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Original Article

Cloning, expression and functional study of translation elongation factor 2 *(EF-2)* in zebrafish

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ABSTRACT We have identified translation elongation factor 2 *(EF-2)* in zebrafish (GenBank Accession No. AAQ91234). Analysis of the DNA sequence of zebrafish *EF-2* shows that the 2826 bp cDNA spans an open reading frame between nucleotide 55 to 2631 and encodes a protein of 858 amino acids. Zebrafish EF-2 protein shares 92%, 93%, 93% and 92% identity with the corresponding amino acid sequence in human, mouse, Chinese hamster and *Gallus EF-2*, respectively. Whole-mount *in situ* hybridization showed that zebrafish *EF-2* was a developmentally regulated gene and might play important roles during the early development of zebrafish embryos. Therefore, we further studied the function of *EF-2* during early embryogenesis. Using morpholino antisense oligo knockdown assays, anti-MO injected embryos were found to display abnormal development. The yolk balls were larger than normal and the melanophores spreading on their bodies became fewer. Furthermore, their tails were incurvate and their lenses were much smaller than those of the normal embryos. However the *EF-2* overexpression data showed that extra EF-2 protein had no obvious effect on zebrafish embryonic development.

KEY WORDS: *EF-2, zebrafish, whole-mount in situ hybridization, overexpression, knockdown*

Introduction

Translation elongation factor 2, EF-2 and its eubacterial homolog, EF-G, act sequentially after eEF1a/EF-Tu to catalyze the GTP hydrolysis-dependent translocation of the ribosome during protein synthesis, to allow the peptidyl-tRNA to move from aminoacyl site to the peptidyl site on a ribosome, liberating the former site to accept a new mRNA triplet and its cognate ternary complex (eEF1-aminoacyl-tRNA-GTP) (Kohno K *et al.*, 1986; Mendoza A *et al.*, 1999). EF-2 is a GTPase; it binds and hydrolyzes GTP and forms a ternary complex with GTP and ribosomes (Rao S *et al.*, 1996).

Although regulation of EF-2 is well known to include phosphorylation and ADP-ribosylation, developmental regulation of *EF-2* isoforms has not been fully explored. The reported developmentally regulated *EF-2* isoforms constitute a family of genes in *Tetrahymena thermophila,* which encode proteins homologous to EF-2 and are expressed only during sexual reproduction (Malave TM *et al.*, 2004). *Drosophila* contains two nearly identical *EF-2* genes (Lasko, 2000). Nevertheless, there are many examples in multicellular eukaryotes of differential expression of isoforms of translation elongation factor 1a (*eEF-1a*). *Drosophila* has two copies (*F1* and *F2*) of the *EF-la* gene, which are expressed at different times during development. While *F2* is transcribed only in the pupal stage, *F1* is a housekeeping gene expressed in all cells during development and in adulthood (Hovemann B *et al.*, 1988). Three *EF-la* genes have been characterized in *Xenopus* - (*42Sp50*, *EF-laO* and *EF-laS*), *42Sp50* is expressed exclusively in oocytes, while *EF-laO* is active from fertilization until early onset of neurulation (Krieg P *et al.*, 1989; Dje MK *et al.*, 1990). In mammals, the *EF-S* (*eEF1a2*) gene is expressed in heart and muscle tissue and eventually becomes the only form expressed in these tissues (Knudsen *et al.*, 1993). Disruption of the *EF-S* (*eEF1a2*) gene in mouse results in deficiencies in muscle and neuronal function within a few weeks after birth (Chambers *et al.*, 1998).

Since zebrafish embryos develop externally and are optically transparent, this system may cast light on the function of *EF-2*. In this report, we cloned zebrafish *EF-2* gene and examined its

Abbreviations used in this paper: EF-2, translation elongation factor 2; MO, morpholino modified antisense oligonucleotide; PTU, 1-phenyl-2-thiourea; PFA, paraformaldehyde; ORF, open reading frame; hpf, hours post-fertilization.

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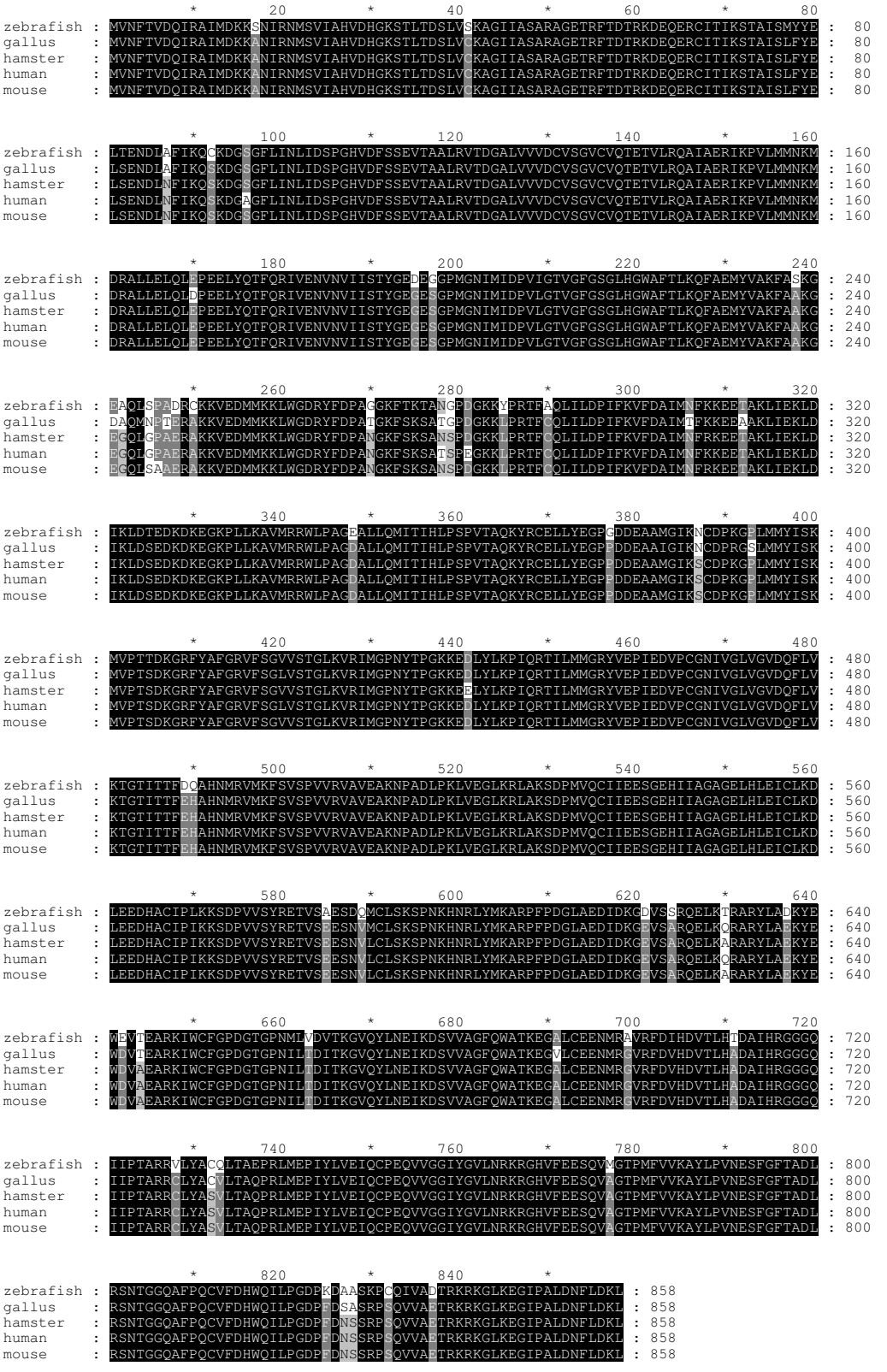

Fig. 1. Alignments of zebrafish, Chinese hamster, *Gallus gallus*, human and mouse *EF-2*. *Amino acids are shaded according to the degree of conservation using GeneDoc: black (100% similarity), gray (80% similarity) and light gray (60% similarity). The amino acid sequences of Chinese hamster,* Gallus gallus, *Human and Mouse* EF-2 *were obtained from the GenBank database with Accession Nos. A25440, NP-990699, CAA77750 and NP-031933, respectively.*

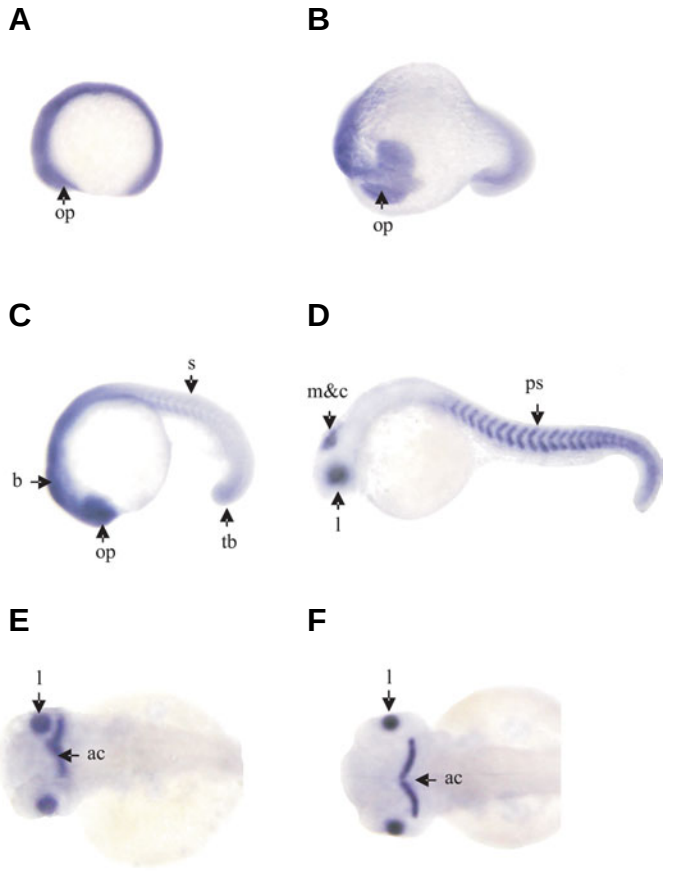

Fig. 2. Expression patterns of *EF-2* in the zebrafish embryo. *Side view(A,B,C,D); dorsal view (E,F).***(A)***5-somite stage;* **(B)***17-somite stage;* **(C)***22 -somite stage;* **(D)** *prim-15 stage;* **(E)** *prim-25 stage;* **(F)***protruding-mouth stage. Abbreviations: op, optic primordial; b, brain; tb, tail bud; s, somite; m&c, midbrain and cerebellum; l, lens; ps, posterior somites; ac, anterior portion of the cerebellum.*

expression pattern by whole-mount *in situ* hybridization. The result of whole-mount *in situ* hybridization showed that zebrafish *EF-2* was a developmentally regulated gene just like *EF-S* and might play important roles during the early development of zebrafish embryos. Therefore, we further studied the function of *EF-2* during early embryogenesis.

Results

Sequence analysis of the zebrafish EF-2 *gene*

The full-length zebrafish *EF-2* gene was sequenced. The result shows that the 2826 bp cDNA spans an ORF between nucleotide 55 to 2631 and encodes a protein of 858 amino acids (GenBank Accession No. AAQ91234).

To figure out the structural features of the zebrafish EF-2 protein, its amino acid sequence was compared with those of other EF-2 proteins using clustalw version 1.82 online (http://www.ebi.ac.uk/clustalw/). The zebrafish EF-2 protein is highly conserved sharing identity with overall 92%, 93%, 93% and 92% of amino acid sequence in human (CAA77750), mouse (NP_031933), Chinese hamster (A25440) and *Gallus gallus* (NP_990699) *EF-2*, respectively. Then the alignment was done with software GeneDoc (shown in Fig. 1). The data shows that the zebrafish EF-2 protein is highly conserved.

Spatiotemporal expression pattern of the zebrafish EF-2 *gene*

EF-2 transcript was not detected during somitogenesis using sense probe (data not shown). However, the results of whole-mount *in situ* hybridization using antisense probe showed that a large amount of *EF-2* transcripts existed during somitogenesis. *EF-2* transcripts originally appeared strongly throughout envelope at 5-somite stage (Fig. 2A). At 17-somite stage (Fig. 2B) and 22–somite stage (Fig. 2C), *EF-2* transcripts were detected still throughout the embryo with especially significant in the optic primordia, the whole brain, the trunk and tail bud. From 22-somite stage the stained cells began to concentrate strongly in the eyes, brain and somites. At prim-15 stage, the stained cells concentrated mainly in the lens, retina, midbrain and cerebellum (Fig. 2D) and posterior somites expression is especially evident. But after prim-25 stage the expression of *EF-2* only appeared in the lens and the anterior portion of the cerebellum (including the proliferation zone at the midbrain-hindbrain – boundary) (Fig. 2 E-F).

Overexpression of EF-2

Expressing vector pcDNA3.0-EF2 with various amounts (1.0ng/embryo, 0.67ng/embryo and 0.33ng/embryo) and *EF-2* capped mRNA with the amount of 4.5ng/ embryo were injected into each of 1–4-cell-stage embryos, respectively. After injecting pcDNA3.0-EF2 expression vector with the amount of 1.0ng/embryo, most of the zebrafish embryos died (Table 1), showing that the amount of 1.0ng/ embryo was fatal to the zebrafish embryos. However, no abnormal phenotypes appeared when they were injected by other amounts of pcDNA3.0-EF2 (0.67 ng/embryo and 0.53 ng/embryo) or *EF-2* capped mRNA (Table 1). So, overexpression of *EF-2* had no obvious effect on the zebrafish embryonic development (Fig. 3).

Gene knockdown with morpholino antisense oligo

The results of gene knockdown assay with morpholino antisense oligo showed that most embryos injected with anti-MO displayed abnormal phenotypes during embryonic development while the same dose of sense-MO had no obvious effect on the embryos (Table 1). From 24-hpf EF-2-MO-treated embryos began to show developmental growth arrest. The EF-2-MO treated embryos were shorter, yolk balls were larger, tails were incurvate, lenses were much smaller and the melano-

Fig. 3. Overexpression of the *EF-2* gene (48 hpf). *There is no obvious difference between the* pcDNA3.0-EF2 *injected zebrafish and the wild type zebrafish.*

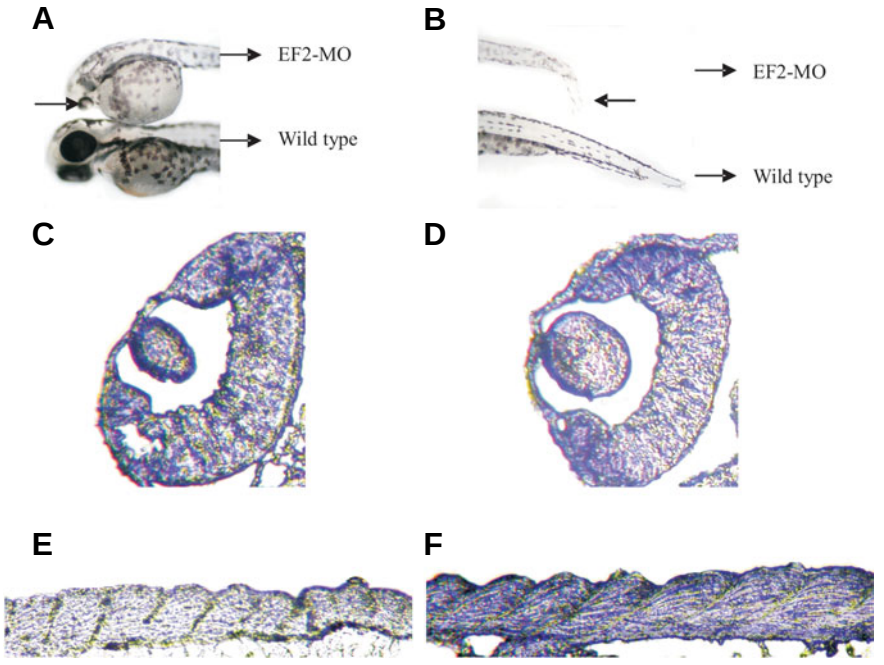

Fig. 4. Morphological and histological analysis of EF2-MO injected embryos at 48 hpf. (A,B) *Morphological comparisons of EF2-MO injected embryos and control embryos.* **(C,D)** *Histological analysis of EF2-MO injected embryos through cross-sections.* **(E,F)** *The cross-sections of sense-MO injected embryos. It is obvious that in the EF-2-MO injected embryo, the lens is much smaller and the tail is incurvate and incompact.*

phore spreading on their bodies became lighter than normal level (Fig. 4 A,B). Furthermore cross-sections also revealed that their tails were incurvate and their lenses were much smaller than those of normal embryos (Fig. 4 C–F).

Discussion

We have identified a translation elongation factor 2 (*EF-2*) in zebrafish with high identity to EF-2 proteins of other animals. From the results of the whole-mount *in situ* hybridization, it can be presumed that the zebrafish *EF-2* gene is developmentally regulated. Because zebrafish *EF-2* transcripts appear strongly throughout envelope before prim-15 stage, we consider the *EF-2* gene is expressed as a maternal transcript. After prim-15 stage, the expression of zebrafish *EF-2* is limited to irreversibly growth-arrested cells such as lens, cerebellum and somites, so we think zebrafish EF-2 is a terminal differentiation-specific protein just like EF-S protein (Knudsen *et al.*, 1993; Chambers *et al.*, 1998). And we can also accordingly presume that zebrafish *EF-2* is potentially a multi-gene family and the one which we are reporting is only one isoform of this family and its main function is maintaining the development of lens, cerebellum and somites but not protein synthsis.

This seems to be the first functional report on the developmental regulation of zebrafish *EF-2* gene. Overexpression of *EF-2* had no obvious effect on the zebrafish embryonic development. The data of gene knockdown approved that *EF-2* surely plays important roles in maintaining the development of zebrafish embryos. As EF-2 proteins are expressed in posterior somites and the tail bud, so in the EF-2-MO treated embryos the low level of EF-2 could not maintain the production of new somites in the tail buds and finally result in incurvate tails. Similarly, EF-2 proteins are also expressed in the lenses and were important to maintain their development, so probably hypoplasia appeared at those places when the level of the EF-2 could not maintain normal lens development. Although EF-2 expressed in the anterior portion of the cerebellum, no obvious abnormalities were found here upon the treatments, so we presume that some other important genes participate in the development of the anterior portion of the cerebellum and EF-2 only had a subsidiary role.

Above all, from the results of the whole-mount *in situ* hybridization, overexpression and gene "knock-down" experiments, we can believe that the zebrafish *EF-2* gene regulates primarily specific aspects of development.

TABLE 1

OVEREXPRESSION AND MORPHOLINO KNOCKDOWN OF THE *EF-2* GENE

	Amount of microinjection (ng/embryo)	Whole mount embryos	Live embryos (%)	Injected and phenotype for over-expression studies (%)
pcDNA3.0-EF2	1.0	263	12(4.6)	2(16.7)
	0	88	59(67.0)	0(0)
	0.67	248	120(48.4)	7(5.8)
	0	60	39(65.0)	0(0)
	0.53	169	83(49.1)	3(3.6)
	0	64	37(57.8)	0(0)
EF-2 capped mRNA	4.5	211	126(59.7)	7(5.6)
	0	73	48(65.8)	0(0)
EF-2 morpholino	4.5	116	87(75)	52(60)
	0	96	78(82)	0(0)
control sense-MO	4.5	167	130(77.8)	0(0)
	0	155	125(80.6)	0(0)

Materials and Methods

Zebrafish and embryo maintenance

Zebrafish were raised and maintained under standard laboratory conditions at 28°C, as described by Westerfield *et al.* (Westerfield *et al.,* 1995). Embryos used in whole-mount *in situ* hybridization were raised in 0.003% PTU (1-phenyl-2-thiourea, Sigma, St.Louis, MO, USA) to prevent pigment formation. The stage of the embryos was determined by morphological features and fixed with 4% PFA (paraformaldehyde) according to Kimmel *et al.* (Kimmel *et al.*, 1995).

Cloning and sequence analysis of EF-2

A zebrafish cDNA clone RK115A2C01 containing the *EF-2* full-length cDNA was isolated from the zebrafish adult kidney cDNA library (Song *et al.,* unpublished data). The isolated full-length *EF-2* cDNA was subcloned into pBK-CMV vector and the two inserted enzyme sites were EcoRI and XhoI, respectively. Then the full-length *EF-2* gene was sequenced. Its DNA and amino acid sequences were analyzed on National Center for Biotechnology Information (NCBI) blast server.

Multiple alignments were performed with clustalw version 1.82 (http://www.ebi.ac.uk/clustalw/) as well. The graphic presentations were prepared by software GeneDoc.

Whole-mount* in situ *hybridization

To study the expression pattern of the *EF-2* gene during embryogenesis, the zebrafish embryos were processed for whole-mount *in situ* hybridization after fixation in 4% PFA for 24 h at 4ºC. As described previously, the isolated full-length *EF-2* cDNA sequence was subcloned into pBK-CMV vector, which contains T7 and T3 promoters. Using T3 and T7 RNA polymerases, the sense and antisense RNA probes were synthesized labelled with digoxigenin (Roche) *in vitro*, respectively. The whole-mount *in situ* hybridization procedure was carried out as described by Westerfield *et al.* (Westerfield *et al.,* 1995).

***Overexpression of* EF-2**

Vector pBK-CMV-EF2 was cut by Xho I and used as transcription template. Finally the capped *EF-2* mRNA was transcribed *in vitro* using T3 RNA polymerase.

Expressing vector pcDNA3.0-EF2 was constructed as following: *EF-2* ORF was cut by Xho I and EcoR I from pBK-CMV-EF2 vector, then inserted into pcDNA3.0 expression vector (bought from Invitrogen company) that was also digested by Xho I and EcoR I. Capped *EF-2* mRNA and various amounts of expression vector pcDNA3.0-EF2 were injected into each of 1–4 cell stage embryos with a fine glass needle connected with an automatic injector (IM-300, Narishige, Japan), respectively. Phenotypes were observed at 48 hpf (hours post-fertilization). We selected three microinjection amounts of expression vector pcDNA3.0-EF2: 1.0ng/embryo, 0.67 ng/embryo and 0.53 ng/embryo. And the amount of capped *EF-2* mRNA microinjection is 4.5 ng/embryo.

Gene knockdown with morpholino antisense oligo

Both *EF-2* gene-specific antisense and sense morpholino oligonucleotides were purchased from Gene Tools LLC (Philomath, OR, USA), as following: anti-MO, 5'-CACCATTTTGACAGATGTTCTTGG-3'; sense-MO, 5'-CCAAGAACATCTGTCAAAATGGTG-3'.

Morpholino oligonucleotides were injected into each of 2-8-cell-stage embryos with a fine glass needle connected with an automatic injector (IM-300, Narishige, Tokyo, Japan). The amount of microinjection was about 4.5ng/embryo (Nasevicius *et al.*, 2000). Some anti-MO and sense-MO treated embryos at 48 hpf were fixed, dehydrated, embedded in resin and cross-sectioned to 5–10 μm thick with a glass knife. Slides were dyed by 1% toluidine blue.

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