAs. (A) *m/z* 900–2700; (B) *m/z* 2700–6000. (C) MALDI mass spectra obtained from the RNase A digestion of *E. coli* tRNAs. Signature products are labeled with assignments listed in Tables 3 and 4 for RNases T1 and A, respectively.

nature digestion products of RNase T1, with 80 ng of RNA sample required for RNase A. At the higher end, ~5–10 μg of sample can be spotted on the MALDI target with no evidence of detector saturation. Of course, concentrated samples can always be diluted to the suitable range as required prior to MALDI analysis in this process as the endonuclease digestion reaction is irreversible.

To determine the reproducibility of the method, three separate RNase T1 digestions of an *E. coli* cell lysate were analyzed by MALDI-MS three times each. As noted in Supplemental Figure S1, reproducible information and identifications can be obtained from RNase signature digestion

TABLE 3. Experimentally detected tRNAs from mass spectral data in Figure 2, A and B, as identified by their RNase T1 signature digestion products

(Isoaccepting) tRNAs	m/z signature peak	Type[a]	Signature peak sequence
tRNA$^{Ala\ I/III}$	2427.4	III	CUC CAC CA
tRNA$^{Arg\ III}$	1327.2	I	AUA Gp
tRNA^{Asn}	3214.4	I	UAU m^{7}GUC ACU Gp
tRNA^{Asp}	2572.4	I	AAU ACC UGp
tRNA^{Cys}	2685.4	I	CAms^{2}i^{6}A AΨC CGp
tRNA^{Cys}	2941.4	I	Us^{4}UA ACA AAGp
tRNA$^{Gln\ I}$	1928.3	I	m^{2}AΨΨ CCGp
tRNA$^{Gln\ II}$	1951.3	I	m^{2}AΨA CCGp
tRNA$^{Gln\ II}$	2266.4	I	AAU CCA Gp
tRNA$^{Glu\ II/III}$	3182.5	III	AAU CCC CUA Gp
tRNA$^{Glu\ I/II/III}$	1936.3	II	ACA CCGp
tRNA$^{Glu\ I/II/III}$	1961.3	II	UAA CAGp
tRNA$^{Gly\ III}$	3796.0	I	CUA UUA CCU CAGp
tRNA$^{Gly\ I/II/III}$	1487.2	II	CUC CA
tRNA^{His}	2426.4	I	CCA CCC CA
tRNA^{His}	3512.5	I	AAU CCC AUU AGp
tRNA$^{Ile\ I/II}$	1801.3	III	AUt^{6}A AGp
tRNA$^{Ile\ I/II}$	2121.4	III	CCU ACC A
tRNA$^{Ini\ I/II}$	3197.4	II	TΨC AAA UCC Gp
tRNA$^{Leu\ I}$	2289.4	I	ACA CAA Gp
tRNA$^{Leu\ II}$	3721.6	I	UCC CCC CCC UCGp
tRNA$^{Leu\ III}$	2571.4	I	CCC AAU AGp
tRNA$^{Leu\ II/III}$	1996.3	III	AAD DGmGp
tRNA^{Lys}	2450.5	I	ACC CAC CA
tRNA^{Met}	2242.4	I	AAU CCC Gp
tRNA^{Phe}	3319.6	I	AAms^{2}i^{6}A AΨC CCC Gp
tRNA$^{Ser\ I/IV}$	2403.5	III	Ams^{2}i^{6}AA ACC Gp
tRNA$^{Ser\ III/IV/V}$	1350.1	III	AAA Gp
tRNA^{Trp}	3944.0	I	UCmU CCA ms^{2}i^{6}AAA CCGp
tRNA$^{Tyr\ I/II}$	1960.3	II	CCA AAGp
tRNA$^{Tyr\ I/II}$	4100.6	II	ACU QUA ms^{2}i^{6}AAΨ CUGp
tRNA$^{Tyr\ I/II}$	5857.7	II	AAU CCU UCC CCC ACC ACC A
tRNA$^{Tyr\ II}$	1937.3	I	UCA CAGp
tRNA$^{Val\ III}$	4821.4	I	CAC CUC CCU cmo^{5}UACm^{6}AAGp
tRNA$^{Val\ II/III}$	4002.6	III	UCA UCA CCC ACC A
5S rRNA	1655.2		AAA CGp
5S rRNA	2829.4		UCC CAC CUGp
5S rRNA	3135.4		UCU CCC CAU Gp

[a]Type I—a signature product unique to one tRNA; Type II—a signature product specific to one tRNA family; and Type III—a signature product selective for two or more, but not all, of the members of one tRNA family.

products. Although most *E. coli* tRNAs have multiple signature digestion products, all are not typically detected in a MALDI mass spectrum (Supplemental Fig. S1). The most likely reason is the peak suppression effect, which is common in MALDI-MS (Knochenmuss 2003).

DISCUSSION

Signature digestion products

A list of all the theoretical signature digestion products of *E. coli* tRNAs are presented as supplemental material (Supplemental Table S1). Reviewing these data, from the 46 *E. coli* tRNAs, 31 yield RNase T1 signature digestion

products, 40 yield RNase U2 signature digestion products, and 32 yield RNase A signature digestion products. Individually, 89% of the *E. coli* tRNAs can be identified solely by using RNase U2. For *E. coli*, the only tRNAs isoacceptors that do not yield at least one signature digestion product are tRNA$^{Ala\ III}$, tRNA$^{Glu\ II}$, tRNA$^{Glu\ III}$, and tRNA$^{Tyr\ I}$.

While several tRNA isoacceptors have only a single Type I signature product for any particular RNase chosen (Table 2), besides the four tRNA isoacceptors with no signature products for any RNase, all other tRNA isoacceptors except Ini I, Ini II, and Tyr II yield Type I signature products from at least two of the three RNases investigated. Thus, an improvement in tRNA coverage and a reduction in the possibility of false positives can be realized when multiple RNases are used (Fig. 3). When feasible, it is recommended that RNases T1, U2, and A be used to provide maximal coverage and allow for multiple, independent identifications of tRNA isoacceptors.

The present approach has several similarities to the peptide mass fingerprinting approach popular in proteomics. A unique difference, however, is that here positive identification of a tRNA isoacceptor is made on the basis of detection of any one of its signature products as opposed to a comparison of all RNase fragment ions with database predictions. In this way, our approach is most analogous to the accurate mass tag method described for protein identification (Conrads et al. 2000), although those stringent levels of mass accuracy are not necessary for the success of the present approach.

E. coli cell lysate

Applying this approach for tRNA identification to a complex mixture of tRNAs, such as those obtained from an *E. coli* cell lysate, reveals the potential of this method. tRNAs identified by both endonucleases through Type I signature products include Asp, Gln II, Gly III, Phe, Trp, and Val III. tRNAs identified by both endonucleases through a combination of Type I–III signature products include Ala III, Arg III, Gly I/II/III, Ini I/II, Ser I/III/IV, and Tyr I/II. tRNAs detectable only in RNase T1 digests

TABLE 4. Experimentally detected tRNAs from mass spectral data in Figure 2C as identified by their RNase A signature digestion products

(Isoaccepting) tRNAs	*m/z* signature peak	Type[a]	Signature peak sequence
tRNA$^{Ala\ II/III}$	1048.4	III	o^{5}UGCp
tRNA$^{Ala\ II/III}$	2376.3	III	AGG AGm^{7}G Up
tRNA$^{Arg\ V}$	2046.3	I	Gm^{1}GA GGCp
tRNA$^{Arg\ III/IV}$	672.1	III	mnm^{5}UCp
tRNA^{Asp}	2392.3	I	AGG GGm^{7}G Up
tRNA$^{Gln\ II}$	519.0	I	NUp
tRNA$^{Gly\ I}$	2016.3	I	GAG AGCp
tRNA$^{Gly\ II}$	1672.2	I	AAG GUp
tRNA$^{Gly\ III}$	518.1	I	NCp
tRNA$^{Ini\ I/II}$	1678.2	II	GGG CmUp
tRNA^{Phe}	2404.3	I	GAA ms^{2}i^{6}AAΨ Cp
tRNA^{Sec6}	1379.4	I	A+A Up
tRNA^{Sec6}	1703.2	I	GGG GCp
tRNA$^{Ser\ I}$	2098.4	I	GAms^{2}i^{6}A AACp
tRNA$^{Ser\ II}$	1984.3	I	AAA AGCp
tRNA$^{Ser\ III}$	2998.4	I	DGA AGG AGCp
tRNA$^{Ser\ V}$	1686.6	I	AGA GTp
tRNA$^{Ser\ V}$	2691.3	I	GAA AGG GUp
tRNA$^{Thr\ I/II}$	1012.3	II	AGTp
tRNA^{Trp}	1753.3	I	Ams^{2}i^{6}AA ACp
tRNA$^{Tyr\ I/II}$	3019.4	II	AAA GGG AGCp
tRNA$^{Val\ III}$	3754.6	I	m^{6}AAG GAG GGG m^{7}GUp
5S rRNA	2345.8		AGG GAA Cp

[a]Type I—a signature product unique to one tRNA; Type II—a signature product specific to one tRNA family; and Type III—a signature product selective for two or more, but not all, of the members of one tRNA family.

during evolution (Ikemura 1981, 1985; Ikemura and Ozeki 1983). It should be pointed out that bacterial tRNA populations differ greatly from that of yeast, for instance, *Saccharomyces cerevisiae* (Ikemura 1982).

The isoaccepting tRNAs present in a typical *E. coli* cell can be grouped into two main categories: major (those present in >2% of total tRNAs) and minor (<2% of total tRNAs). With the exception of Arg I and II, all major tRNAs were detected by the signature digestion peak approach. Arg I and II share a common RNase T1 signature peak, and their RNase A signature peaks were not detected in the MALDI-MS data. Among the 21 minor tRNAs, 86% were detected by at least one of the RNase digestions, if not both. No bias can be detected with this method toward only the most abundant tRNAs within the sample.

While not reported here, the signature digestion product approach can be combined with RNase-mediated labeling for the relative and absolute quantification of tRNAs from a cell lysate (Berhane and Limbach 2003b; Meng and Limbach 2005). The approach as developed cannot be used for the de novo identification of RNAs. Moreover, some signature digestion products arise from the presence of post-transcriptional modifications. For example, among the detected *E. coli* RNase T1 signature digestion products (Table 3), the signature products for Asn, Cys, Gln I, Gln II, Ini I/II, Leu II/III, Phe, Ser I/IV, Trp, Tyr I/II, and Val III contain post-transcriptionally modified nucleosides. Reviewing the data in Supplemental Table S1, slightly more than half (54%) of the RNase T1 signature
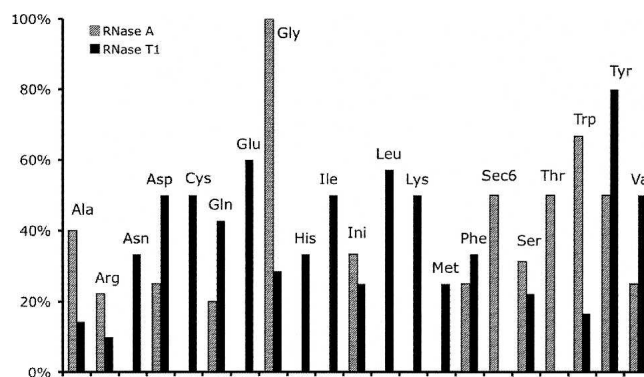
included Ala I, Asn, Cys, Gln I, Glu I/II/III, His, Ile I/II, Leu I/II/III, Lys, Met, and Val II (Table 3). tRNAs detectable only in RNase A digests include Ala II, Arg IV/V, Sec6, Ser II, and Thr I/II (Table 4). Altogether, 21 tRNA families were detected by either RNase T1 or RNase A, with 11 tRNA families represented in both endonuclease digestions (Fig. 3). At the individual level, 40 of 46 possible tRNAs were identified, although with the two RNases used in this study, only 62% were identified using the more stringent Type I signature products. Further improvements are expected when RNase U2 becomes available and experimental data can be incorporated into the approach.

While there are 46 possible *E. coli* tRNAs that could be present in this mixture, the codon bias of *E. coli* leads to the possibility of widely varying tRNA abundances within the sample. Supplemental Table S2 lists the frequency of tRNA isoacceptors in a typical *E. coli* cell and the results found in the MALDI-MS approach developed here. The abundance of tRNA isoacceptors listed are from *E. coli* strain W1485, a derivative of *E. coli* K12 (Dong et al. 1996), which is an appropriate representation of the MRE 600 strain used here, as it has been previously reported that the tRNA contents of the two enterobacteriaceae (*E. coli* and *Salmonella typhimurium*) are quite similar, indicating that the populations of tRNA molecules have been conserved

FIGURE 3. Signature digestion product coverage of tRNA families analyzed from an *E. coli* cell lysate using RNases T1 and A.

products contain modified nucleosides. However, only tRNA isoacceptors Arg IV, Arg V, Val II, Val II, and Sec6 contain modified nucleosides in each of their Type I signature products. In general, therefore, it appears that undermodification of tRNAs or the lack of modification status would not preclude the use of this approach. It is clear that additional applications of this approach will be enabled by more comprehensive RNA sequence databases, which incorporate the modification status of the RNAs.

MATERIALS AND METHODS

Materials

Escherichia coli strain MRE 600 was purchased from American Type Culture Collection (ATCC). *E. coli* tRNA$^{Val\ III}$, tRNA$^{Tyr\ I}$, tRNA$^{Tyr\ II}$, tRNA$^{Glu\ II}$, tRNA^{Phe}, diammonium hydrogen citrate (DAHC), and 2,4,6-trihydroxyacetophenone (THAP) were purchased from Sigma-Aldrich and used as received. RNase T1 and A were obtained from Roche Molecular Biochemicals. Sep-Pak C18 cartridges were obtained from Waters. MOPS minimal media was purchased from Teknova. Synthetic oligonucleotides (dT$_3$, dT$_5$, dT$_{15}$, and dT$_{20}$) were obtained from the University of Cincinnati DNA Core Facility. Nanopure water (18 MΩ), from a Barnstead (Dubuque, IA) nanaopure system, was autoclaved before use.

Bacterial cultures

E. coli strain MRE 600 was grown in MOPS minimal media, which is a modification of Neidhardt supplemented MOPS minimal media (Neidhardt et al. 1974). *E. coli* cells were grown aerobically at 37°C with vigorous shaking (300 rpm) in an incubator shaker (Innova 4000, New Brunswick Scientific). Cells were harvested at a cell density corresponding to an A$_{540}$ of 0.50.

Isolation of small RNAs

Total tRNA from *E. coli* was isolated using the *mirVana* miRNA isolation kit from Ambion. Briefly, *E. coli* cells with culture media were centrifuged at 10,000 rpm for 10 min in a Sorvell RC 5C centrifuge. After centrifugation, the media solution was discarded and pellets were collected. After washing with phosphate-buffered saline at pH 7.2 several times, lysis/binding buffer solution was added to the disrupted cells.

For immediate analysis, one-tenth volume of the *mirVana* homogenate solution was added to the lysis/binding buffer-added disrupted microbial cells and the mixture was incubated for 10 min on ice. A volume of acid-phenol:chloroform equal to the initial lysate volume was added. The aqueous phase was carefully recovered and was transferred to a fresh tube. One-third volume of 100% ethanol was added and mixed thoroughly. The lysate/ethanol mixture was passed through a filter cartridge and the filtrate was collected. A two-thirds volume of 100% ethanol was added to the filtrate. The filtrate/ethanol was passed through a second filter cartridge and the flow-through was discarded. The filter was washed with *mirVana* wash solution I, and the flow-through was discarded. The filter cartridge was transferred into a fresh collection tube and nuclease-free water was added to the

center of the filter. The solution was spun for 20–30 sec at 10,000 rpm to recover the small RNA. The eluent, which contained the RNA, was collected and stored at −20°C prior to further analysis. The purity and concentration of the isolated RNA were determined from the A$_{260}$/A$_{280}$ absorbance ratio (Cherayil 1990). If the RNA isolation processes were done later, then the disrupted cells were submerged in an RNA$_{later}$ solution and stored at −20°C.

Enzyme purification and digestion

Ribonuclease T1 (E.C. 3.1.27.3) was precipitated from its original suspension by the use of acetone. The precipitate was resuspended and eluted in 1 mL of 75% aqueous acetonitrile from a Sep-pak C18 cartridge. The solution was taken to dryness and resuspended in sterile water. Approximately 10 μg of RNA was added to 500 units of RNase T1 and 5 μL of 220 mM ammonium acetate buffer. The reaction mixtures were incubated for an hour at 37°C. The minimum enzyme-to-substrate ratio was estimated to be ∼50 units per μg of RNA. For RNase A (E.C. 3.1.27.5) digestions, ∼10 μg of RNA was added to 0.1 units of RNase A in ammonium acetate buffer, followed by incubation for 2 h at 37°C.

Mass spectrometry

All mass spectrometry experiments were performed on a Bruker Reflex IV MALDI-TOF from Bruker Daltonics having a 3-m effective flight path, a two-stage gridless ion reflector, pulsed ion extraction, and a nitrogen laser (λ=337 nm). All MALDI spectra were acquired in negative polarity and in reflectron mode.

A two-layer sample spotting approach was used. The matrix components were 250 mM THAP in acetonitrile and 300 mM DAHC in water, with each being prepared fresh before use. Approximately 0.5 μL of the THAP matrix was first spotted onto the MALDI sample target plate and allowed to dry. Equal volumes of THAP and DAHC were combined and then mixed with an equal volume of the sample solution. Approximately 1 μL of this sample/matrix solution was spotted on top of the previously dried THAP.

Vendor-supplied Flex control and X-mass software were used for data acquisition and processing, respectively. Typically, 200 laser shots were co-added per spectrum. Each spectrum was smoothed using a five-point Savitsky–Golay algorithm and background subtracted. The instrument was calibrated externally with synthetic oligonucleotides that bracketed the *m/z* range of interest.

Signature digestion products

tRNA sequences were obtained from the tRNA sequence database (Sprinzl and Vassilenko 2005). Published sequences were digested in silico using the Mongo Oligo Mass Calculator v2.06 (Rozenski 2006).

SUPPLEMENTAL DATA

Supplemental material including a complete listing of RNase signature digestion products from a larger number of organisms is available for download in Excel file format at http://bearcatms.uc.edu/signature/.

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