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Selective inhibition of divergent seryl-tRNA synthetases by serine analogues

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Abstract **Seryl-tRNA synthetases (SerRSs) fall into two distinct evolutionary groups of enzymes, bacterial and methanogenic. These two types of SerRSs display only minimal sequence similarity, primarily within the class II conserved motifs, and possess distinct modes of tRNA^{Ser} recognition. In order to determine whether the two types of SerRSs also differ in their recognition of the serine substrate, we compared the sensitivity of the representative methanogenic and bacterial-type SerRSs to serine hydroxamate and two previously unidentified inhibitors, serinamide and serine methyl ester. Our kinetic data showed selective inhibition of the methanogenic SerRS by serinamide, suggesting a lack of mechanistic uniformity in serine recognition between the evolutionarily distinct SerRSs.**

Keywords: Seryl-tRNA synthetase; Substrate analogue; Selective inhibition; Serinamide; Evolution

1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) catalyze acylation of transfer RNAs with their cognate amino acids, and thus possess a central role in assuring faithful transmission of genetic information. Essentiality of their function has made aaRSs attractive targets for drug design, while their structural and evolutional diversity provides a constructive foundation that is readily exploited in the identification of species-specific enzyme inhibitors.

Evident lack of phylogenetic uniformity is observed among seryl-tRNA synthetases (SerRSs), which diverge into two major and disparate types of enzymes. The bacterial-type SerRS is present in bacteria, eukaryota and a majority of archaea, whereas methanogenic archaea, with the exception of *Methanosarcina mazei* and *Methanosarcina acetivorans*, possess the methanogenic-type SerRS [1] (Fig. 1). Most conspicuous differences between the two types of SerRSs include insertions in the N-terminal domain of methanogenic proteins and a deletion within the motif 2 loop, which was demonstrated to participate in both tRNA^{Ser} and serine recognition in *Thermus thermophilus* [2]. Such dissimilarity suggests that modes of substrate recognition may differ between the two types of SerRSs. In this respect it is also noteworthy that only a single residue involved in serine recognition of the bacterial-type SerRSs (Glu279 of *T. thermophilus*, which interacts with both serine hydroxyl and amino groups) preserves its identity in the methanogenic sequences (Fig. 1). On the other hand, despite the conservation of the active site residues within the bacterial-type SerRSs, eukaryotic enzymes are characterized by the presence of a C-terminal extension that affects optimal serine recognition: reportedly, C-terminal deletion in the yeast SerRS impaired tRNA-facilitated serine recognition, as shown by a 3-fold increase of the serine K_M value [3]. Moreover, functional implications of the structural differences between methanogenic, bacterial and eukaryotic SerRSs may be observed in their respective modes of tRNA^{Ser} recognition, which display notable disparities [1,4].

In order to assess whether the differences between the SerRS enzymes also pertain to serine recognition we analysed the sensitivity of the coexisting bacterial and methanogenic-type *Methanosarcina barkeri* SerRSs to serine hydroxamate, a known inhibitor of the *Escherichia coli* and yeast SerRSs [5–9], and two previously unidentified inhibitors, serine methyl ester and serinamide. Additionally, the *E. coli* and *S. cerevisiae* enzymes were examined as representative bacterial-type SerRSs of bacterial and eukaryotic origin, respectively. We show that distinct types of SerRSs display differential inhibitory profiles, and propose that such dissimilarity is both consequential and informative in respect of their evolutionary origin.

2. Materials and methods

2.1. Cloning and expression of seryl-tRNA synthetases

Methanogenic and bacterial-type SerRS genes were cloned as described [1]. *E. coli serS* was amplified by PCR using Expand High Fidelity polymerase (Roche), and cloned into the pET15b vector (Novagen). N-terminally His_6-tagged *M. barkeri* and *E. coli* proteins were produced according to the published procedure [10]. The yeast SerRS gene *SES1* was previously cloned into vector pCJ11 for the expression in the *Saccharomyces cerevisiae* strain S2088 [3]. *S. cerevisiae* SerRS was purified on a series of chromatographic columns, as described [11].

2.2. Aminoacylation assay

Aminoacylation was performed in 50 mM HEPES–KOH pH 7.2, 50 mM KCl, 15 mM $MgCl_2$, 5 mM dithiothreitol, with 10 mM ATP, 20 μg/μL total tRNA, and [^{14}C] serine at concentrations varying between 0.2 and 5 times K_M serylation with the *M. barkeri* and *E. coli* enzymes was performed at 37 °C with *E. coli* unfractioned tRNA, whereas

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Abbreviations: aaRS, aminoacyl-tRNA synthetase; SerRS, seryl-tRNA synthetase (EC 6.1.1.11)

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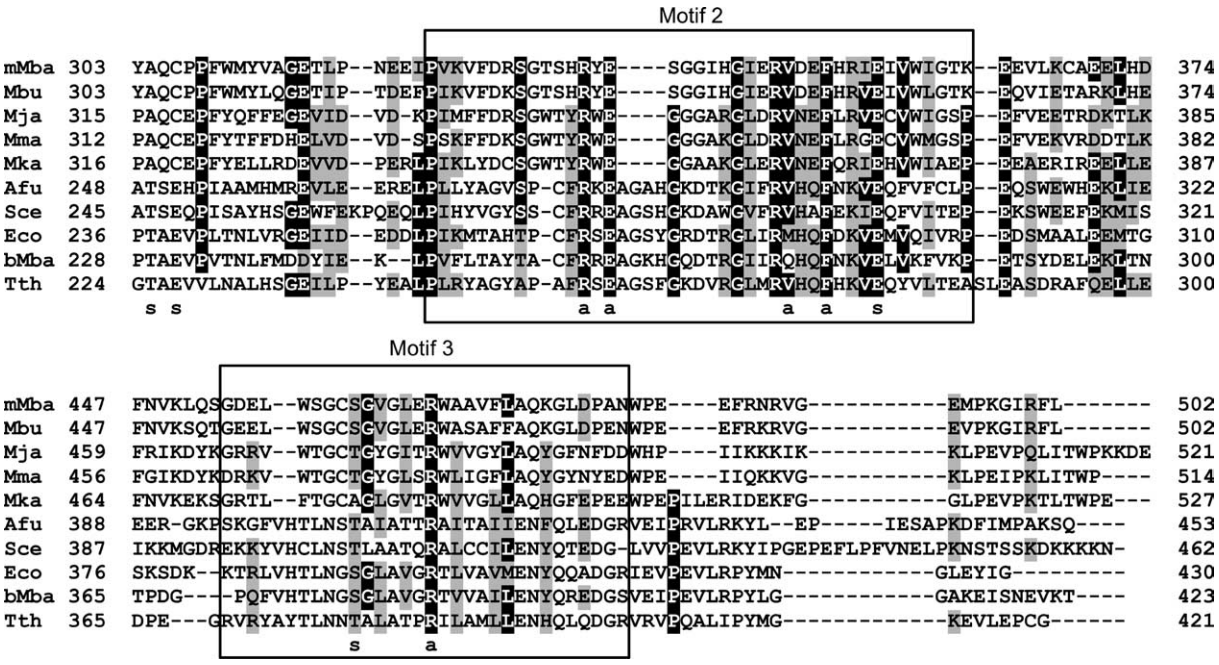

Fig. 1. Alignment of motifs 2 and 3 from a number of representative SerRSs. The sequences are from *Methanosarcina barkeri*-methanogenic-type SerRS (mMba), *Methanococcoides burtonii* (Mbu), *Methanocaldococcus jannaschii* (Mja), *Methanococcus maripaludis* (Mma), *Methanopyrus kandleri* (Mka), *Archaeoglobus fulgidus* (Afu), *Saccharomyces cerevisiae* (Sce), *Escherichia coli* (Eco), *Methanosarcina barkeri*-bacterial-type SerRS (bMba) and *Thermus thermophilus* (Tth). "s" and "a" denote residues in *Thermus thermophilus* sequence that participate in serine and adenylate recognition, respectively (Val272 and Arg386 form only main chain interactions). Note that residues involved in adenylate recognition are strictly conserved, whereas those involved in serine recognition are not.

yeast unfractioned tRNA was used for aminoacylation with the yeast SerRS at 30 °C. Enzyme concentrations were experimentally determined in order to obtain linear velocities. Radioactive aminoacyl-tRNA synthesized after 2–8 min was quantified as described previously [10]. The kinetic constants were derived from Hanes–Woolf plots.

2.3. Selection of serine analogues

The following serine analogues (Sigma) were analysed as potential inhibitors of the methanogenic and bacterial-type *M. barkeri* enzymes: serinamide, serine methyl ester, serine hydroxamate, serinol,-methyl serine, *O*-methyl serine, phosphoserine, serine ethyl ester and *N*-acetyl serine. Aminoacylation was performed at 37 °C with 5 μM [^{14}C] serine, 1 mM serine analogue and 125 nM methanogenic SerRS or 30 nM bacterial SerRS. The level of inhibition was determined as a ratio of initial velocities of inhibited and uninhibited reactions. Analogues that showed different profile of inhibition by the two *M. barkeri* enzymes were kinetically analysed with all representative SerRSs.

2.4. Kinetic analysis of inhibition

To determine K_I values for serine inhibitors, at least five different concentrations were first screened in the aminoacylation reaction under standard conditions with 5 μM [^{14}C] serine. Inhibitor concentrations were then established, at which the initial rate of aminoacylation was decreased by 20–60% when compared with the uninhibited reaction; these levels were used for K_I determinations (three different concentrations for each inhibitor). In all cases, stock solutions of inhibitors were maintained at neutral pH. The enzyme concentrations were 400 nM for the methanogenic, 225 nM for the bacterial *M. barkeri* SerRS, 53 nM for the *E. coli* SerRS and 203 nM for the yeast enzyme. The kinetic constants were derived from Hanes–Woolf and K_{Mi}/V_{Mi} versus I plots [12]. Final individual kinetic parameters are the average of three independent determinations.

3. Results

3.1. Divergence of SerRSs

Our phylogenetic analysis of available SerRS sequences (Fig. 2) confirmed the known division between the methanogenic and bacterial-type sequences [13]. Bacterial-type SerRSs exhibit lower evolutionary distances, but still preserve a basic canonical pattern with grouping of archaeal and eukaryotic proteins and distinction of bacterial sequences. Nonetheless, horizontal gene transfers are evident within this group: bacterial-type SerRSs in *Methanosarcina* species and Halobacteria are almost certainly results of such events, as these enzymes group with bacterial, rather than archaeal proteins. Accordingly, the original methanogenic-type SerRSs may have been replaced by the bacterial enzymes in these organisms; in this respect, *M. barkeri* may represent an intermediate stage in this process with two types of SerRSs.

3.2. Differential sensitivity to serine analogues

Since the binding of tRNA^{Ser} induces conformational changes [2] that facilitate binding of serine to the active site of bacterial-type SerRS [11], we chose to measure the steady-state aminoacylation kinetics of representative SerRS enzymes. Initially, various serine analogues were used as probes for identification of possible structural and functional differences between the two major types of SerRSs represented by the two *M. barkeri* SerRSs. Our results show that only serine hydroxamate, serine methyl ester and serinamide inhibited serylation by the methanogenic SerRS to a considerable level (Fig. 3). Moreover, these compounds showed a seemingly differential effect on the methanogenic and bacterial SerRSs, and were accordingly examined in a kinetic analysis with representative SerRSs of methanogenic, bacterial and eukaryotic origin.

Kinetic analysis revealed increased K_M values upon inhibition, thus confirming the competitive nature of serine analogues. The data also showed a marked variation of serinamide K_I values between the representative SerRSs (Fig. 4): the sensitivity to serinamide is significantly more

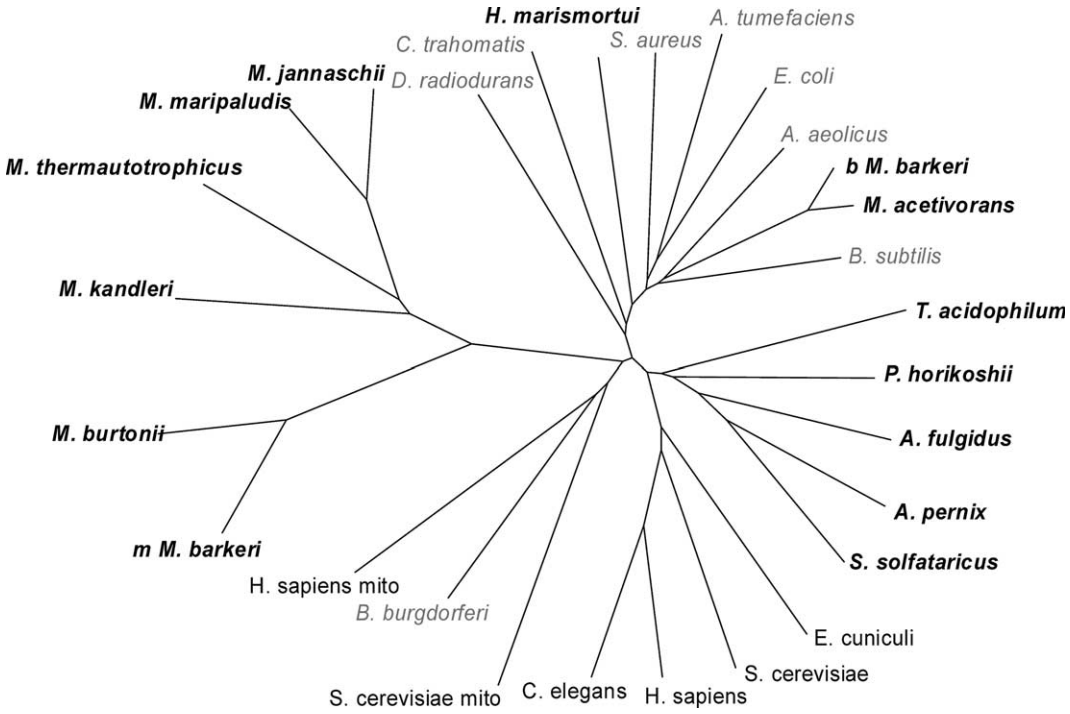

Fig. 2. Unrooted phylogenetic tree of seryl-tRNA synthetases implied by the neighbor-joining method. Archaeal sequences are shown in bold italics, bacterial in grey italics, and eukaryotic as plain text. *m M. barkeri* denotes the methanogenic, and *b M. barkeri* bacterial-type protein.

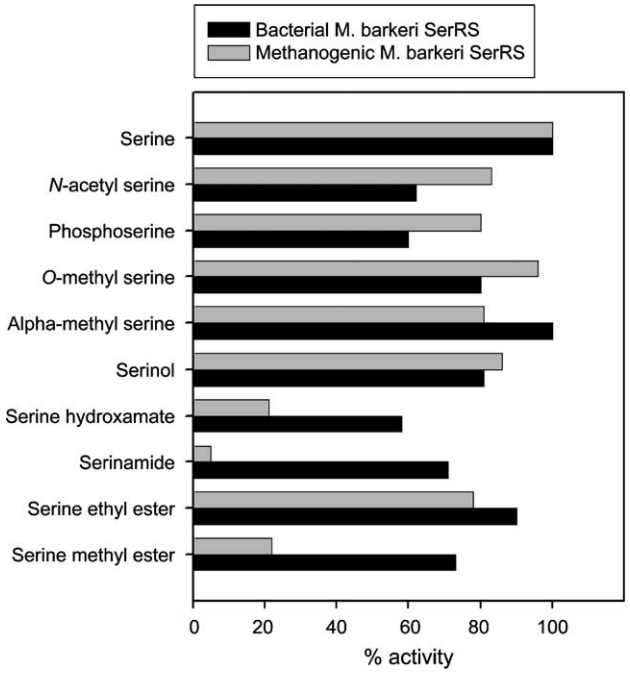

Fig. 3. Activity of the methanogenic and bacterial *M. barkeri* seryl-tRNA synthetases in the presence of serine analogues. Aminoacylation was performed with 5 μM serine and 1 mM analogue. % activity is calculated from initial velocity, relative to the uninhibited reaction.

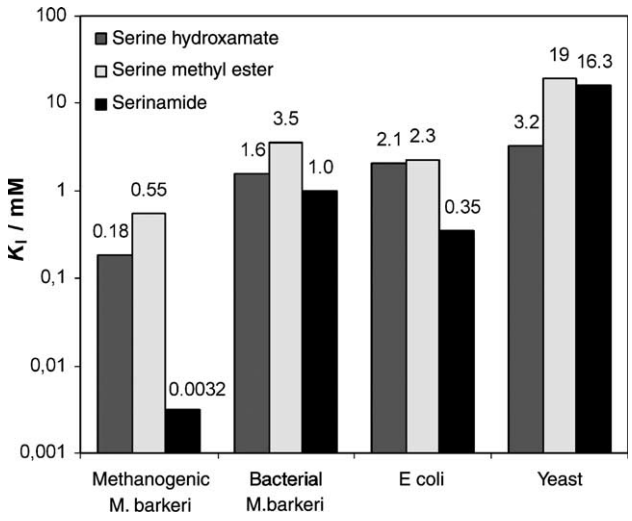

Fig. 4. Inhibition profiles of representative seryl-tRNA synthetases. K_I values are plotted on logarithmic scale.

pronounced in the methanogenic *M. barkeri* SerRS than in other, bacterial-type enzymes. The most striking differences are observed for the yeast and the bacterial *M. barkeri* SerRS, whose K_I values differ from the methanogenic *M. barkeri* SerRS inhibition constant by several orders of magnitude. Thus, the relative serinamide K_I values (Fig. 4) reveal an approximately 5000-fold difference between the yeast and methanogenic *M. barkeri* SerRSs, and a 300-fold distinction in the case of the bacterial and methanogenic *M. barkeri* enzymes. On the other hand, the K_I values of serine hydroxamate and serine methyl ester display a lesser degree of variation between different SerRSs (18-fold for serine hydroxamate and 35-fold for serine methyl ester at the most) (Fig. 4). Our current studies do not reveal a notable difference in sensitivity to serine hydroxamate between by the *E. coli* and yeast enzymes, as reported previously [8]; the discrepancy is mostly due to the significantly higher K_I value for the *E. coli* SerRS, determined

recently in our laboratory (2.1 ± 0.3 mM), in comparison with the published data (30 μM) [6].

Apparently, the methanogenic SerRS is more susceptible to inhibition by all three serine analogues than are the bacterial-type enzymes from *M. barkeri*, *E. coli* or yeast (although the difference is much less pronounced for serine hydroxamate and serine methyl ester; Fig. 4). However, with respect to the efficacy of the inhibition, neither serine hydroxamate nor serine methyl ester displayed affinities for the target enzymes that are comparable to the affinities of the natural substrate serine (Fig. 4): whereas the K_M values of all four enzymes are in micromolar range (25 μM for the methanogenic *M. barkeri* SerRS, 34 μM for the bacterial *M. barkeri* SerRS, 60 μM for the *E. coli* SerRS and 62 μM for the yeast SerRS), the K_I values of serine hydroxamate and serine methyl ester fall into the (sub-)millimolar range. The same applies to serinamide; the only exception is the methanogenic *M. barkeri* SerRS, whose K_I value of 3.2 μM signifies that serinamide is apparently preferable to serine (K_M = 25 μM) with respect to the binding affinity.

4. Discussion

4.1. Mechanistic implications of inhibition studies

Among the serine analogues used in this study, only serine hydroxamate, serine methyl ester and serinamide were successful in conferring inhibition. As confirmed by the crystallographic evidences of the *T. thermophilus* SerRS, the binding pocket effectively assures serine specificity by disallowing oversized side chains, and we may speculate that such strategy is unanimous among the SerRSs, despite their evolutionary divergence. Presumably, whereas steric clashes preclude binding of α-methyl serine, *O*-methyl serine, phosphoserine and *N*-acetyl serine, causing their inhibitory incompetence, serinol is unlikely to induce steric hindrance; the fact that it is inefficient in inhibiting serylation activity suggests that its initial binding to the SerRS pocket is disfavoured due to the reduced capacity of hydrogen bond formation, i.e., the absence of the carbonyl oxygen.

Not surprisingly, differences in the inhibitory profile of the tested SerRSs are most striking between the methanogenic-type and any of the bacterial-type enzymes (Fig. 4). In particular, binding affinities of the bacterial and methanogenic *M. barkeri* SerRSs for serinamide differ by more than two orders of magnitude. Although the yeast enzyme displays a more discernible distinction compared to the methanogenic *M. barkeri* SerRS, the difference between the two *M. barkeri* enzymes is more intriguing because these SerRSs coexist in the same cellular environment. Such differential binding of serinamide could likely be correlated to the fundamentally different modes of serine recognition in the methanogenic and the bacterial-type SerRSs. This assumption is supported by the minimal conservation of the residues crucial for serine recognition in bacterial-type enzymes among their methanogenic analogues (Fig. 1).

Among the bacterial-type enzymes, SerRSs from *M. barkeri* and *E. coli* show apparent similarity in their inhibition profiles, and their K_I values do not differ substantially (Fig. 4). However, in the case of the yeast enzyme, the pattern of inhibition is slightly changed: while serinamide seems to be more potent than hydroxamate against the *E. coli* and *M. barkeri* bacterial-type SerRSs, the situation is reversed for the yeast enzyme. Such grouping is well supported by the phylogenetic data (Fig. 2): evidences of horizontal gene transfers in *Methanosarcina* species clearly account for the similar inhibition pattern of the *M. barkeri* and *E. coli* SerRSs, whereas the noted distinction in the profile between the bacterial versus eukaryotic enzymes appears to reflect the apparent evolutionary distance. Alternatively, this difference could be attributed to the eukaryote-specific C-terminal extension, as it was proposed to be important for serine recognition [3].

Analogously to our results, indolmycin, pseudomonic acid, thialysine and borrelidin, naturally existing inhibitors of tryptophanyl-, isoleucyl-, lysyl- and threonyl-tRNA synthetases, respectively, were shown to preferentially inhibit only certain subtypes of their corresponding aminoacyl-tRNA synthetase targets [14–17]. In this context, evaluation of sensitivity to substrate analogues appears to be a valuable strategy for identifying differences between the evolutionary divergent synthetases, as well as a means of probing more subtle differences between related enzymes. Furthermore, it may also provide a more general outlook on the biological significance of duplicated aaRS genes, as discussed below.

4.2. Duplicated aaRSs

Available data do not suggest functional complementarity of the two *M. barkeri* enzymes, and it remains unclear why they coexist in this organism. As suggested, the duplicity of SerRSs in *M. barkeri* may represent an evolutionary interphase that precedes the loss of the redundant *serS* gene. On the other hand, additional functions pertaining to either of these enzymes cannot be excluded; in this respect it seems noteworthy that the bacterial SerRS is less stringent in recognition of $tRNA^{Ser}$, as indicated by our study [1]. Moreover, the two *serS* genes may be regulated differently, and expression profiles of these SerRSs may be revealing in terms of their functionality.

Furthermore, differential sensitivity of the two enzymes to the inhibitory effect of the serine analogues possibly indicates that the two dissimilar SerRS forms may provide resistance to a wider spectrum of potential natural or synthetic inhibitors, and hence be of great advantage to their host. It has also been suggested that the divergence of the LysRS forms prevents infiltration of non-canonical amino acids into the genetic code, and thus contributes to quality control of protein synthesis [18]. Considering that the duality of aaRS systems in *M. barkeri* includes both unrelated SerRS and LysRS enzymes, we may speculate that such diversity is maintained as a result of selective pressure. Interestingly, *M. barkeri* thrives in a variety of environments, such as anoxic freshwater mud or rumen of cattle [19]. As the intestinal system of grazing animals involves dramatic changes of concentrations of amino acids, their analogues and other potential inhibitors, it likely creates the turbulent and potentially detrimental surrounding to which essential enzymes, such as aaRSs, are challenged to adjust. In this respect, alternative SerRS and LysRS systems may be presumed to facilitate the adaptation of *M. barkeri* to the distinct ecological niches.

Lastly, existence in natural environments involves competition between co-inhabitants that have developed a diversity of targeted inhibition-resistance strategies. In view of that, it was reported that *Pseudomonas fluorescens* produces a specific inhibitor of the bacterial-type IleRS (pseudomonic acid) to kill

competing bacteria, whereas it avoids suicide by maintaining a second copy of IleRS that is of eukaryotic origin and resistant to pseudomonic acid [15]. Whether *M. barkeri* uses an analogous strategy with the SerRS system remains an attractive speculation; size and diversity of its genome certainly leave this option feasible.

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