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Transcription of the Archaeal *trkA* Homolog in *Methanosarcina mazei* S-6

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Transcription of the archaeal *trkA* gene homolog in *Methanosarcina mazei* S-6 was studied at the optimal growth temperature of 37°C and after heat shock at 45°C. Northern (RNA) blotting results (transcript size) and data from primer extension experiments to map the transcription initiation site indicate that *trkA* is cotranscribed with another gene. The latter, *orf11*, encodes a protein of 94 amino acids (10,611 Da) and is located upstream of *trkA*, with which it overlaps: the translation stop codon of *orf11*, TGA, shares the bases T and G with the translation start codon of *trkA*, ATG. These genes' transcription was decreased by heat shock to the point of making the transcript undetectable by Northern or dot blotting procedures. *orf11* and *trkA* differ in codon usage patterns, and the proteins coded by them, i.e., Orf11 and TrkA, are dissimilar in amino acid sequence and composition.

The only archaeal *trkA* gene homolog known so far is from the anaerobic methanogen *Methanosarcina mazei* S-6 and was described in 1993 (17). At that time, there was only one *trkA* gene sequence in the databases, and it was from the eubacterium *Escherichia coli* (36). More recently, two additional homologs have been found also in eubacteria: one in *Vibrio alginolyticus* (24), and the other, named *sapG*, in *Salmonella typhimurium* (30). The physiological role of this gene's product, TrkA in *E. coli* and *V. alginolyticus* and SapG in *S. typhimurium*, has been studied, and it is understood to a considerable extent (2, 4, 10, 13, 24, 30, 36, 37). There is, however, less information on transcription of *trkA* (or *sapG*) in eubacteria, and none is available for the archaeal homolog. Studies with the eubacterial gene have shown that its protein product is part of the K^+ uptake system and that it is important for the cell under normal, physiological conditions. It remains to be established whether *trkA* is involved in cell survival under conditions of physical or chemical stress. Participation of *trkA* in the response to stress is suggested by the role of TrkA in K^+ transport (2, 10, 24, 30), and the cell's need for this cation in stressful situations, and by the involvement of K^+ in the activity of the heat shock (stress) proteins DnaK, DnaJ, and GrpE via the chaperone complex (15, 22, 27, 29, 34, 39). In this work, transcription of the archaeal *trkA* homolog has been studied in *M. mazei* S-6 maintained at the optimal growth temperature and after heat stress of various durations.

MATERIALS AND METHODS

Cell cultures. *M. mazei* S-6 (19) was grown as packets in alpha medium with 200 mM methanol and as single cells in MS medium with 80 mM trimethylamine (3, 6) as described previously (21).

Heat shock. Culture bottles were transferred from the incubator at 37°C to a water bath at 45°C and were incubated with gentle shaking for various time periods, from 15 to 60 min, as specified for each experiment, as described previously (8).

Plasmid, DNA insert, DNA sequencing, probes, and primers. A plasmid with a ~12-kbp insert containing the *dnaK* locus and adjacent regions was constructed by using (i) *M. mazei* S-6 genomic DNA digested with *Sac*I and (ii) pBluescript (Stratagene, La Jolla, Calif.) (18). The nucleotide sequence of the DNA was determined with the dideoxy-chain termination method according to standard protocols (1). A portion of the insert containing *orf11* and *trkA* is shown in Fig. 1, which also depicts the probe and primers used in this work.

RNA preparation and Northern (RNA) and dot blotting. Northern and dot blottings were done as described previously (8), with total RNA extracted by the guanidinium method and a probe for *trkA* (Fig. 1), produced by PCR and radiolabeled by random priming (1).

Mapping of the 5′ end of the *orf11* transcript. Primer extension experiments were carried out according to standard protocols (1), using a commercial kit (Promega) and the 18-mer oligonucleotide primers complementary to bases −6 to 15 [PE (−6/15)] of *orf11* and bases 19 to 36 [PE (19/36)] and 110 to 127 [PE (110/127)] of *trkA* (Fig. 1) as described previously (8).

Sequence and codon usage analyses. The nucleotide and deduced amino acid sequences of *orf11*, including flanking regions, were analyzed by using the Genetics Computer Group package (12). The inverted repeats were determined by using the Stemloop program with a minimum stem length of 8, minimum bonds per stem of 16, a maximum loop size of 20, and a minimum loop size of 3. Comparative analyses of codon usage and amino acid sequences were done as previously described (8).

Nucleotide sequence accession number. The sequence shown in Fig. 2 has been deposited in the EMBL-GenBank database with accession number X77992.

RESULTS

Analysis of the *trkA* 5′-flanking region. To map the transcription initiation site for *trkA*, it was necessary to study the nucleotide sequence upstream of this gene, beyond what was already known. The sequence of 431 nucleotides upstream of *trkA* is shown in Fig. 2. In this DNA segment, *orf11* was identified, with its translation start codon 281 bp from the start codon of *trkA* and with its translation stop codon, TGA, overlapping the start codon of *trkA*, ATG. There is a putative promoter 100% identical to the consensus sequence for promoters of genes in methanogens (5), including box A (40). This promoter is centered around a base at position −28 with respect to the transcription initiation site, which is also typical of genes in methanogens (32, 40). Two inverted repeats, between the transcription initiation site and the translation start codon, have the potential to form two hairpins in tandem (−23.0 kJ/mol) in the transcript and/or in single-stranded DNA.

Mapping of the transcription initiation site. Initial primer extension experiments, aimed at mapping the 5′ end of the *trkA* transcript, were carried out with primers designed to detect it nearby, upstream of the *trkA* protein-coding region. Primers PE (19/36) and PE (110/127) (Fig. 1) were used in two

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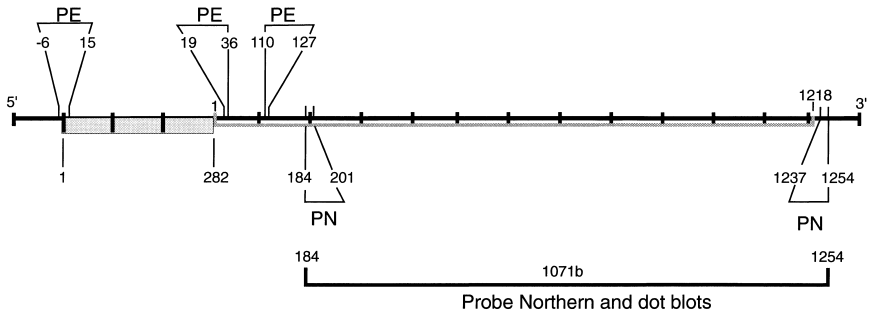

FIG. 1. Graphic representation of the *M. mazei* S-6 chromosomal segment that contains *orf11* and *trkA* and that served as the template for preparation of the probes used to characterize the transcript. Short vertical lines separate 100-base intervals. The protein-coding region of *orf11* is represented by the shaded horizontal rectangle between positions 1 (first base) and 282 (last base). The protein-coding region of *trkA* is represented by the double horizontal line between positions 1 (first base) and 1218 (last base). PE, primers used in the primer extension experiments; PN, primers used to generate, by PCR, the probe used in the Northern and dot blotting experiments. −6, base number counting from the first base upstream of the translation start codon in the 3′-to-5′ direction.

separate experiments each. The results of these four independent experiments were negative. It was then decided to extend the sequence analysis further, in the 5′ direction, beyond the point already examined (17). When *orf11* was identified, primer extension experiments were carried out with primers designed to detect the transcription initiation site upstream of the translation start codon of *orf11*. The results obtained with primer PE (−6/15) (Fig. 1) are shown in Fig. 3. The transcription initiation site was mapped to a purine (A), located 70 bp upstream of *orf11* (Fig. 2). The same site was found for the two morphologic stages of *M. mazei* S-6 tested, i.e., packets and single cells, at the optimal growth temperature of 37°C or after heat shock at 45°C. However, the signal (i.e., the amount of transcript) was lower for heat-shocked single cells than for cells grown at the optimal temperature.

Effect of heat shock on *trkA* transcription. Northern blotting experiments with the probe described in Fig. 1 showed that the transcript (~1.6 kb) decreased considerably after heat shock at 45°C for 15 min or longer. Illustrative results are shown in Fig. 4a. Dot blotting experiments confirmed these results (Fig. 4b). When RNA from packets was tested, the transcript also decreased after heat shock (data not shown).

Orf11. The protein Orf11, encoded in the gene *orf11* and whose deduced amino acid sequence is depicted in Fig. 2, has 94 residues and a calculated molecular mass of 10,611 Da. Its amino acid composition differs from that of TrkA (Table 1). Orf11 has a high content of the aromatic amino acids Phe and Tyr but no Trp and only two acidic amino acids.

The amino acid sequence (Fig. 2) also displays noteworthy features. (i) Aromatic amino acids are more abundant at the end of the second third of the molecule, in which there are six of the seven Tyr residues and three Phe residues. (ii) The only two acidic amino acids, one Asp and one Glu, are near the C terminus, while five of the seven basic residues are in the N-terminal third of the molecule. (iii) Likewise, the seven Asn residues of Orf11 are in its N-terminal third. (iv) Phe and Leu are the predominant hydrophobic residues, while Ser is the most abundant hydrophilic amino acid. (v) Orf11 is relatively rich in Gly residues, 50% of which are in the C-terminal third. (vi) The only two His residues are adjacent to each other near the N terminus. (vii) There are no potential glycosylation sites (sequons; Asn-Xaa-Thr/Ser, where Xaa is any amino acid except Asp or Pro). (viii) There are virtually no identifiable alpha helices and very few potential β sheets (data not shown).

Homology in the TrkA family of proteins. A pairwise comparison of Orf11 with the TrkA family of proteins showed 23% or less identity (Table 2). Since *M. mazei* S-6 TrkA is 52 amino acids shorter than the eubacterial homologs, it was necessary to check whether Orf11 was not the missing portion in the archaeal molecule. The two *M. mazei* S-6 sequences, Orf11 and TrkA, were joined C to N terminus and then compared with the sequences of the eubacterial proteins. No homolog segment matching Orf11 was found in any of the longer, eubacterial TrkAs, and no similarity increase was observed in comparison with those obtained by using the archaeal TrkA sequence alone (Table 2). TrkA molecules were compared with each other and with two hypothetical proteins found in the databases with identity of 30% or higher (Table 2). The

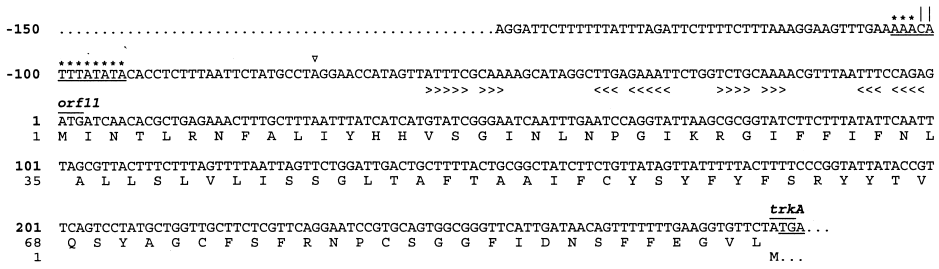

FIG. 2. Nucleotide sequence of *orf11*. The deduced amino acid sequence of the encoded protein Orf11 is also shown, using the single-letter designation for each residue placed under the first base of the corresponding codon. The putative promoter with box A is underlined; the asterisks above the bases indicate identity with the consensus promoter for genes in methanogens, while the vertical lines indicate the two positions at which there is no base preference (5). The transcription initiation site is shown by an open triangle. Arrowheads show inverted repeats that could form hairpins in the transcript and/or in single-stranded DNA. The translation start codon is overlined, while the translation stop codon is underlined. The translation start codon of *trkA* is overlined, and the first amino acid of TrkA (M) is shown. Numbers at the left indicate nucleotide (boldface) and amino acid numbers. Nucleotides are numbered from the first base (inclusive) of the *orf11* translation start codon in the 3′ direction (positive values) and from the first base (inclusive) upstream of the start codon in the 5′ direction (negative values).

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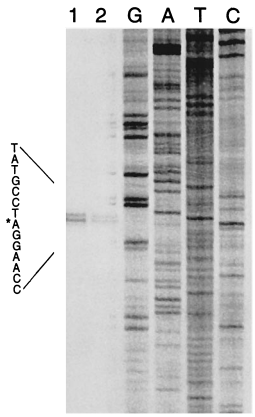

FIG. 3. Mapping of the transcription initiation site by primer extension. Total RNAs from *M. mazei* S-6 single cells grown at 37°C (lane 1) or heat shocked at 45°C for 30 min (lane 2) were tested with primer PE (−6/15) (Fig. 1). GATC, sequence ladder of the antisense (noncoding) DNA strand determined with the same primer. The initiation site is shown by an asterisk on the sense (coding) strand to the left and is indicated by an open triangle in Fig. 2.

proteins from *E. coli* and *S. typhimurium* differ in only three amino acids, and both are closer to the *V. alginolyticus* TrkA than to the *M. mazei* S-6 protein. The similarity of the three eubacterial molecules with Orf2 (incomplete) of the *ntrY/ntrX* locus of *Azorhizobium caulinodans* (31) and with Orfr (incomplete) of the *redD* locus of *Streptomyces coelicolor* A3(2) (25) is higher than that of the *M. mazei* S-6 TrkA. All these proteins have a putative NAD^+/NADH-binding site similar to that of the *Bacillus subtilis* dehydrogenase (30, 36). This site occurs twice in the TrkA molecules because they are composed of two homologous halves, suggesting that they arose by gene duplication (30, 36). Partially overlapping the NAD^+/NADH-binding site in the N-terminal half of the archaeal TrkA, we identified a 13-residue motif which is highly conserved in the subunits I, II, and III of the α chain of a rat brain sodium channel (accession numbers p04774, p04775, and p08104 [16, 26]); this motif is also present in the eubacterial TrkA proteins (Fig. 5). In the archaeal TrkA, the motif spans residues 239 through 251, while the NAD^+/NADH-binding site extends between amino acids 231 and 250. In the eubacterial proteins, the structural relationship is the same but displaced two amino acids toward the C terminus.

Codon usage. The codon usage patterns of the *orf11* and *trkA* genes of *M. mazei* S-6 are depicted in Fig. 6. Codons ending in G or C are used more (44.6%) by *trkA* than by *orf11* (38.3%).

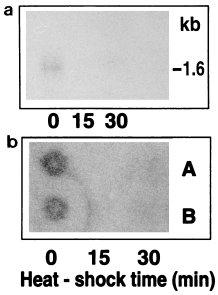

FIG. 4. Effect of heat shock on *trkA* transcription, determined by Northern blotting (a) and dot blotting (b; A and B represent two independent experiments). The probe used is depicted in Fig. 1. The RNA was from single cells maintained at the optimal growth temperature of 37°C or heat shocked at 45°C for 15 or 30 min, as indicated.

TABLE 1. Main contrasting features between the proteins Orf11 and TrkA of *M. mazei* S-6

Protein	Total aa[a]	pI	Mol%							
			Aromatic aa	Charged aa	Basic aa	Acidic aa	Met	Cys	Ser	Trp
Orf11	94	8.8	21.3	9.6	7.4	2.1	1.1	3.2	14.9	0.6
TrkA	106	4.7	2.9	29.3	13.1	16.3	3.4	1.7	9.9	1.7

[a] aa, amino acids.

DISCUSSION

Transcription of the archaeal *trkA* gene identified in *M. mazei* S-6 (17) was investigated to determine this gene's response to heat shock. The hypothesis was that the *M. mazei* S-6 *trkA* gene might respond to stress by heat like the adjacent genes of the *dnaK* locus, i.e., 5′-*grpE-dnaK-dnaJ*-3′ (7, 8), considering that K^+ is necessary for an adequate response to stress and for chaperone function (15, 27, 29, 34). A transcript that was longer than expected from the size of this gene was detected with a probe for *trkA* in non-heat-shocked cells. A transcription initiation site within the expected region upstream of *trkA* was not found. Subsequently, a single transcription initiation site was mapped upstream of the translation start codon of *orf11*. This site is preceded at the expected distance by a putative promoter identical to the consensus promoter for genes in methanogens (5, 40). These findings, along with the transcript size, suggest that the *trkA* gene in *M. mazei* S-6 is cotranscribed with *orf11*. Transcription decreases and is undetectable by Northern and dot blotting methods after a heat shock of 15 min or longer. We thus conclude that the *M. mazei* S-6 *trkA* gene does not respond to heat stress as the upstream genes, 5′-*grpE-dnaK-dnaJ*-3′, do. Transcription termination may be predicted to occur at a short series of contiguous U's, right after inverted repeats in what would be the untranslated 3′ segment of the transcript, 43 bases downstream of the *trkA* translation stop codon (17). The inverted repeats have the potential to form a hairpin in the transcript, and could thus serve as a termination signal, as in other archaeal heat shock and non-heat shock genes (7, 8, 18, 23, 32, 38). Transcription termination would, therefore, be like the rho-independent termination in eubacteria. In contrast, the potential transcription initiation signals upstream of *orf11* resemble not the eubacterial but the archaeal signals, in line with findings on archaeal transcription factors, which suggest a preinitiation complex of eucaryotic type (14, 20, 33).

orf11 and the deduced protein, Orf11, are different from *trkA* and TrkA, respectively, in sequence and amino acid composition. The differences in codon usage may reflect differences in the rates of translation and half-lives of the mRNAs for these two genes, as is the case for other genes (11, 35). The hydrophobicity of Orf11 suggests that it may serve to keep TrkA attached to the cytoplasmic membrane, where it is found in *E. coli* (2, 4).

The archaeal TrkA is unmistakably related to the eubacterial homologs, but it is less similar to them than they are to each other. A 13-amino-acid motif found in a eucaryotic sodium channel, and also observed in the TrkA molecules, illustrates the degree of relatedness of the archaeal TrkA with regard to the eubacterial homologs, which coincides with that shown by comparing entire sequences. Considering the motif

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TABLE 2. Comparison of the deduced amino acid sequences of Orf11, Orf11 plus TrkA (*M. mazei* S-6), and other similar molecules[a]

Protein[b]	% Similarity or identity[c]							
	Orf11, *M.m.* (94[d])	TrkA, *M.m.* (406)	Orf11 + TrkA, *M.m.* (500)	TrkA, *E.c.* (458)	SapG, *S.t.* (458)	TrkA, *V.a.* (458)	Orf2 NtrY/X, *A.c.* (111)[e]	Orfr, *S.c.* (183)[e]
Orf 11, *M.m.* [X77992] (this report)		47.8	ND[f]	46.3	47.4	43.2	34.4	39.0
TrkA, *M.m.* [X74307] (17)	20.7		ND	59.6	59.6	59.2	59.3	49.2
Orf11 + TrkA, *M.m.*	ND	ND		59.6	59.6	59.2	59.3	ND
TrkA, *E.c.* [X52114, X77091] (36)	23.2	33.3	33.3		99.8	90.0	64.0	56.3
SapG, *S.t.* [X80501] (30)	23.2	33.3	33.3	99.3		90.0	64.0	56.3
TrkA, *V.a.* [D28477] (24)	15.8	33.3	33.3	79.0	79.0		64.0	53.0
Orf2 NtrY/X, *A.c.* [Q04856] (31)	15.0	40.7	40.7	49.5	49.6	48.6		54.2
Orfr, *S.c.* [M29790] (25)	15.8	27.7	ND	32.2	32.2	27.9	31.8	

[a] Abbreviations: *M.m.*, *M. mazei*; *E.c.*, *E. coli*; *S.t.*, *S. typhimurium*; *V.a.*, *V. alginolyticus*; *A.c.*, *A. caulinodans*; *S.c.*, *Streptomyces coelicolor.*
[b] Accession numbers are in brackets; references are in parentheses.
[c] Numbers above the diagonal row of spaces indicate percent similarity (identities plus conservative substitutions). Values below 50 are underlined. Numbers below the diagonal row of spaces indicate percent identity. Values below 30 are underlined.
[d] Total number of amino acids.
[e] Fragment (incomplete open reading frame; end not yet determined).
[f] ND, not done.

alone, one can see that the archaeal TrkA shares characteristics with the eubacterial homologs, but it also differs from them in other properties. For instance, the archaeal protein is more similar to the eucaryotic α-chain subunits I to III than to the eubacterial TrkA at motif positions 2, 3, 5, 6, 10, and 11 (Fig. 5). However, the only two positions at which there is no agreement between the archaeal and eubacterial TrkA motifs are 2 and 10. The *M. mazei* S-6 TrkA has Ile and Leu (i.e., hydrophobic amino acids with apolar R groups) at positions 2 and 10, respectively, whereas the eubacterial proteins have Asn (i.e., hydrophilic amino acid with uncharged polar R groups) at position 2 and Arg (i.e., hydrophilic amino acid with charged R groups) at position 10. The archaeal TrkA motif is more similar to the eubacterial than to the eucaryotic homologs at positions 1 and 9 and differs significantly from the latter eucaryotic molecules at position 9 (the archaeal protein has Lys, i.e., an amino acid with basic R groups, whereas the eucaryotic molecules have Glu, i.e., an amino acid with acidic R groups). Overall, the proteins coincide in having an amino acid with charged R groups at position 9 and an amino acid with uncharged polar R groups at position 1. Thus, there is consensus among the seven proteins shown in Fig. 5 at all positions except 2 and 10. In the eucaryotic proteins, the motif is located in one of the putative transmembrane domains (16). It remains to be determined whether the same motif in the archaeal proteins plays a role in a TrkA-membrane interaction.

The organization of the *dnaK* locus of *M. mazei* S-6 (9) was compared with that of the corresponding locus of the eubacterium *Staphylococcus aureus* (28), the only other *dnaK* locus fully sequenced in which a gene upstream of *grpE* and a gene downstream of *dnaJ* have also been characterized. Interestingly, while the triad 5′-*grpE-dnaK-dnaJ*-3′ in *M. mazei* S-6 is similar in organization to that of *S. aureus*, and to those of some other eubacteria, the genes preceding (18) and following (reference 17 and this report) the triad show no relation with the eubacterial counterparts.

	POSITION												
	1	2	3	4	5	6	7	8	9	10	11	12	13
***R.n.* subunits I-III**	S	I	V	G	M	F	L	A	E	L	I	E	K
***M.m.* TrkA**	G	I	V	G	M	Y	L	A	K	L	I	D	K
CONSENSUS 4 proteins	**U.p.R-g.**	**I**	**V**	**G**	**M**	**Ar**	**L**	**A**	**C.R-g.**	**L**	**I**	**Ac**	**K**
***E.c.* TrkA**	G	N	I	G	A	G	L	A	R	R	L	E	K
***S.t.* SapG**	G	N	I	G	A	G	L	A	R	R	L	E	K
***V.a.* TrkA**	G	N	I	G	A	S	L	A	K	R	L	E	Q
CONSENSUS 3 proteins	**G**	**N**	**I**	**G**	**A**	**U.p.R-g.**	**L**	**A**	**Ba**	**R**	**L**	**E**	**Hi**
CONSENSUS overall	**G (U.p.R-g.)**	**X**	**Ho**	**G**	**Ho**	**U.p.R-g.**	**L**	**A**	**Ba (C.R-g.)**	**X**	**Ho**	**E (Ac)**	**K (Hi)**

FIG. 5. Thirteen-amino-acid sequence stretch of *M. mazei* S-6 (*M.m.*) TrkA (amino acids 239 through 251) found to be conserved in the α-chain subunits I, II, and III (accession numbers p04774, p04775, and p08104, respectively) of a rat (*Rattus norvegicus* [R.n.]) brain sodium channel protein (16, 26) and in the known eubacterial TrkA homologs, which are from *E. coli* (*E.c.*), *S. typhimurium* (*S.t.*), and *V. alginolyticus* (*V.a.*) (see Table 2 for accession numbers and text for references). U.p.R-g., uncharged polar R groups; Ar, aromatic; C.R-g., charged R groups; Ac, acidic; Ba, basic; Hi, hydrophilic; X, no consensus; Ho, hydrophobic. The amino acids are designated by single-letter notation.

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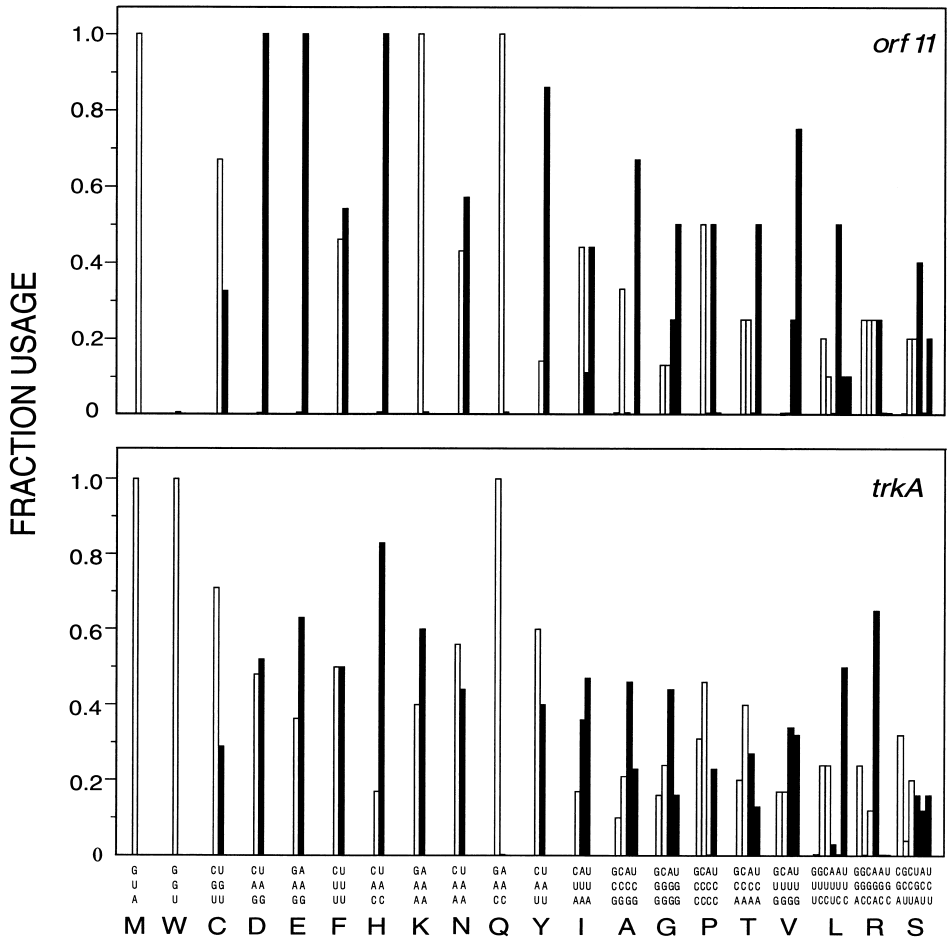

FIG. 6. Codon usage patterns of *orf11* and *trkA* from *M. mazei* S-6. Open and solid bars represent the fraction of codons used (vertical axis) with G or C and A or U, respectively, in the third position. The codons possible for each amino acid are shown along the horizontal axis and are to be read upwards (first base at bottom). Amino acids (single-letter designation) are listed at the bottom.

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