

Amino Acid Substitution in *Trichophyton rubrum* Squalene Epoxidase Associated with Resistance to Terbinafine

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There has only been one clinically confirmed case of terbinafine resistance in dermatophytes, where six sequential *Trichophyton rubrum* isolates from the same patient were found to be resistant to terbinafine and cross-resistant to other squalene epoxidase (SE) inhibitors. Microsomal SE activity from these resistant isolates was insensitive to terbinafine, suggesting a target-based mechanism of resistance (B. Favre, M. Ghannoum, and N. S. Ryder, *Med. Mycol.* 42:525–529, 2004). In this study, we have characterized at the molecular level the cause of the resistant phenotype of these clinical isolates. Cloning and sequencing of the SE gene and cDNA from *T. rubrum* revealed the presence of an intron in the gene and an open reading frame encoding a protein of 489 residues, with an equivalent similarity (57%) to both yeast and mammalian SEs. The nucleotide sequences of SE from two terbinafine-susceptible strains were identical whereas those of terbinafine-resistant strains, serially isolated from the same patient, each contained the same single missense introducing the amino acid substitution L393F. Introduction of the corresponding substitution in the *Candida albicans* SE gene (L398F) and expression of this gene in *Saccharomyces cerevisiae* conferred a resistant phenotype to the transformants when compared to those expressing the wild-type sequence. Terbinafine resistance in these *T. rubrum* clinical isolates appears to be due to a single amino acid substitution in SE.

Dermatophytosis is a common infection of the keratinized tissues skin, hair, and nails caused by dermatophytes. Among the three known genera of dermatophytes, *Epidermophyton*, *Microsporum*, and *Trichophyton*, *Trichophyton* species, especially *T. mentagrophytes*, *T. tonsurans*, and *T. rubrum*, are the most common pathogens, with *T. rubrum* being the most prevalent isolated organism. *T. rubrum* is particularly involved in tinea pedis and tinea unguium (onychomycosis). While most superficial infections can be effectively cured with different topical agents with various mechanisms of action, tinea capitis and onychomycosis require the use of oral drugs such as fluconazole, itraconazole, griseofulvin, and terbinafine to be successfully treated. Nail infections require extended periods of therapy with at least 3 months of daily or intermittent dosing regimens.

Despite the high incidence of dermatophytosis and the difficult and long-term treatment of some of these infections, with associated uneven patient compliance, antifungal resistance in dermatophytes appears to be rare. This is in contrast with candidiasis and aspergillosis, where numerous isolates resistant to various antifungals have been identified and then characterized (1, 18, 24, 27, 29, 30). Systematic susceptibility testing of clinical isolates from patients with onychomycosis who failed on therapy with terbinafine did not reveal any correlation between the MIC of terbinafine against these isolates and clinical failure (20). Nevertheless, in one case terbinafine-resistant *T.*

rubrum was identified (20). All isolates collected from the same patient, including the baseline, were about 1,000-fold less susceptible than normal strains to terbinafine as well as other squalene epoxidase (SE) inhibitors such as naftifine or tolnaftate. However, they displayed normal susceptibility to antifungals acting via a different mechanism, such as the azoles, amorolfine, griseofulvin, and amphotericin B. This selective cross-resistance to squalene epoxidase inhibitors suggested a target-specific mechanism of resistance. This hypothesis was reinforced by the finding that microsomal SE activity from these resistant isolates was indeed insensitive to all selective SE inhibitors (8).

We report here (i) the cloning of *T. rubrum* SE from normal and terbinafine-resistant isolates, (ii) the identification of an amino acid substitution in the sequence from resistant strains, and (iii) the demonstration that expression in *Saccharomyces cerevisiae* of *Candida albicans* SE, containing the same amino acid substitution, results in a terbinafine-resistant phenotype.

MATERIALS AND METHODS

Strains and culture. Strains tested were from the Novartis Fungal Index (NFI) collection. *T. rubrum* strains NFI 1895 and NFI 5182 are reference strains. NFI 1895 is a clinical strain, and NFI 5182 corresponds to strain ATCC 18759. *T. rubrum* terbinafine-resistant isolates NFI 5146 to NFI 5151 were previously described (20). Stock inoculum, culture, and mycelium were prepared exactly as previously described (4). *S. cerevisiae* strain INVSc1 (*MAT α his3- Δ 1 leu2 trp1-289 ura3-52*) was purchased from Invitrogen and grown on yeast extract-peptone-dextrose medium (Difco Laboratories) or synthetic medium (28).

Antifungal testing. A microdilution assay using a 96-well plate (Greiner, Kremstunster, Austria) was used to test drug susceptibility of *S. cerevisiae*. Terbinafine was dissolved in dimethyl sulfoxide (DMSO; 12.8 mg/ml), serially diluted 2 \times in dimethyl sulfoxide, and then diluted 50 \times in synthetic complete medium without uracil and glucose but supplemented with 2% galactose. To 100 μ l of these dilutions distributed in a 96-well plate, 100 μ l of yeast culture in galactose synthetic medium, optically adjusted to 2 \times 10⁴ CFU/ml, was added. Plates were incubated at 30°C for 2 days. Cells were resuspended, and optical density read at 620 nm with a microtiter plate reader. 50% MIC and 90% MIC

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corresponded to the lowest terbinafine concentrations leading to a reduction of growth of $\geq 50\%$ and $\geq 90\%$, respectively, in comparison with solvent controls. For plates, terbinafine, 100 \times concentrated in DMSO, was added in molten 2% agar galactose synthetic medium.

Cloning and sequencing of *T. rubrum* squalene epoxidase. Genomic DNA and total RNA were isolated from frozen *T. rubrum* mycelium using the DNeasy plant minikit (QIAGEN, Valencia, CA) and SV total RNA isolation system (Promega, Madison, WI) as per the supplied protocol. Initially, a fragment of the *T. rubrum* SE gene was amplified by PCR with *Taq* polymerase (Invitrogen, Carlsbad, CA) from genomic DNA using the degenerate primers, 5'-CCWGA YCGIATYGTGGTGARCTYATGCAACC and 5'-ACRGTCATACCACCA CCRGTRAGWGGRTG (W = A/T, Y = C/T, R = A/G), with an annealing temperature of 44°C, cloned into the vector pCR2.1 (Invitrogen), and sequenced with BigDye terminator kit (Applied Biosystems, Foster City, CA). Subsequently the 5' and 3' ends were amplified from total RNA by rapid amplification of cDNA ends with the GeneRacer kit (Invitrogen) according to the manufacturer's instructions and using the specific primers 5'-TCTGAGGAAGGAATTTGGC ATTGACCGAAA and 5'-AACGCAGCTTCAAATGAGGGCCGAATA for the 5' end and 5'-CTCTGAACAACCTCGGCTGAGAGATT and 5'-ATAG ATGCCGTGAGGACATATGGATA for the 3' end at an annealing temperature of 50°C during PCR. Fragments were cloned and sequenced. A genomic fragment covering the whole coding region of the SE gene was then amplified with the primers 5'-CCTCTAGACCCCATCAATAAGTTACTACAATG and 5'-CCTCTAGAGAGTTAGAGATAAGCCTATCTGCCTA, cloned, and sequenced as well.

Constructs with *C. albicans* SE gene. All constructs were derived from the one described previously (6). Point mutations were introduced with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Nucleotides were numbered from the translation start codon ¹ATG corresponding to nucleotide (nt) 1290 in U69674 (GenBank).

Transformation of *S. cerevisiae*. The INVSc1 strain was transformed with the various constructs in pYES2 with EasyComp transformation kit (Invitrogen). Transformants were selected on glucose synthetic medium. Expression of *C. albicans* SE cloned in pYES2 was induced by transferring isolated colonies either on agar or in liquid medium consisting of galactose synthetic medium.

Western blot analysis. Protein extracts were prepared using the trichloroacetic acid method (13). Western blot analysis using polyclonal anti-SE peptide antibodies was performed as described previously (6).

Nucleotide sequence accession numbers. The complete sequence data for the *T. rubrum* SE gene open reading frame (ORF) has been assigned GenBank accession number AY282411. The complete sequence data for the SE genes of strains NFI 5146 and NFI 5150 have been assigned GenBank accession number AY282412.

RESULTS

Cloning of *T. rubrum* SE. Initially, a fragment of the *T. rubrum* SE gene was amplified by PCR from NFI 1895 genomic DNA using degenerate primers designed from two stretches of amino acids, which are well conserved among yeast and mammalian SEs: PDRI(V/L)GE(L/C)(M/L)QP and RHPLTGG GMTV (6). The resultant amplicon was cloned and sequenced, and the deduced protein sequence was homologous to yeast and mammalian SEs, indicating that it was indeed a fragment of SE gene. Subsequently, the 5' and 3' ends were amplified from total RNA by rapid amplification of cDNA ends. Assembly of the overlapping sequences revealed an ORF coding for 489 residues, as confirmed by the reverse transcription-PCR (RT-PCR) of the full ORF with primers annealing outside the coding sequence. Comparison of *T. rubrum* SE protein sequence with those of *S. cerevisiae*, *C. albicans*, *Schizosaccharomyces pombe*, *Neurospora crassa*, and humans showed it to be 43%, 40%, 44%, 50%, and 41% identical, respectively. The alignment of these proteins is presented in Fig. 1. It shows that the mammalian SE has an NH₂-terminal extension absent in fungal proteins and that SEs from *S. cerevisiae*, *C. albicans* and *N. crassa* have a stretch of 32 to 34 residues missing in *S. pombe*, *T. rubrum*, and *Homo sapiens* proteins. All of them

contain the three consensus sequences considered as the hallmark of flavoprotein hydroxylases (framed in Fig. 1) (2).

Amplification of SE gene directly from genomic DNA revealed the presence of an intron of 62 nt in between nt 1200 and 1201 (Gly400 and Asp401) of the ORF. The donor and acceptor splicing sites contained the canonical dinucleotides GT and AG, respectively (19).

The SE sequences from related resistant strains contain one amino acid substitution. Having cloned SE cDNA and gene from one *T. rubrum* strain, it was possible to amplify the gene from other strains. First we sequenced the SE gene from a second terbinafine-susceptible strain, NFI 5182, and found its nucleotide sequence to be identical to that of NFI 1895. In contrast, the SE sequences from the terbinafine-resistant strains NFI 5146 and NFI 5150 (which were sequentially isolated from the same patient) contained one mismatch, ¹¹⁷⁷TTA→TTT, in comparison with the NFI 1895 (and NFI 5182) sequence, leading to the amino acid substitution L393F. This result suggests that this identified amino acid substitution in *T. rubrum* SE could negatively affect the affinity of the enzyme for the antifungal agent and consequently the susceptibility of the whole organism to the drug.

The corresponding amino acid substitution in *C. albicans* SE negatively affects the potency of terbinafine against *S. cerevisiae* expressing the altered protein. Our initial attempts to express *T. rubrum* SE in *S. cerevisiae* were unsuccessful. Therefore, to further investigate the impact of amino acid substitutions in SE on the susceptibility of a whole organism expressing this altered protein, we used the *C. albicans* protein as a model SE and *S. cerevisiae* as a recipient organism. We had previously cloned *C. albicans* SE and shown that it could be overexpressed in an active form in *S. cerevisiae* (6). We first replaced the triplet ⁵¹⁴CTG in the *C. albicans* SE gene, coding for a Ser residue in *C. albicans* but for a Leu in *S. cerevisiae* (23) by TCT, a canonical Ser codon. Then, in this modified gene, we introduced the single point mutation leading to the amino acid substitution L398F, corresponding to the alterations L393F identified in *T. rubrum* SEs from resistant isolates (Fig. 1). Finally, we transformed the *S. cerevisiae* INVSc1 strain with these constructs made in the galactose-inducible expression vector pYES2, selected the transformants based on uracil auxotrophy complementation by the vector, and induced the expression of the cloned gene by replacing glucose by galactose in the medium. Western blot analysis of the expression of each SE protein indicated that the expression levels were similar in all transformants irrespective of the amino acid substitution introduced in the sequence (Fig. 2). Susceptibility of the transformants to terbinafine was tested on agar plates and in liquid medium. *S. cerevisiae* strain INVSc1 transformed with pYES2 was poorly susceptible to terbinafine on agar galactose synthetic medium, and only a concentration of 128 μ g/ml was able to prevent almost any growth. Transformation of INVSc1 with the constructs pYES2-CaSE-S172L (corresponding to wild-type *C. albicans* sequence) or pYES2-CaSE (corresponding to the corrected *C. albicans* sequence) reduced the susceptibility of cells to terbinafine, indicating that overexpression of SE was sufficient to confer a semiresistant phenotype to this strain when grown on agar medium. Overexpression of CaSE-L398F in INVSc1 conferred complete resistance to 128 μ g/ml terbinafine (data not shown).

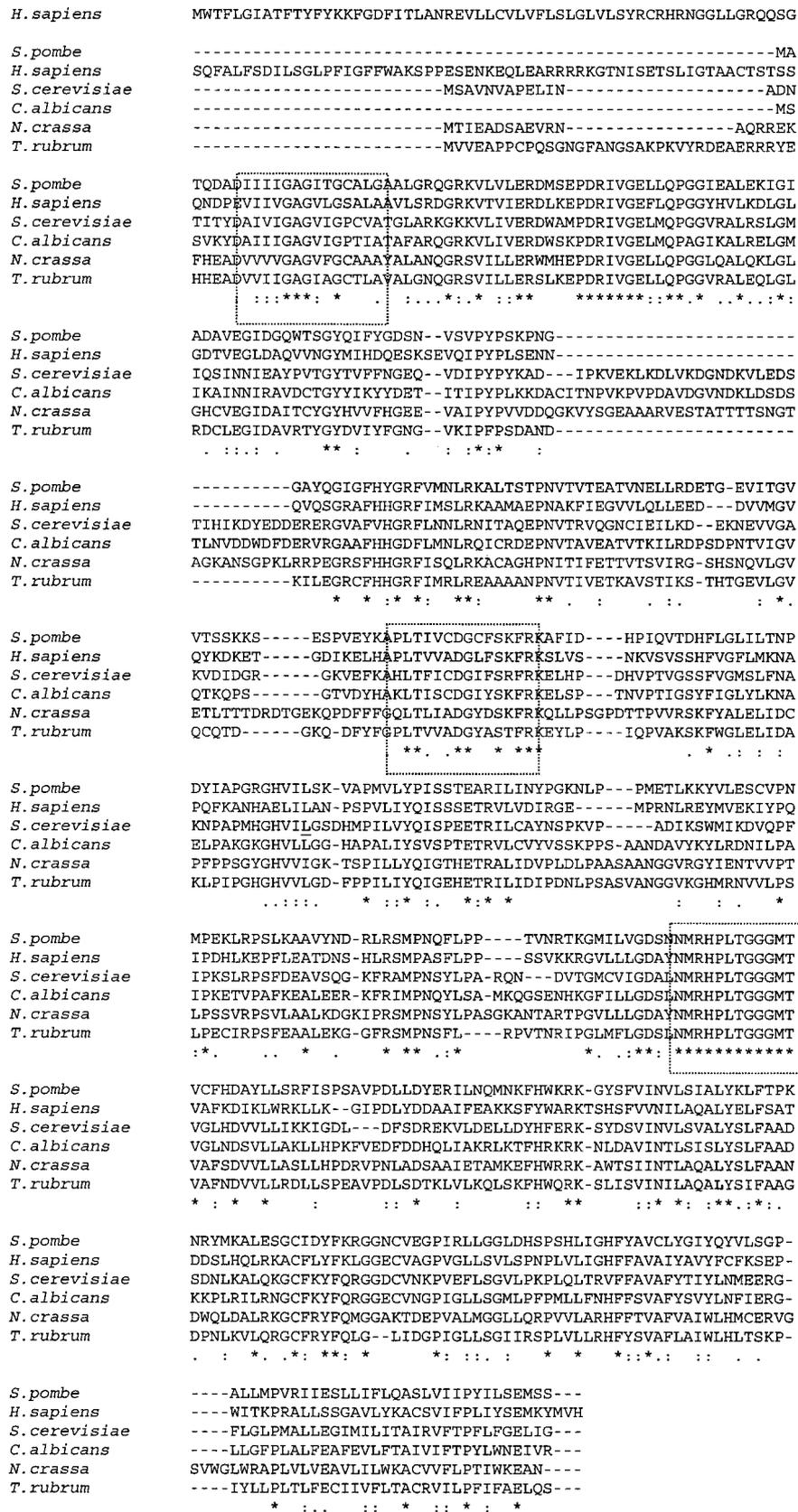


FIG. 1. Alignment of the SE sequences from *S. cerevisiae*, *C. albicans*, *Schizosaccharomyces pombe*, *Neurospora crassa*, and humans. The three consensus sequences considered as the hallmark of flavoprotein hydroxylases have been framed. The position of the amino acid substitution L393F in the terbinafine-resistant *T. rubrum* is shown in boldface.

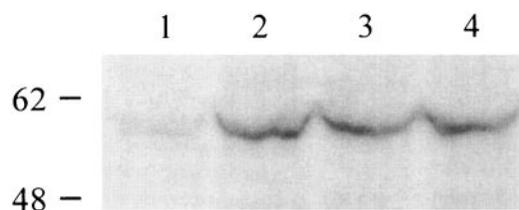


FIG. 2. Expression of *C. albicans* SE in *S. cerevisiae*. Western blot analysis of whole-cell trichloroacetic acid extracts from *S. cerevisiae* transformed with the vector pYES2 (lane 1) or the pYES2 constructs consisting of CaSE-S172L (lane 2), CaSE (Ser¹⁷²) (lane 3), and CaSE-L398F (lane 4). Transformants (2×10^4 CFU/ml) were cultured in synthetic medium containing 2% galactose for 2 days at 30°C. Proteins were size fractionated on a sodium dodecyl sulfate–8% polyacrylamide gel, transferred onto an Immobilon-P membrane, and analyzed with rabbit polyclonal anti-SE peptide antibody (6). The positions of the size markers (kDa) are indicated on the left. The faint band seen in lane 1 probably corresponds to endogenous SE.

In liquid galactose synthetic medium, INVSc1 transformed with pYES2 was much more susceptible to terbinafine than on agar medium (Table 1) and the susceptibility patterns resulting from overexpressing various *C. albicans* SEs (CaSEs) were more differentiated than those observed on agar medium. Overexpression of CaSE (or CaSE-S172L) did not affect MIC of terbinafine, whereas expression of the mutated CaSE-L398F strongly decreased the susceptibility of the transformants (Table 1), demonstrating that this point mutation in SE could confer a terbinafine-resistant phenotype to a microorganism.

DISCUSSION

In this study we have cloned *T. rubrum* SE and identified an amino acid substitution in SE from terbinafine-resistant isolates. Introduction of the corresponding amino acid substitution in *C. albicans* SE and expression of this protein in *S. cerevisiae* revealed that this point mutation indeed conferred a resistant phenotype to the transformants. These results suggest that this residue is part of the allylamine binding site on the enzyme.

Dermatophytes are the major clinical target of terbinafine. *T. rubrum* SE had been characterized biochemically (5) but not at the molecular level. Cloning of *T. rubrum* SE reveals that the deduced protein sequence is most similar with SE from the

filamentous ascomycete *N. crassa*. However, a stretch of about 30 extra residues found specifically in most fungal SEs except *S. pombe*, as well as mammalian SEs, is also absent in the *T. rubrum* enzyme. The role of this domain, which obviously is not essential for the enzymatic activity or related to the specificity of antifungal SE inhibitors, is unknown. Nevertheless, we wonder if the absence of this domain in *T. rubrum* SE is responsible for our unsuccessful attempts to express the protein in *S. cerevisiae*. In agreement with this hypothesis, mammalian SE was elegantly cloned by complementation of fungal SE, which was selectively inhibited by terbinafine in *S. pombe* but not in *S. cerevisiae* (26). A second unsolved enigma concerning the primary structure of SE is the function of the N-terminal extension of mammalian SEs, which is not required for their enzymatic activity (21).

The first mutation reported to confer resistance to terbinafine was identified in *S. cerevisiae* SE-L251F (10). Leber et al. (14) observed a significant resistant phenotype in *S. cerevisiae* expressing the L251F mutated protein. We did not observe such a dramatic effect when the homologous mutation was introduced in CaSE (C. S. Osborne, B. Favre, and N. S. Ryder, unpublished data).

Sequencing of SE from *T. rubrum* isolates resistant to terbinafine identified a single amino acid substitution L393F towards the carboxyl end, exactly in the same region in *S. cerevisiae* SE where several point mutations were found to confer resistance to terbinafine (12, 14). These results strongly suggest that this region bears the high-affinity binding site for terbinafine on fungal SEs. This domain is located downstream from the highly conserved stretch of residues present in all SEs, which is considered to be the substrate binding site. This hypothesis is strengthened by the recent labeling of the protein with a competitive SE inhibitor (16), which in turn built on the work of previous studies in this area (15). The competitive SE inhibitor was covalently linked to a peptide situated just upstream to this highly conserved stretch of amino acids (16).

The relationship between the terbinafine and substrate binding sites is less clear. Terbinafine inhibits fungal SEs in a noncompetitive manner while being a competitive inhibitor of mammalian SEs at much higher concentrations (25). However, biochemical properties of terbinafine analogs are complex, and some results suggest the presence of more than one binding site on SEs, perhaps because of a multimeric tertiary structure of the protein (7).

Owing to the high selectivity of terbinafine for fungal SEs versus mammalian ones it is very surprising, as already noted by Leber et al. (14), that all identified crucial residues for the high sensitivity of fungal SEs to terbinafine are conserved in mammalian SEs. The same paradox had previously been observed for another major target of antifungals, the enzyme lanosterol 14 α -demethylase, which is selectively inhibited by azole, such as fluconazole, itraconazole, and ketoconazole. Most of the residues involved for the binding of fluconazole on lanosterol 14 α -demethylase are conserved in the mammalian enzymes (3, 17). Therefore, it appears that only subtle structural differences both in SE and lanosterol 14 α -demethylase govern the specificity of chemical compounds to these proteins. The present paradigm in antimicrobial research, the need to target enzymes unique to a particular kingdom to find specific compounds, is challenged by the two major classes of antifun-

TABLE 1. Susceptibility to terbinafine of *S. cerevisiae* strain INVSc1 transformed with three different variants of the *C. albicans* SE gene cloned into the galactose-inducible expression vector pYES2^a

Construct	Nucleotide mutation	Amino acid mutation	50% MIC	90% MIC
None			1	8
pYES2			0.5	4–8
CaSE	None (wt seq ^b)	S172L	1	8
CaSE	⁵¹⁴ CTG→TCT	None	1	8
CaSE	¹¹⁹¹ TTA→TTC	L398F	>128	>128

^a 50% MIC and 90% MIC correspond to the lowest terbinafine concentrations (μ g/ml) leading to reductions of growth of $\geq 50\%$ and $\geq 90\%$, respectively, in comparison with controls.

^b wt seq, wild-type sequence.

gals, the allylamines and azoles. Determination of the three-dimensional structure of SEs should finally explain the molecular specificity of antifungal SE inhibitors.

The low incidence of terbinafine resistance in *T. rubrum* observed both in vitro ($\leq 5 \times 10^{-9}$) (22) and in vivo (9) is in agreement with the mode of resistance identified in this study, single nonsilent nucleotide substitution in a protein-encoding gene, which is a rare event in all living organisms (11). Kasuga et al. (11) estimated the nonsynonymous nucleotide substitution rates in *Eurotiomycetes* at 1×10^{-9} to 1×10^{-10} per site and per year in protein-coding genes (about 20 times smaller than silent nucleotide substitution rates). Nevertheless, the overall natural resistance incidence will be influenced by the number of SE mutations which can confer insensitivity to terbinafine, the biological cost of SE mutation(s), selection pressure, and population size (11).

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