Iron uptake and molecular recognition in *Pseudomonas putida*: receptor mapping with ferrioxamine B, coprogen B and their biomimetic analogues

Edouard Jurkevitch,¹ Yitzhak Hadar,¹ Yona Chen,² Pnina Yakirevitch,³ Jacqueline Libman³ and Abraham Shanzer³

Author for correspondence: Yitzhak Hadar. Tel: +972 8 481129. Fax: +972 8 468785.
e-mail: CHET%HUJIAGRI@VMS.HUJI.AC.II.

^{1,2} Departments of ¹Plant Pathology & Microbiology, and ²Soil & Water Sciences, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel

³ Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

This study shows that *Pseudomonas putida* possesses active uptake systems for Fe³⁺-ferrioxamine B (FOB) and Fe³⁺-coprogen B (Cop. B). These systems were characterized using natural and synthetic siderophores as structural probes. The synthetic analogues p178, p191, p239, p254 and p271 are a family of systematically modified linear retro-trishydroxamates that have shorter links between the ion binding groups relative to the natural compounds and possess chiral centres. They form a lower number of isomeric Fe³⁺ complexes relative to the natural compounds, and may be regarded as their specific conformers. Growth promotion and facilitated ⁵⁵Fe³⁺ uptake using both natural and synthetic siderophores were studied. The results obtained, along with those from competition experiments between the natural and the synthetic analogues demonstrate that: (i) the FOB and Cop. B uptake systems share common transport determinants; (ii) FOB and Cop. B make use of separate receptors; (iii) the Cop. B receptor is conformationally more demanding than the FOB receptor; and (iv) the FOB receptor has preference for the Δ -cis configuration although the natural siderophore is achiral. These results also demonstrate the usefulness of the synthetic analogues as structural probes. Some of these analogues simulate the natural counterparts as Fe³⁺ carriers, while others merely inhibit the action of the natural compounds by competing for the respective siderophore receptor.

Keywords: *Pseudomonas putida*, iron uptake, ferrioxamine B, coprogen B

INTRODUCTION

Fluorescent pseudomonads are widely found in the rhizosphere (Curl & Truelove, 1986). They can promote plant growth and act as biocontrol agents (Bakker *et al.*, 1990; Loper & Buyer, 1991). Since Fe forms insoluble ferric hydroxides in solution, the amount of soluble Fe in the environment is minute (Lindsay, 1979). One of the mechanisms by which fluorescent pseudomonads exert their beneficial effects is by scavenging Fe via their own siderophore or via siderophores produced by other micro-organisms (Bakker *et al.*, 1990; Kloepper *et al.*, 1980). Fluorescent pseudomonads are thus able to use various hydroxamate and catecholate siderophores produced by

other bacteria and fungi, such as those of the ferrichrome (Fc) family (Jurkevitch *et al.*, 1992a). The ability to utilize the siderophores of other micro-organisms probably confers an ecological advantage in the competition for Fe (Buyer & Sikora, 1990; Crowley *et al.*, 1991). A knowledge of the structural requirements for siderophore uptake in fluorescent pseudomonads thus provides a means to enhance or inhibit specific microbial populations.

Siderophore-mediated Fe uptake in bacteria involves several steps: First, the Fe complex is recognized by a receptor. It is then transported through one or two membranes, the Fe separated from the ligand, then the Fe³⁺ is metabolized or stored (Winkelmann, 1991). Recognition of the metal complex is usually receptor specific, a certain receptor being able to identify and

Abbreviations: Cop., coprogen; Fc, ferrichrome; FOB, ferrioxamine B.

transport one (or a few) siderophore(s) (having many structural features in common). In some cases, the domains that are important for recognition and transport have been determined. Relevant features involve the chirality of the coordination geometry around the metal centre (which can either be of a Δ or a Λ configuration), the Fe-surrounding residues, and the three-dimensional structure of the metal complex (Bergeron & Weimar, 1990; Jurkevitch *et al.*, 1992b; Winkelmann & Braun, 1981). Some hydroxamate siderophores, which are produced by a wide range of fungi, actinomycetes and bacteria, adopt a Δ configuration around the Fe centre (coprogen); others are left-handed (Fc), while still others form racemic mixtures (ferrioxamine).

Coprogen (Cop.) is a modified trimer of *N*⁵-hydroxy-*N*⁶(5-hydroxy-3-methyl-1-oxo-2-pentyl)-L-ornithine which adopts a Δ -*trans* configuration around the metal centre (Wong *et al.*, 1983). Coprogen B (Cop.B) is a closely related compound that differs from Cop. by substitution of the 3-acetyl residue with a hydrogen atom (Ernst & Winkelmann, 1974). Both compounds have been shown to be actively taken up by fungi (Ernst & Winkelmann, 1974) and bacteria (Braun & Hantke, 1991).

Ferrioxamine B (FOB), produced by *Streptomyces pilosus*, is a linear trishydroxamic acid composed of alternating units of 1-amino-5-(hydroxyamino)pentane and succinic acid (Tufano & Raymond, 1981). FOB represents an extreme case of configurational variability, in that it can form a total of five isomers when binding trivalent metal ions, each as racemic mixture (Leong & Raymond, 1975). FOB mediates Fe uptake in fungi, actinomycetes and bacteria (Mor & Barash, 1990; Muller & Raymond, 1984; Rabsch & Winkelmann, 1991).

Uptake of FOB and Cop. proceeds through two different receptors in *Pantoea agglomerans*, but it is unclear whether *Escherichia coli* possesses one receptor with dual function (Braun & Hantke, 1991), or two distinct receptors, one for each compound (Berner *et al.*, 1991; Nelson *et al.*, 1992).

In a previous study, we showed that Fc promotes the growth of *P. putida* and mediates ⁵⁵Fe incorporation (Jurkevitch *et al.*, 1992b). Using an original series of biomimetic analogues, we demonstrated that the Λ configuration is an essential, but not sufficient feature for receptor recognition and further transport; the three-dimensional shape and the bulkiness of the projecting side chains are also of importance. In this study, we show that Cop. B and FOB act as siderophores for *P. putida*. In order to probe for the structural requirements of the FOB receptor and their relation to Cop. recognition, we designed and synthesized synthetic FOB analogues that would preferentially (if not exclusively) adopt the *cis* configuration, exhibit significant chiral preference for either the right- or the left-handed configurations, possess minimal conformational freedom, and thereby 'freeze out' specific conformations of the natural Fe³⁺-FOB complexes.

METHODS

Bacterial strains. *P. putida* WCS358 and its siderophore minus (Sid⁻) Tn5-induced mutant JM218 were obtained from P. Weisbeek in Utrecht, The Netherlands. *P. putida* B10 was a kind gift from J. Buyer, Beltsville, MD, USA. *P. putida* St3 was isolated in our laboratory.

Siderophores. Fc and Cop. B were from laboratory stock. FOB was purchased in the methylsulfonate form (Desferal) from Ciba-Geigy. Synthesis of the Fc analogues B5 and B9 has been described previously (Jurkevitch *et al.*, 1992b).

Synthesis. The ferrioxamine analogues were prepared in essentially four steps as described by Yakirevitch *et al.* (1993).

Plate tests. The antibiosis plate test was carried out as described by Buyer & Leong (1990). Soft agar (0.6 ml) inoculated with approximately 10³ c.f.u. ml⁻¹ of the test bacterium was poured over modified King's B (MKB) medium in 5 cm diameter Petri dishes. Twenty μ l of a 10⁻³ M solution of the test desferri compound was dropped onto a 6 mm diameter Whatman paper disk. The plates were incubated at 30 °C and examined after 24 h.

For the Fe-siderophore complex utilization test, the bacteria were grown in liquid MKB medium and about 10³ c.f.u. were plated on MKB plates containing 400 μ M α , α '-dipyridyl. The bacteria were also grown in a liquid rhizosphere simulating medium (RSM) and plated on RSM containing 201 μ M ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDHA) (Buyer *et al.*, 1989). Ten μ l of a 10⁻⁴ M solution of the Fe-siderophore complex was dropped on a Whatman paper disk. The plates were incubated as above.

Growth curves. The Sid⁻ strain JM218 was used to test the efficiency of bacterial growth promotion in an Fe-free liquid medium. HEPES-succinate medium [HSM, containing (g l⁻¹): succinate, 4; K₂HPO₄, 0.2; (NH₄)₂SO₄, 1; MgSO₄·7H₂O, 0.2; and HEPES, 11.91] was batch-treated for 4 h with Chelate N resin Serva, 10 g l⁻¹], decanted and filtered through a 0.45 μ m filter. The pH was then raised to 7.2 by addition of 10 M NaOH and the medium was autoclaved. Double-distilled water was used, and the glassware was washed with 6 M HCl followed by thorough rinsing with double-distilled water.

Polypropylene test-tubes (50 ml) were filled with 10 ml of the Chelate-treated HSM. The Fe³⁺-siderophores were added to a final concentration of 10⁻⁶ M, the tubes were inoculated with about 4 $\times$ 10⁷ c.f.u. ml⁻¹ and shaken at 28 °C. Samples were taken at appropriate intervals and optical density at 620 nm was recorded. Each treatment was performed in triplicate.

⁵⁵Fe uptake. The procedure for determining ⁵⁵Fe uptake described by de Weger *et al.* (1988) was used, with minor modifications. The mutant JM218 was used in all experiments. The bacterium was grown in a half-strength standard succinate medium (SSM) to an OD₆₂₀ of 0.4, centrifuged for 15 min at 2500 r.p.m. then resuspended in fresh half-strength SSM to a final OD₆₂₀ of 0.3 and incubated for 1 h in a water bath at 28 °C. The labelled ⁵⁵Fe³⁺ complex was added to a final concentration of 1 μ M. When appropriate, dinitrophenol (DNP) and potassium cyanide (CN⁻) were added 20 min before the labelled compound to a final concentration of 2 and 5 mM, respectively. Aliquots (0.5 ml) were taken in duplicate, layered onto a mixture of octyl/dibutyl phthalate (1:2, v/v Sigma) and centrifuged. Counting was performed on a Beckman LS1801 counter.

Fig. 1. Ferrioxamine analogues [left P178, $m = 0$, $R = iBu$ (L-Leu); P239, $m = 0$, $R = iBu$ (D-Leu); P191, $m = 0$, $R = Me$ (L-Ala); P254, $m = 2$, $R = CONEt_2$ (L-Glu); P271, $m = 2$, $R = CONEt_2$ (D-Glu)] and ferrichrome analogues [right: B9, $R = H$; B5, $R = Me$ (L-Ala)].

RESULTS

Design

The synthesized ferrioxamine analogues are trimers of three monomers, each composed of a natural amino acid linked via hydroxamate bonds to *N*-hydroxyaminopropionic acid (Fig. 1). This design allows for a large variability by relying on natural amino acids as building blocks. Shortening the spacers between the binding sites relative to those of FOB ($m = 0$ and $m = 2$, respectively; Fig. 1) restricts the number of possible coordination isomers to those possessing *cis* geometry. Incorporation of asymmetric centres induces chiral preference for either the right- or left-handed *cis* configuration.

Circular dichroism spectroscopy of the Fe^{3+} complexes demonstrated preferred right-handedness when L-amino acid components were used. Quite remarkably, the extent of chiral preference for the Δ -*cis* configuration (when L-amino acids were used) varied substantially with the nature of the amino acid. While the chiral preference was small for the Ala and Leu derivatives, it was larger for the Asp derivative, and particularly large for the Glu derivatives. The enhanced chiral preference of the Asp derivative suggests the presence of specific non-covalent interactions between the projecting amide groups CH_2CONEt_2 and the backbone and/or Fe^{3+} centre of the molecule. The greater optical purity of the Glu derivatives is attributed to the extended amino acid bridges, which tend to lower the strain in the complex. A more detailed description of the compounds, their synthesis and coordination chemistry is given in Yakirevitch *et al.* (1993).

Growth promotion and antibiosis plate tests

Plate tests showed that *P. putida* St3, WCS358, B10 and JM218 (the *Sid*⁻ derivative of WCS358) are able to grow in the presence of the siderophores Cop.B and FOB.

When Cop.B or FOB were used as sole Fe source in plates containing EDDHA or α, α' -dipyridyl, growth halos of bacteria 25 to 45 mm and 35 to 50 mm in diameter, respectively, were measured after 18 to 24 h incubation at 30 °C. None of the Fe complexes of the synthetic siderophores p178, p191, p239, p254 and p271 could sustain bacterial growth.

Inhibitory activity of the desferri-compounds was also tested. Application of 20 μ l of a 10^{-3} M solution of Cop.B or FOB as free ligands resulted in normal growth of the colonies around the paper disks, again indicating that the bacteria were able to use the Fe-siderophore complex formed with residual Fe from the growth medium. The synthetic siderophore analogues p178, p191 and p239 did not exhibit antibiotic activity towards strain JM218. However, application of the desferri compounds p254 and p271 resulted in a clear zone of inhibition around the paper disks. This indicates that these latter compounds are totally unavailable as Fe sources for strain JM218. Since the control treatments, including the Fe complexes of the compounds did not show any deleterious effects, it can be concluded that Fe immobilization was the cause of the inhibition.

Growth in liquid medium

The growth-promoting ability of Fe-FOB and its biomimetic analogues was studied in a liquid medium treated with Chelate N to remove residual Fe. As seen in Fig. 2, the cells grew very poorly in the control treatment, while Fe-FOB promoted strong growth of the bacterium, with a doubling time of about 1.75 h. The Fe complexes of the synthetic analogues p178, p191 and p239 promoted the growth of JM218, but not as efficiently as the natural siderophores. Although the lag period was longer the slope of the curves differed little and generation times of 1.8 to 2 h were recorded. In agreement with the plate

Fig. 2. Growth of mutant JM218 in Chelate-treated HSM with ferrioxamine B (■), and the ferrioxamine B analogues p178 (○), p191 (□), p239 (▽), p254 (▼) and p271 (●) as iron sources. ◇, Control (no added iron source).

experiments, the Fe complexes of p254 and its enantiomer p271 were totally ineffective in promoting bacterial growth. When Cop. B was tested in identical experiments, it behaved similarly to FOB.

⁵⁵Fe uptake

⁵⁵Fe uptake mediated by Cop. B and FOB was demonstrated (Fig. 3). The two compounds mediated ⁵⁵Fe uptake similarly, showing a linear uptake response during the 20 min experiment. About 90 and 120 pmol Fe (mg dry wt)⁻¹ were incorporated after 20 min for Cop. B and FOB uptake, respectively. Uptake was strongly reduced by the inhibitory compounds potassium cyanide and dinitrophenol, as in Fc- and B9-mediated uptake (Table 1). The *K_m* values for ⁵⁵Fe uptake mediated by both FOB and Cop. B were calculated using data from concentration dependent experiments. The apparent *K_m* values were 0.09 μM and 0.22 μM for Cop. B- and FOB-

Table 1. Inhibition of ⁵⁵Fe uptake mediated by ferrichrome (Fc), coprogen B (Cop. B), and ferrioxamine B (FOB) and their biomimetic analogues by the respiratory inhibitors dinitrophenol (DNP) and potassium cyanide (CN⁻)

Inhibitor	Percentage inhibition						
	⁵⁵ Fe-siderophore:						
	Fc	Cop. B	FOB	B9	p178	p191	p239
DNP (2 mM)	96	86	71	93	—	—	—
CN ⁻ (5 mM)	95	91	72	99	78	89	81

mediated uptake, respectively. The synthetic analogues p178, p191 and p239 mediated ⁵⁵Fe uptake, but showed a different uptake pattern (Fig. 3). After 3 min, the amount of ⁵⁵Fe associated with the cells was about three times higher than that for FOB- or Cop. B-mediated uptake (Fig. 3). The final concentration of Fe was about 110 pmol (mg dry wt)⁻¹ resulting in a flatter uptake curve. Uptake of ⁵⁵Fe mediated by these three analogues was reduced by the addition of CN⁻ to the incubation medium, demonstrating active uptake (Table 1). Compounds p254 and p271 did not promote appreciable ⁵⁵Fe uptake (Fig. 3).

Competition experiments

Competition experiments between the natural and the synthetic siderophores were performed to: (i) determine whether Fe uptake mediated by the siderophores Fc, FOB and Cop. B shares common components of the transport system; (ii) trace the structural requirements for recognition by these components. Uptake of ⁵⁵Fe-Cop. B was inhibited by increasing concentrations of cold Fe-Fc and of the ferrichrome analogue Fe-B9 derived from

Fig. 3.

Fig. 4.

Fig. 3. ⁵⁵Fe uptake mediated by ferrioxamine B (■), Cop. B (▲), p178 (○), p191 (□), p239 (△), p254 (▼) and p271 (●).

Fig. 4. Percentage of ⁵⁵Fe uptake mediated by Cop. B in the presence of increasing concentrations of Fe-ferrichrome (○) and Fe-B9 (●).

Table 2. Inhibition of ferrioxamine B (1 μ M)-mediated ^{55}Fe uptake by 'cold' Fe-coprogen B (Fe-Cop.B), Fe-ferrichrome (Fe-Fc) and the ferrichrome analogue Fe-B9

Competing siderophore	Percentage inhibition			
	Concn (μM)...	1	3	5
Fe-Cop. B		47	50	59
Fe-Fc		49	58	71
Fe-B9		18	31	40

Table 3. Inhibition of ferrioxamine B (FOB)- and coprogen B (Cop.B)-mediated ^{55}Fe uptake (1 μ M) by 'cold' Fe-FOB analogues (10 μ M)

	Percentage inhibition				
	p178	Biomimetic analogue:			
		p191	p239	p254	p271
Fe-Cop.B	46	53	42	0	2.5
Fe-FOB	51	46	65	22	52

glycine (Jurkevitch *et al.*, 1992b) (Fig. 4). Equimolar concentrations of Fe-Fc and Fe-B9 reduced ^{55}Fe uptake mediated by Cop.B by 55% and by 22%, respectively. Similarly, FOB-mediated ^{55}Fe uptake was strongly affected by increased concentrations of the competing siderophores Fe-Fc, Fe-Cop.B and Fe-B9 (Table 2). The data suggest that these siderophores use at least one common component in their uptake systems.

The Fe complex of the optically pure ferrichrome analogue B5 (Δ) which blocks the Fc receptor (Jurkevitch *et al.*, 1992b) was shown to have no effect on ^{55}Fe uptake mediated by either Cop.B or FOB. These siderophores must therefore mediate Fe uptake through one or two receptors that are different from that of Fc. Differential effects of the synthetic Fe-p254 (Δ) and Fe-p271 (Δ) on the extent of Fe uptake mediated by Cop.B and FOB shows that these make use of different receptors: neither Fe-p254 nor Fe-p271 inhibited ^{55}Fe uptake mediated by Cop.B (Table 3). However, both Fe-p254 and Fe-p271 affected FOB-mediated ^{55}Fe uptake with greatly different efficiencies. The former reduced FOB-mediated ^{55}Fe incorporation by 22% and the latter by 52% (Table 3). The Fe complexes of compounds p178, p191 and p239, which are linear Cop. and ferrioxamine analogues of mixed chirality, inhibited both FOB- and Cop.B-mediated ^{55}Fe uptake, although at various levels (Table 3).

DISCUSSION

This study focused on the uptake of Fe via the linear siderophores Cop.B and FOB. We showed that these siderophores are active in fluorescent pseudomonads,

being able to promote the growth of cells when supplied as the sole Fe source. Using natural siderophores and synthetic analogues as probes, and examining both their action as Fe carriers and their mutual inhibitory properties, we obtained qualitative information on the structural requirements of several hydroxamate siderophore Fe transport systems.

Uptake of ^{55}Fe mediated by FOB and Cop.B, as well as by Fc, was shown to be an active process, probably involving the protonmotive force since it is inhibited by DNP as shown earlier for pseudobactin-mediated Fe uptake (de Weger *et al.*, 1991). FOB- and Cop.B-mediated uptake of Ge showed high K_m values (0.22 and 0.09 μ M, respectively). The uptake systems of these natural siderophores (Fc, Cop.B and FOB) must share a common component since ^{55}Fe uptake mediated by any of these compounds was decreased in the presence of 'cold' complexes of each of the other siderophores. This observation is reminiscent of the Fe uptake system of *Escherichia coli* where the periplasmic FhuD protein is able to bind siderophores with widely differing chemical structures such as Fc, Cop. and aerobactin (Koster & Braun, 1990). The siderophores are then presumably passed to the cytoplasmic membrane-bound FhuB protein (Koster, 1991). Whether FhuD is free in the periplasmic space is unknown (Koster & Braun, 1990). Huschka *et al.* (1985) have demonstrated that although the transport system for Fc and Cop. is shared in *Neurospora crassa* each compound has a specific receptor. More recently, Dori *et al.* (1990) have shown that in *Gaeumannomyces graminis* var. *tritici*, the siderophores dimerium acid, Cop.B, rhotulic acid and Fc share a common transport system.

Competition experiments between FOB and Cop.B and synthetic analogues have enabled us to distinguish between different components of the siderophores' transport systems as well as to trace structural requirements for recognition by these components.

Compounds B5 and B9 are C_3 -symmetric Fc analogues (Fig. 1). B5 is chiral, adopts preferentially the Δ -*cis* configuration and has been shown to block the Fc receptor of *P. putida*. B9 is achiral and acts as an Fc substitute by promoting bacterial growth and ^{55}Fe uptake (Jurkevitch *et al.*, 1992b). Inhibition of FOB- and Cop.B-mediated ^{55}Fe uptake by natural Fc and analogue B9 (Table 2) but not by analogue B5 – which merely blocks the Fc receptor – demonstrates the involvement of common components, but separate receptors for Fc and FOB/Cop.B transport systems.

Compounds p178, p191 and p254 are linear trishydroxamates possessing L-Leu, L-Ala and L-Glu amino acid residues respectively. p239 (D-Leu) is enantiomeric to p178, and p271 (D-Glu), is enantiomeric to p254. Although the Fe complexes of the shorter homologues p178, p191 and p239 were unable to promote growth in plate experiments, they supported growth enhancement in liquid medium. Furthermore, they effectively facilitated ^{55}Fe uptake which could be inhibited by the presence of cyanide. This demonstrates their availability as Fe carriers and their action to be dependent upon an energy-driven

process. The Fe chelators used in the plates might therefore have interfered with the synthetic analogues (Berner *et al.*, 1991). The fact that ^{55}Fe uptake did not follow first-order kinetics suggests some non-specific absorption of the complexes at the initial stages. This does not however, change the overall conclusions.

Both transport systems, that of FOB and that of Cop.B may be used by the shorter analogues (p178, p191 and p239), as shown by the inhibitory effects of these on FOB- and Cop.B-mediated ^{55}Fe uptake (Tables 2 and 3). This may be attributed to their small diameter which facilitates adaptation to their respective recognition sites. The longer homologues (p254 and p271) fit to the FOB receptor, but not to the Cop.B receptor. They inhibited FOB-mediated ^{55}Fe uptake without acting as Fe carriers, but had no effect on Cop.B-mediated ^{55}Fe uptake. Moreover, the complex adopting a preferential Λ configuration (derived from p271) was found to be a two- to threefold better inhibitor than its enantiomer derived from p254. The latter observations indicate: (i) the presence of different receptors for the FOB and Cop.B transport systems, and (ii) higher structural discrimination by the Cop.B receptor. Most remarkable is the chiral preference for the Λ configuration (p271) exhibited by the FOB receptor, which might imply that an 'archaic' FOB possessed chiral centres.

In conclusion, we have shown that the Fc, FOB and Cop.B transport systems of *P. putida* contain distinct receptors, but common transport proteins. Furthermore the Fc and FOB receptors may specifically be blocked with selected synthetic analogues (B9 and p271). In this respect, synthetic analogues may exceed the specificity of the natural siderophores, opening the possibility of selective growth inhibition. On the other hand, some synthetic compounds – like p178, p191 and p239 – may exhibit dual activity, substituting both FOB and Cop.B as carriers and introducing 'broad spectrum' growth promoters. The effect of the synthetic compounds may, however, differ between micro-organisms. Thus, the shorter analogues p178, p191 and p239 exerted FOB-like activity towards *Hafnei alvei*, Coprogen-like activity towards *E. coli* and both activities towards *Pantoea agglomerans* (Berner *et al.*, 1991) as towards *P. putida*. However, the longer homologues p254 and p271 also show dual FOB and Cop. activities independently of their chirality in *P. agglomerans* (G. Winkelmann, personal communication) in contrast to *P. putida*. These findings demonstrate that species-dependent differences in the structural characteristics of related siderophore uptake systems may prevail. Whether these differences can be employed for the synthesis of species-specific growth promoting or growth inhibiting agents remains to be established.

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