

Molecular Analysis of the *Trichosporon cutaneum* DSM 70698 *argA* Gene and Its Use for DNA-Mediated Transformations

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Genomic clones capable of complementing a previously isolated arginine auxotrophic mutant strain of the filamentous yeast *Trichosporon cutaneum* DSM 70698 have been identified by DNA-mediated transformation, and a complementing 4,082-bp subfragment was sequenced. This analysis revealed an intact gene (*argA*) showing a high degree of homology with the *Saccharomyces cerevisiae* CPA2 gene encoding the large subunit of carbamoyl-phosphate synthetase (CPS-A). The inferred amino acid sequence of the *T. cutaneum argA*-encoded protein contains 1,168 residues showing 62% identity with the sequence of the *S. cerevisiae* CPA2 protein, and the comparison of the two sequences uncovered a putative intron sequence of 81 nucleotides close to the 5' end of the coding region of the *T. cutaneum argA* gene. The presence of this intron was confirmed by nuclease protection studies and by direct DNA sequence analysis of a cDNA fragment which had been obtained by PCR amplification. The *T. cutaneum* intron shares the general characteristics of introns found in yeasts and filamentous fungi. A major transcript of around 4 kb was found in Northern (RNA) blots. The *T. cutaneum argA* coding region was expressed in *Escherichia coli* under the control of the regulatable *tac* promoter. A roughly 130-kDa protein which was found to cross-react with an anti-rat CPS antibody in Western blots (immunoblots) was observed. Two putative ATP-binding domains were identified, one in the amino-terminal half of the *argA*-encoded protein and the other in the carboxy-terminal half. These domains are highly conserved among the known CPS-A sequences from *S. cerevisiae*, *E. coli*, and the rat. From these results we conclude that the *T. cutaneum argA* gene encodes the large subunit of CPS. This is the first gene to be identified and analyzed in the *T. cutaneum* DSM 70698 strain.

Trichosporon cutaneum belongs to genus *Trichosporon* Behrend, which was first described by Behrend in 1890 (3) and which is composed of a heterogeneous group of basidiomycetous yeasts containing organisms that differ from each other in a number of morphological, physiological, and genetic characteristics (25, 27, 28, 45). Since no sexual reproduction is known in *Trichosporon* yeasts, this genus belongs to the Fungi Imperfecti. The phenotypic and DNA base composition analysis of 28 species of yeasts classified in the genus *Trichosporon* Behrend revealed two major groups (27). The first group, with 13 species, harbors yeasts which are unable to hydrolyze urea, with *Trichosporon margaritifera* being an exception. All species of this group have a G+C content between 34.7 and 48.8 mol% and appear to be related to the ascomycetous yeasts. The second group, containing 15 species, has a G+C content between 56.9 and 64.2 mol% and produces urease. The high G+C content and urease activity are typical for basidiomycetous yeasts. With G+C contents of 59 and 63.5 mol%, respectively, *Trichosporon pullulans* and *T. cutaneum* (one of the synonyms of *Trichosporon beigelii*) belong to the basidiomycetous yeasts. Furthermore, the diazonium blue B test (83) has been applied to a number of *T. beigelii* strains (39). The positive test results which were obtained are in agreement with the described basidiomycetous affinity of these strains. *Trichosporon* yeasts have been isolated from a number of sources including soil, industrial wastewater, wood pulp, sludge, and clinical specimens, and *T. beigelii* has been found to be the causative agent of white piedra, which is a relatively inconse-

quential infection of the hair. However, this organism does not appear to be part of the skin flora in healthy subjects. Certain strains have also been found as opportunistic pathogens causing deep-seated and disseminated infections in immunocompromised patients (reviewed in references 32 and 89a). Previous findings suggest that strains that cause invasive disease are distinct from the superficial and nonclinical isolates and that isolates from the skin and mucosae represent a number of different organisms and include some environmental forms (39). The same study also revealed that *T. beigelii* is a complex of genetically distinct organisms and that more than one type is found in clinical samples. Only recently the genus *Trichosporon* was revised once more on the basis of a number of characteristics such as morphology, ultrastructure of septal pores, coenzyme Q system, G+C content of DNA, DNA-DNA reassociation, and 26S rRNA partial sequences (24, 26). This work separates the *Trichosporon* isolates from soil into three species: *T. pullulans*, a psychrophilic species which is clearly different from all other *Trichosporon* species; *T. dulcitum*, a mesophilic species; and *T. moniliiforme*. *T. cutaneum* seems to be a rare species never isolated from soil (26). A preliminary inspection of the *T. cutaneum* ATCC 46490 and DSM 70698 strains using culture studies and biochemical characteristics classifies them into the *T. moniliiforme* group (23, 26).

Trichosporon yeasts have the potential to use a very large variety of carbon sources (41) and have been shown to grow on various monosaccharides including pentoses and hexoses, on disaccharides, and on a number of polysaccharides. These yeasts have also been shown to degrade and utilize cyclohexane, lipids, uric acid, and other purine derivatives (reviewed in references 65 and 66). Furthermore, *T. cutaneum* and *T. beigelii* are capable of using various aromatic compounds as sole carbon and energy sources (reviewed in references 8 and 53). These properties indicate the extraordinary potential of

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Source or reference(s)
<i>E. coli</i> strains		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Bethesda Research Laboratories
XL-1 Blue	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 Lac⁻ F' [proAB⁺ lacI^q lacZΔM15 Tn10(Tet^r)]</i>	6
carB8	<i>thr31 carB8 relA1 metB1</i>	49
Yeast strains		
<i>T. cutaneum</i> DSM 70698		36, 37
<i>T. cutaneum</i> DSM 70698 arg70	Arginine auxotrophic	59
<i>T. cutaneum</i> ATCC 46490		85
<i>T. cutaneum</i> ATCC 58094		76
<i>T. cutaneum</i> ATCC 20509		93
<i>T. cutaneum</i> ATCC 62975		86
<i>T. beigelii</i> CBS 5790		95
<i>T. pullulans</i> ATCC 10677		13
<i>Cryptococcus elinovii</i>		51
<i>Candida tropicalis</i> ATCC 750		29
Plasmids		
pBluescript SK+	<i>E. coli</i> cloning vector	72
pTrc99A	<i>E. coli</i> expression vector	1
p1.1	7.7-kb <i>T. cutaneum</i> DNA fragment containing <i>argA</i> in pBluescript SK+	This study
pCFII	Contains <i>A. nidulans argB</i>	84, 90
pTrc-D	pTrc99A containing <i>argA</i> lacking the intron sequence	This study
pTrc-E	pTrc99A containing <i>argA</i> with the intron sequence present	This study

these organisms for the efficient conversion of various cheap carbon sources into biomass. Such an application has indeed been feasible, e.g., by converting whey into microbial lipids using *T. beigelii* (78).

The abilities to clone specific gene sequences and to transform them back into the organism are prerequisites for exploring the biotechnological potential of this group of yeasts, and it may also be useful for investigating the pathogenic behavior of some of the *Trichosporon* strains. With these goals in mind we have succeeded in setting up a genetic transformation procedure based on dominant selection markers (19, 20, 66). The transforming DNA was found to consist of multiple tandem plasmid copies of high molecular weight. However, this polymeric structure, in nonselective medium, was mitotically unstable, indicating that it existed in an episomal state.

A transformation system for *T. cutaneum* based on auxotrophic markers, and techniques for the induction, isolation, and characterization of mutants have been described by Ochsner et al. (59) and by Reiser et al. (66). A number of auxotrophic mutants were isolated and characterized by using biosynthetic precursors and/or inhibitors. A mutant unable to grow in the presence of ornithine could be complemented successfully in protoplast transformation experiments using the cloned *Aspergillus nidulans* ornithine carbamoyltransferase (OCTase) (*argB*) gene. In these transformants, the heterologous *argB* gene was also present in multiple tandem copies, but the transforming DNA was found to remain stable after more than

50 generations in nonselective medium. The same mutant strain could be complemented by a *T. cutaneum* cosmid library, and a complementing cosmid was subsequently isolated from this library by a sib selection strategy. This cosmid transformed *T. cutaneum* protoplasts with an efficiency of 50 to 200 colonies per μ g of DNA, and the transforming sequences became integrated in the host genome at the homologous site.

On the basis of this cosmid clone we have isolated a number of complementing subclones, and we describe in this article the isolation and molecular characterization of a complementing genomic sequence from *T. cutaneum* DSM 70698 and the use of this DNA for transformation studies.

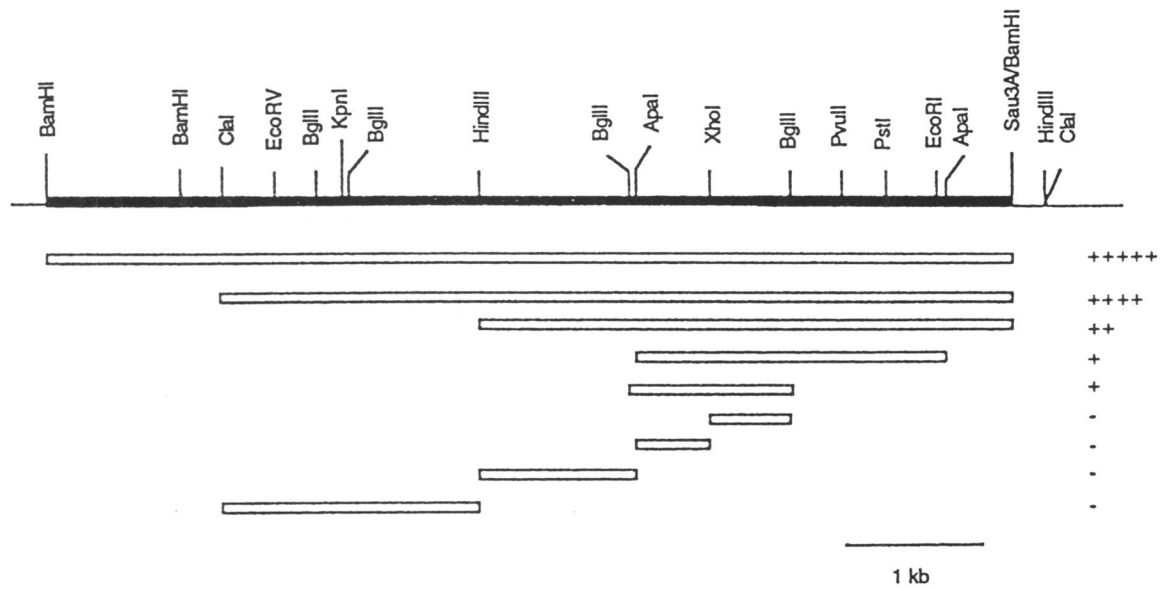
MATERIALS AND METHODS

Chemicals, radiochemicals, and enzymes. All chemicals were commercial preparations, of reagent or sequencing grade whenever possible. [α -thio-³⁵S]dATP (1,000 Ci mmol⁻¹), [α -³²P]dCTP (3,000 Ci mmol⁻¹), and [α -³²P]UTP (800 Ci mmol⁻¹) were from Amersham.

Strains, media, and culture conditions. The bacterial and yeast strains and plasmids used in this study are listed in Table 1. The bacterial strains were grown in Luria-Bertani medium or 2 \times YT medium (48) at 37°C, and the yeast strains were cultivated in YEPD medium (1% yeast extract, 2% peptone, and 2% glucose) or in minimal medium D containing 1%

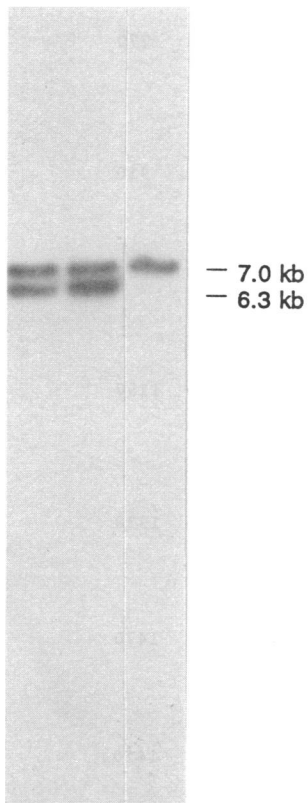
FIG. 1. Transformation of the *T. cutaneum* DSM 70698 arg70 mutant strain. (A) Identification of the minimal functional unit capable of complementing the *T. cutaneum* DSM 70698 arg70 mutant strain. Relative transformation efficiencies are indicated on the right. +++++, about 1,000 transformants per μ g of plasmid DNA; -, 0 transformants. Only the restriction enzyme cleavage sites relevant for the complementation study are shown. A more complete restriction map is presented in Fig. 2A. Thin lines, pBluescript SK+ vector sequences. (B) Southern blot analysis of chromosomal DNA from two independent transformants (lanes 1 and 2) and from the untransformed *T. cutaneum* strain (lane 3) digested with *Pvu*II. (C) Description of events leading to the hybridization patterns in the Southern blot analysis. A 1.86-kb *Cl*a1-*H*indIII fragment was used as a hybridization probe. The locations of the *Pvu*II (P) fragments hybridizing to the probe are indicated.

(A)

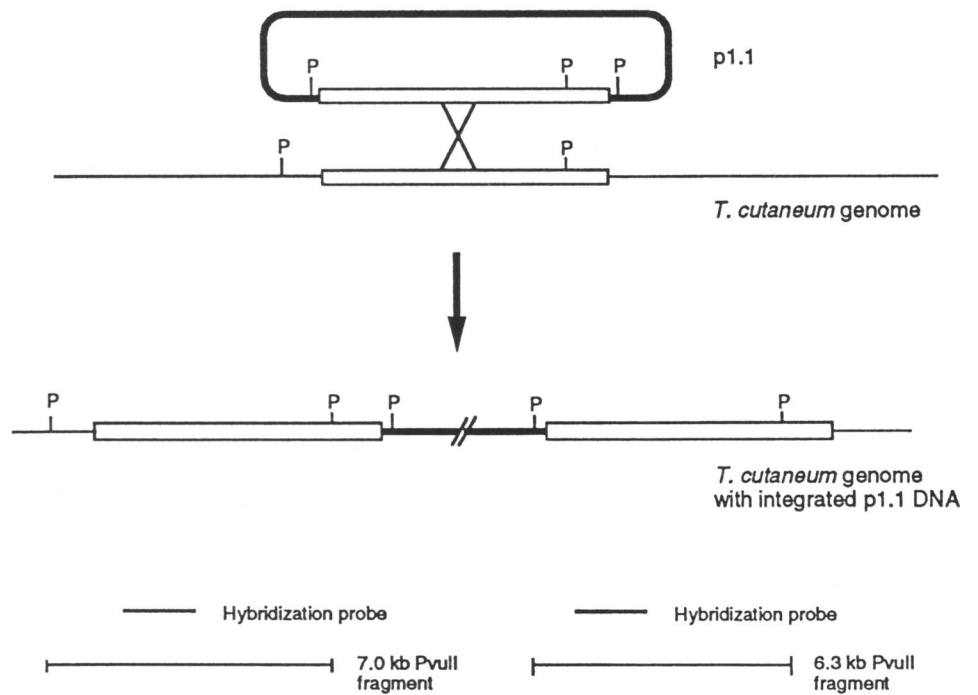


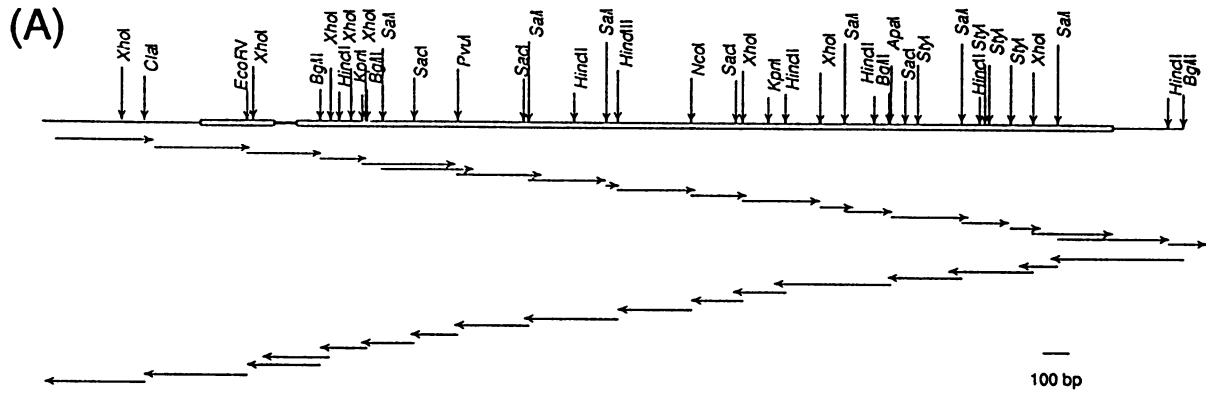
(B)

1 2 3



(C)





(B)

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                                BamHI
                                |
CTCTGGCGCCGATCTGGTCCGATGTCCCAAAATCAAAAATCTCTGCACCAAGGGGATCCAGATTGGCGC      70
GAGATTATCCCGTCGTCCACACGATCAAGCGAAGGCTTGCCAGGCGTTTGACTCGACAAGATCCGGAGT
AAGGTGCTCGCTAGACAAAAGTCTTTTCAGGGTTTTTGGGTATAAGCAATGCAAGGCGGCGAATGGCTCC      210
AGCCTTGTGCCAAGCTGTAGCCCCACAGTGGCATGTAGATGCGCGGCCGAAAGGTGGTTAGTCACTCCGG
TCCGAAAACCCCTTAGGTTGAACAAGTCCACGTAATAAATTTCCGCCAATTCCAACCGAGTCGCGATCTCG      350
AGAGTCGGTATCGACAGGCCAGGAGGTTGGGATGGATTTTTCGAATGAATCGATAAAGAAACAACCACAA
ACACCTTTCAACACCGACCGCTCGGCAACCTCTAACACACCAGAGGCTATCCCACTCGTCTCGCCGGC      490
CTGGCATCAACCCACTCCGGCCACTCATCGCCCTCACCCCTCGCGTTTCCGACCGACGTTTAGTTGGAGG
CCCACAAAACTTAAAAAACCCCGGCTCAAGCCCGCGACACACCCAGCATAACCGCCATGCTCCGTTCAT
                                M L R S I
CTCGATTGCCCTCGCGCGCTGCGGCCCCGTCGCCCAAGTTGCTAGGGCCAGGACCGCTGTGGGTGTCGTC
S I A S R A A G P V R P V A R A R T A V G V V
GCGCCGCGGGTATGCTGTGCGCTCCGGCGGTCCGGCAACTACGGGCAGGTGGACGGTAAGGCGACTC      770
A P P R V C C C A P A V G N Y G Q V D G K A T L
                                EcoRV
TTAACTCGCTTCGGAGATGCCCCGCGGATATCGGCCAGGTCTGCCCCAGCTCGAGAAGCCCCGACGT
N S L R R L P A G Y R P R S C P S S R S P D V
CAAGAAGGTCCTTGTGTCGGCTCGGGTGGTCTCTCTATCGGTGAGGCTGGAGAGTTCGACTACTCTGGT      910
K K V L V V G S G G L S I G Q A G E P D Y S G
GAGTTTATTTGCCAGAAATTTCCCTGGTGGAGGGGCAAGATAGGGGAGATAAGAGAGAAAGAACTGAC
AGGTCACAGGTCGCGAGGCCATCAAGGCTCTCCGTGAGAGCAACATTGAGACTATTCTCATCAACCCCAA      1050
S Q A I K A L R E S N I E T I L I N P N
CATTCGCACCATCCAGACCTCGCACCACTCGCCAGCGAGATCTACTTCTTCCCGTCACCGCCGACTAT
I A T I Q T S H H L A S E I Y F L P V T A D Y
GTCGCTACGTCTCGAGAAGGAGCGCCCGGACGGTATCTGTTGACCTTTGGTGGTCACTCGGCCCTCA      1190
V A Y V L E K E R P D G I L L T F G G Q S A L N
                                KpnI
ACGTCGGTATCCAGCTCGAGAAGATGGGTGTCCTTGAGCGCCTCGGTGCCAGGTCCTTGGTACCCCTAT
V G I Q L E K M G V L E R L G V Q V L G T P I
CCGACCCCTCGAGATCTCGGAGGACCGGACCTCTTCGTCCAGGCTCTTAAAGAGATCGACATCCCTGCC      1330
R T L E I S E D R D L F V Q A L N E I D I P A
GCCAGTCGACTGCGGTCTCGACCATCCAGGACGCCCTCGACGCTGCCAAGACCATCGGCTACCCCATCA
A Q S T A V S T I Q D A L D A A K T I G Y P I I
TTCTCCGTTCCGCTCTCCCTCGGTGGTCTCGGCTCGTTCGCCGACGACGAGGAGGCTCCGCAACCT      1470
L R S A F S L G L G S F P H D E E E L R N L
CGCTGCCAAGTCGCTCTCGCTCTCTCCCGGTCCTCATGAGAAGTCGCTCAAGGCTGGAAGGAGGTG
A A K S L S L S P Q V L I E K S L K G W K E V
GAGTACGAGGTCGTCCGTGACGCGCGGACCAACACCATCAATTTGCTGCAACATGGAGAAGTTGACCCTC      1610
E Y E V V R D A A D N T I I C C N M E N F D P L
                                PvuI
TCGGCACACACTGGTGACTCGATCGTCTGCGCCCTCGCAGACCCTCACCGACGAGTACCACATGCT
G T H T G D S I V V A P S Q T L T D E Y H M L

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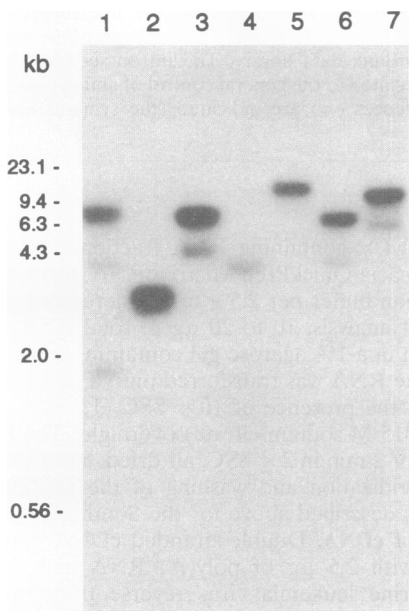



FIG. 4. Relationship of *argA*-related sequences of different *Trichosporon* strains. Chromosomal DNAs (10 μ g) of seven *Trichosporon* strains were digested with *Pvu*II and subjected to a Southern blot analysis. Lanes: 1, *T. cutaneum* ATCC 62975; 2, *T. cutaneum* ATCC 20509; 3, *T. cutaneum* DSM 70698; 4, *T. pullulans* ATCC 10677; 5, *T. beigeli* CBS 5790; 6, *T. cutaneum* ATCC 58094; 7, *T. cutaneum* ATCC 46490. A 32 P-labeled 1.86-kb *Cl*I-*H*indIII fragment derived from the 5' end of the *T. cutaneum* DSM 70698 *argA* gene was used as a hybridization probe (Fig. 2A). The blot was washed twice for 5 min each time in $2\times$ SSC at room temperature, twice for 30 min each time in $2\times$ SSC-1% SDS at 65°C, and twice for 30 min each time in $0.1\times$ SSC at room temperature. Note that there are no *Pvu*II sites within the probe fragment used.

CGTCAAGAAGGTCCTT (primer A) and 5' CGGTGAGG GTCTGCGAGGGCGC (primer B) were used.

RNA mapping of RNA. 32 P-labeled RNA probes were prepared by using T3 or T7 RNA polymerase and [32 P]UTP essentially as described by Sambrook et al. (70) and Krieg (40), except that the final UTP concentration in the reaction was 10 μ M. Hybridizations were carried overnight at 52°C by using 5 to 10 μ g of total RNA in 30 μ l of 80% formamide hybridization buffer (FAHB) (70). RNase ONE (Promega, Madison, Wis.) was used for digestion at 30°C for 60 min (68). Five units of enzyme in 300 μ l of RNase digestion buffer (10 mM Tris-HCl [pH 7.5]-5 mM EDTA [pH 8.0]-200 mM Na-acetate [pH 8.0]) was used. To stop the reaction, 20 μ l of a mixture containing 2% SDS and 1 mg of *E. coli* tRNA per ml was added, and the samples were precipitated with ethanol. The protected RNA hybrids were analyzed on a 6.5% polyacrylamide-8 M urea gel.

SDS-PAGE and Western blot (immunoblot) analysis. Bacteria were cultivated in $2\times$ YT medium at 37°C for 4.5 h and induced by adding 2 mM IPTG (isopropyl- β -D-thiogalactopyranoside). The cells were harvested 18 h after induction and heated for 5 min at 80°C in an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (43). Proteins were separated by SDS-PAGE (54) and transferred electrophoretically onto a nitrocellulose filter (Schleicher and Schuell) by using 15 mM Na-phosphate buffer, pH 6.8, as described by Reiser and Stark (67). After the transfer, the filters were blocked in Tris-buffered saline (20 mM Tris-HCl

[pH 7.5]-150 mM NaCl) containing 5% Bacto skim milk (Difco) for 1 h at room temperature. The primary rabbit anti-rat carbamoyl-phosphate synthetase (CPS) antibody was diluted 1:200 into Tris-buffered saline containing 5% Bacto skim milk and 0.05% Tween 20. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) diluted 1:1,000 in the above buffer was used as the secondary antibody. In situ peroxidase activity was detected by using the conditions of Hawkes et al. (30).

Nucleotide sequence accession number. The nucleotide sequence reported in this article has been entered in the GenBank data library and assigned accession no. L08965.

RESULTS

Isolation of the *T. cutaneum* DSM 70698 *argA* gene by genetic complementation. We have previously described a 39-kb cosmid clone capable of complementing a *T. cutaneum* DSM 70698 arginine auxotrophic mutant strain (*arg70* strain) (59). With a view toward defining the minimal complementing unit, the initial cosmid was partially digested with *Sau*3A to produce shorter fragments which were still able to complement the *arg70* mutant strain. After three cycles of sib selection, a plasmid containing a 7.7-kb *Bam*HI-*Sau*3A subfragment, which will be referred to here as plasmid p1.1, was found to give good complementation, and cloned restriction fragments derived from this subfragment were subsequently tested for their capacity to complement the mutant strain (Fig. 1A). The shortest complementing region was found to be contained in a 1.1-kb *Apa*I-*Bgl*II fragment. The transformation efficiency with this fragment was on the order of 0.1 to 0.2% of that achieved with p1.1. The fate of the transforming p1.1 plasmid DNA was studied by Southern blot analysis. The hybridization pattern observed (Fig. 1B) is consistent with the view that the plasmid had integrated at the homologous site via a single recombination event (Fig. 1C). Interestingly, some of the transformants harbored multiple copies of the transforming plasmid, as judged from the intensity of the 6.3-kb band. Quantification of the various bands seen in the Southern blot revealed that, if the DNA shown in Fig. 1B, lane 1, harbors one plasmid copy as we suspect, then the DNA in lane 2 harbors two plasmid copies. Of the four transformants looked at, two showed the pattern in lane 1 and two showed the pattern in lane 2. This confirms our earlier results (59) which already pointed to multicopy integration events in *T. cutaneum*.

Three subfragments of the complementing 7.7-kb *Bam*HI-*Sau*3A fragment were cloned, and a partial DNA sequence analysis was carried out at the ends of the fragments. Three hundred to 400 nucleotides were analyzed, and data base searches (EMBL and GenBank) were performed. The DNA sequences obtained from all three fragments showed a high degree of homology with a number of genes encoding CPSs.

Features of the *T. cutaneum* DSM 70698 *argA* gene sequence. The DNA sequence of a complementing 4,082-bp subfragment of plasmid p1.1 was obtained by sequencing both strands according to the strategy shown in Fig. 2A. The sequence analysis revealed a complete gene, which will be referred to here as the *argA* gene. The *argA* coding region has the capacity to encode a polypeptide of 1,170 amino acid residues and appears to be interrupted by an 81-bp intron between codons 98 and 99 (see below). Translation of the polypeptide is predicted to initiate at the first ATG codon (nucleotides 617 to 619 [Fig. 2B]), proceeding to a TGA stop codon (nucleotides 4207 to 4209). The *argA* gene contains a possible TATA box (TAATAAATTT) with a limited homology to the *Saccharo-*

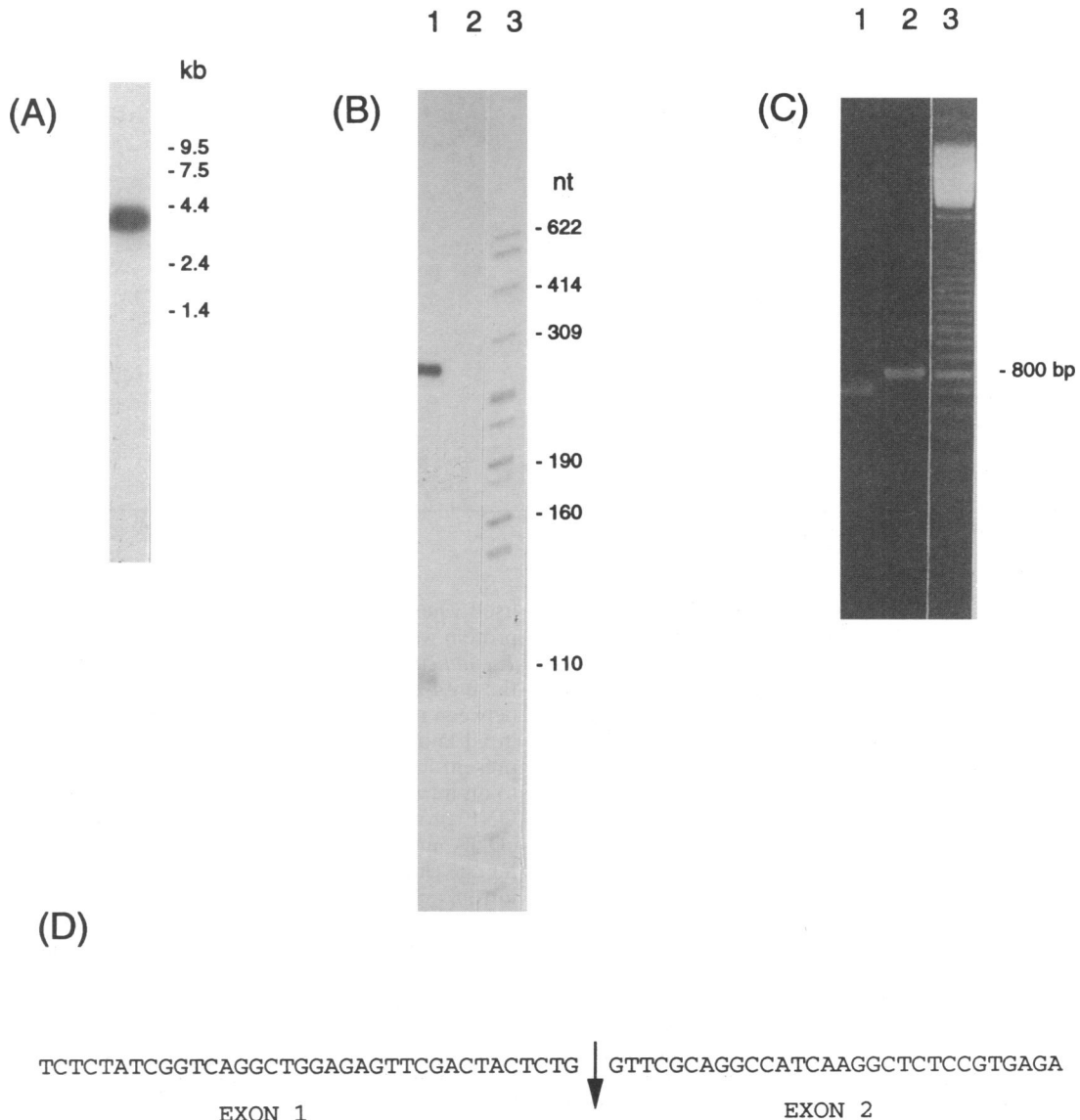


FIG. 5. Analysis of *argA*-encoded RNAs. (A) Sizing the *argA* transcript by Northern blot analysis. Ten micrograms of total RNA was prepared and fractionated as described in Materials and Methods. After electrophoresis, the RNAs were transferred to a GeneScreen Plus membrane, which was then hybridized with a ^{32}P -labeled 1.86-kb *Cla*I-*Hind*III fragment of the *argA* gene. The positions of RNA ladder fragments (Bethesda Research Laboratories) are indicated. (B) Analysis of *argA* transcripts using the RNase ONE protection assay. Lane 1, 5 μg of *T. cutaneum* total RNA. Lane 2, 5 μg of *E. coli* tRNA; the RNA samples were hybridized with an intron-specific probe extending from the *Kpn*I site at position 1255 to the *Eco*RV site at position 801. Lane 3, end-labeled pBR322 *Msp*I fragments; the sizes (in nucleotides [nt]) are indicated on the right. (C) Analysis of the *argA* intron by PCR. The DNA was analyzed by agarose gel electrophoresis. Lanes: 1, PCR product with total *T. cutaneum* cDNA as a template; 2, PCR product with p1.1 as a template; 3, 100-bp ladder (Pharmacia). (D) Partial DNA sequence of the cloned PCR product in panel C, lane 1. Arrow, position of the intron.

myces cerevisiae TATA box consensus sequence TAT(A/T)A(A/T) (21) and two putative CAAT boxes (CAAT) in its 5' noncoding region, but no conserved polyadenylation site is detectable in the 3' noncoding region. The codon usage of the *T. cutaneum argA* gene is similar to what is found in the *T. cutaneum* ATCC 46490 phenol hydroxylase cDNA (35, 66) and in genes from *Neurospora crassa* and *Aspergillus* species (2, 80). Codons ending in A are avoided, and the ones ending in pyrimidines, especially C, are preferred.

Features of the protein encoded by the *argA* gene. The 48 amino-terminal residues of the *T. cutaneum argA*-encoded protein contain six arginine, three serine, one leucine, but no

acidic residues. Such an amino acid composition is typical for mitochondrial targeting signals (88, 89). These have been found to be rich in serine, leucine, and basic amino acids, especially arginine, whereas acidic amino acids are rare. Additionally, the amino acid residues 65 to 70 form a motif (RSCPSS) which is similar to the one found in the processing regions of mitochondrial proteins from other organisms (74, 81, 91). In the mitochondrially imported subunit 9 of the *N. crassa* and *A. nidulans* ATP synthetases, the processing site was found to be ROYSS (91). Moreover, the OCTases of *A. nidulans* and *Pachysolen tannophilus* revealed variations of the above sequence, harboring RSYSS and RFFSS motifs, respec-

TABLE 2. Splice signals within the intron of the *T. cutaneum argA* gene compared with the conserved intron sequences of other organisms

Organism(s) and/or gene	5' splice site ^a	Internal site ^a	3' splice site ^a
<i>T. cutaneum argA</i>	GTGAGTT	AACTGAC	CAG
<i>T. cutaneum phyA</i> (34)	GTGAGTA	AACTGAG	TAG
<i>Cryptococcus neoformans URA5</i> (16)	GTGAGCC T	ATCTGAY G C	YAG
Filamentous fungi (2, 80)	GTUNGY C	NNCTUAY	NAG
<i>S. cerevisiae</i> (44)	GTATGT C	TACTAAC	YYNYAG
<i>Schizosaccharomyces pombe</i> (50)	GTANGN	CTAAC G T	TAG A
Higher eukaryotes (52)	GTAAGT G	CTAAT C C	(T/C) _n NCAG

^a |, intron-exon boundary; Y, pyrimidine; U, purine; N, any nucleotide.

tively (74, 81). Taken together, these results raise the possibility that the *argA*-encoded protein might be a mitochondrial protein.

The alignment of the *T. cutaneum argA*-encoded protein and the *S. cerevisiae* CPS (CPA2) amino acid sequences revealed 62% identity and 77% overall similarity, and two highly conserved regions were evident (Fig. 3). These domains are strictly conserved among the CPS proteins of *E. coli*, *S. cerevisiae*, and the rat (46, 55, 56, 58) and have been suggested to be involved in the binding of ATP. Of the 50 amino acid residues present in the amino-terminal domains of the *S. cerevisiae* CPA2 protein and the *T. cutaneum argA*-encoded protein, respectively, 43 are identical and 6 represent functionally conserved substitutions. The COOH-terminal domains appear to be less conserved, however. Of the 42 residues, 26 are identical and 10 show functionally conserved substitutions.

Genes related to *argA* in other *Trichosporon* yeasts. To test the relationships of the *argA* sequences in a number of different *T. cutaneum* strains, a Southern blot analysis was carried out using parts of the *T. cutaneum* DSM 70698 *argA* gene as a hybridization probe (Fig. 4). This fragment hybridized strongly to the DNAs of *T. cutaneum* ATCC 62975, ATCC 20509, DSM 70698, ATCC 46490, and ATCC 58094 and *T. beigeli* CBS 5790 under conditions of high stringency but less strongly to the DNA from *T. pullulans*. This is consistent with the results of Guého et al. (24, 26) which showed that *T. pullulans* is taxonomically quite distant from other *Trichosporon* species. The strong signals are an indication of the close relationship of the various *Trichosporon* strains despite the fact that the restriction patterns vary quite substantially among the various strains. Hybridization of the same probe to the DNA from *Cryptococcus elinovii* produced a much weaker signal (64), and no hybridization was obtained with DNA from *Candida tropicalis* ATCC 750 (64). The additional, weakly hybridizing fragments in lanes 1, 2, 3, 6, and 7 indicate the presence of additional *argA*-related DNA sequences in these strains.

Analysis of *argA* RNA. The size of the *T. cutaneum argA* transcript was estimated by Northern blot analysis. Hybridization with a 1.86-kb *Clai-HindIII* fragment spanning the 5' end of the coding region of the gene revealed a major transcript of around 4.0 kb (Fig. 5A). A putative intron sequence close to the 5' end of the coding sequence of the *argA* gene revealed

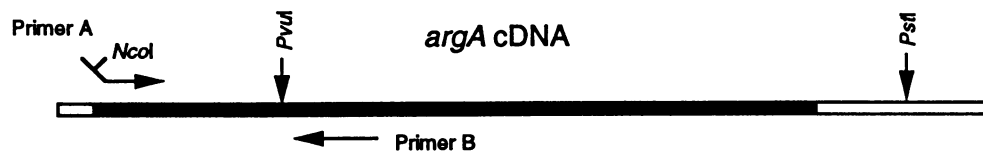
itself when the amino acid sequence of the *S. cerevisiae* CPA2 protein was compared with the one putatively encoded by the *T. cutaneum argA* gene. In the alignment of the two sequences, the *argA*-encoded protein revealed 27 additional amino acids between residues 98 and 99. Since this area is 100% conserved in all known eukaryotic CPSs (73), the extra 81-bp sequence present in the *T. cutaneum argA* gene was assumed to be due to an intron. This was demonstrated experimentally in RNase ONE protection experiments, by PCR using complementary cDNA made from *T. cutaneum* poly(A) RNA and by sequencing the cloned PCR product. For RNase ONE mapping of the intron, an in vitro-made RNA extending from the *KpnI* site at position 1255 up to the *EcoRV* site at position 801 (Fig. 2) was used, and two protected fragments of about 270 and 110 nucleotides, respectively, were apparent (Fig. 5B, lanes 1 and 2). These correspond exactly to the lengths of the predicted exon sequences. The PCR revealed a major band of around 750 bp when total *T. cutaneum* cDNA was being used (Fig. 5C, lane 1) and a band at around 850 bp with p1.1 DNA as a template (Fig. 5C, lane 2). The PCR fragments were cloned into pBluescript SK+, and the sequence in the vicinity of the intron sequence was analyzed. Figure 5D shows that the predicted 81-bp sequence is missing, as expected, in the cloned cDNA. A closer look at the 81-nucleotide DNA fragment present in the *argA* gene revealed all the elements of a typical fungal intron (Table 2). Moreover, the *argA* intron is highly similar to the intron found in the 5' noncoding region of the *T. cutaneum* DSM 70698 phenol hydroxylase (*phyA*) gene (34).

Heterologous expression of *argA*-encoded proteins. To unequivocally demonstrate that the *T. cutaneum argA* gene encodes the large subunit of CPS, expression studies with the *E. coli* carB8 strain, which lacks a functional CPS (18, 49), were carried out. For this purpose the intron sequence was removed by PCR as depicted in Fig. 6B and the 5' end of the protein-encoding region was adjusted, taking into account the conserved amino-terminal sequences of the *E. coli* and *S. cerevisiae* CPSs (Fig. 6A). The gene with the intron sequence removed was cloned into the pTrc99A expression vector (1) downstream of the regulatable *tac* (*trc*) promoter (Fig. 6C) to allow its expression in *E. coli* carB8 cells. These expression studies revealed a protein of around 130 kDa in IPTG-induced cells harboring the expression plasmid lacking the *argA* intron (pTrc-D) (Fig. 6D, lane 1) but not in cells carrying either the

(A)

<i>E. coli</i>	1	PKRTDIKSILILGAGPIVI
		: : :: :
<i>S. cerevisiae</i>	22	QLVEGVNSVLVIGSGGLSI
		: : :: :
<i>T. cutaneum</i>	70	SRSPDVKKVLVVGSGGLSI

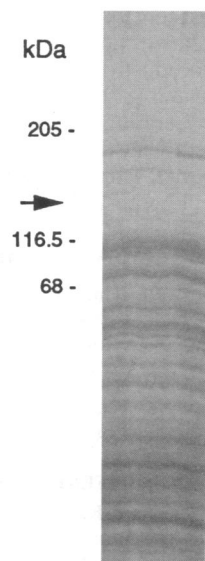
(B)



(C) $\overbrace{\text{AGGAAACACAGACC}}^{\text{NcoI}} \text{ATG GAC GTC AAG AAG GTC CTT}$
M D V K K V L

(D)

1 2 3



(E)

1 2 3



FIG. 6. Expression of *argA*-encoded proteins in *E. coli*. (A) Alignment of the amino-terminal regions of the *E. coli* and *S. cerevisiae* CPSs (46, 57) and of the *T. cutaneum argA*-encoded protein. Identical (|) and similar (:) residues are indicated. (B) Adjustment of the 5' end of the *argA* gene and removal of the intron by PCR. Primer A is complementary to nucleotides 836 to 853 of the *argA* sequence and contains, in addition, an *NcoI* site and an ATG start codon. Primer B binds to nucleotides 1643 to 1664 downstream of the *PvuI* site at position 1635 (Fig. 2B). The 750-bp PCR product was digested with *NcoI* and *PvuI*, purified by electrophoresis in a low-melting-temperature agarose gel, and subsequently ligated to *NcoI*- and *PstI*-cut pTrc99A vector DNA together with a 4.5-kb *PvuI*-*PstI* fragment from plasmid p1.1 (Fig. 1A and 2A). The resulting expression plasmid is referred to as pTrc-D. The expression plasmid pTrc-E was constructed in a similar way, except that p1.1 plasmid DNA was used for PCR. Thus, the intron sequence is still present in pTrc-E. (C) Nucleotide sequence of the fusion between the *tac* promoter and the *argA* sequence. (D) SDS-PAGE analysis of extracts from *E. coli* carB8 cells harboring pTrc-D (lane 1), pTrc-E (lane 2), or pTrc99A (lane 3). The proteins were separated in an SDS-7.5% polyacrylamide gel (54). Arrow, 130-kDa protein in lane 1. (E) Western blot analysis using anti-rat CPS antibodies. Lanes: 1, carB8/pTrc-D extract; 2, carB8/pTrc-E extract; 3, carB8/pTrc99A extract.

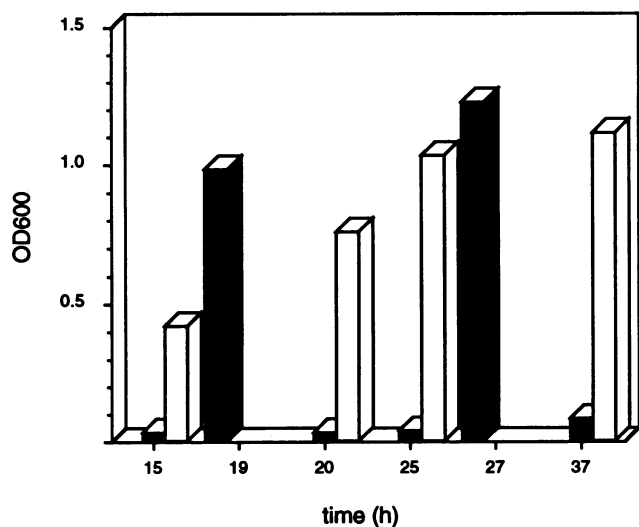


FIG. 7. Growth of a *T. cutaneum* *arg70* transformant harboring the *A. nidulans* *argB* gene in the presence of excess uracil. The cells were cultivated in D medium containing 1% glucose with or without uracil ($100 \mu\text{g ml}^{-1}$) at 30°C . Open bars, growth of transformant in the absence of uracil; hatched bars, growth of transformant in the presence of uracil; solid bars, *T. cutaneum* wild-type cells grown in the presence of $100 \mu\text{g}$ of uracil ml^{-1} . OD600, optical density at 600 nm.

pTrc-E expression plasmid with the *argA* intron still present (Fig. 6D, lane 2) or the pTrc99A expression vector without an insert sequence (Fig. 6D, lane 3) or in uninduced cells (64). An anti-rat CPS antibody (14) was found to cross-react in Western blots with a protein of around 130 kDa present in *E. coli* carB8 cells harboring pTrc-D (Fig. 6E, lane 1) but not in control extracts (Fig. 6E, lanes 2 and 3). This antibody has been found to also cross-react with the *S. cerevisiae* CPA2 protein (63). Attempts to detect CPS activity in carB8/pTrc-D cell extracts by using a coupled enzymatic assay (69) have failed so far (64). Also, growth of such cells in the presence of 100 mM ammonium sulfate but in the absence of arginine and uracil was not possible (64).

Growth of *T. cutaneum* *arg70* transformants harboring the *A. nidulans* *argB* gene in the presence of excess uracil. As shown in this study, the *T. cutaneum* DSM 70698 *arg70* strain is probably affected in the gene encoding CPS-A. Interestingly, however, the strain could be transformed quite readily by using the heterologous *A. nidulans* OCTase-encoding (*argB*) gene, and such transformants were found to carry a high number (up to 200 copies) of the transforming plasmid (59). It is conceivable that some of the OCTase made by these transformants remains in the cytosol and that citrulline is made from cytosolic (pyrimidine-specific) carbamoyl phosphate and ornithine leaking from the mitochondria in derepressed conditions (5, 38). The high OCTase concentration would be essential in this case, as the homologous *T. cutaneum* *argB* gene present at low copy numbers did not complement the mutant (19, 59). This hypothesis was tested by adding uracil to the cultures of the *argB*⁺ transformants. In *S. cerevisiae*, the growth of *cpa2* mutants was found to be inhibited by uracil, because of the feedback inhibition of CPS-P by UTP (61). The same model also appears to apply to *T. cutaneum* *argB*⁺ transformants, as they did not grow in uracil-supplemented medium (Fig. 7).

DISCUSSION

Yeasts, filamentous fungi, and higher eukaryotes have two separate enzymes involved in carbamoyl phosphate synthesis (10, 11). One enzyme is specific for the pyrimidine pathway (CPS-P) and is located in the nucleus. The other enzyme (CPS-A) functions in the arginine pathway and is usually found in mitochondria, although in certain organisms, such as *S. cerevisiae*, it appears to be cytoplasmic (82). In *N. crassa*, the products of both enzymes (CPS-P and CPS-A) are maintained in separate pools, so that mutants lacking a given CPS require the end product of the deficient pathway (94).

In fungi, CPS-A is composed of a small and a large subunit, which are encoded by unlinked genes (9, 42, 47). For example, the small subunit of the *S. cerevisiae* CPS-A is encoded by the *CPA1* gene, and the large subunit is encoded by the *CPA2* gene. In *N. crassa*, the small subunit is encoded by the *arg-2* gene and the large subunit is encoded by the *arg-3* gene. The molecular mass of the holoenzyme has been estimated by gel filtration to be about 175 kDa in both organisms (12, 62). A size of 45 kDa for the small subunit was deduced from the nucleotide sequences of the cloned genes of *S. cerevisiae* and *N. crassa* (58, 60). The large subunit of *S. cerevisiae* CPS-A consists of 1,117 amino acids, and the calculated molecular mass is 124 kDa (46). The CPS-A of the rat has been found to be a fusion polypeptide composed of the small and large subunits with a size of 165 kDa (56). In *N. crassa* (92) and in some obligatory aerobic yeasts (82), the large subunit of CPS-A is found in the mitochondrial matrix. In aerobic yeasts and filamentous fungi, citrulline biosynthesis takes place in the mitochondria (4, 82). The *T. cutaneum* *argA*-encoded protein is probably targeted to the mitochondria, since the proposed NH₂ terminus contains all of the characteristics found in mitochondrial targeting sequences. This would be consistent with the fact that *Trichosporon* yeasts have a purely oxidative metabolism (36, 37). In many yeasts possessing an oxidoreductive metabolism OCTase and CPS are cytoplasmically located (87).

The observed integration patterns of the transforming fragments are consistent with the view that homologous recombination is frequent in *T. cutaneum* DSM 70698, indicating that gene disruption will be possible in this strain in the future.

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