# Supporting Information

## Funes et al. 10.1073/pnas.0809951106

### SI Materials and Methods

**Sequence Analysis.** To compute a distance matrix for the phylogenetic tree, alignments were generated for all possible pairwise sequence combinations using ClustalW 1.83 (1) with default settings for protein alignments. Maximum likelihood distances based on these pairwise alignments were calculated using the program PROTDIST from the PHYLIP package (2), implementing a JTT matrix (3). This matrix was used to calculate a neighbor-joining tree with NEIGHBOR (2). To assess phylogenetic uncertainty, bootstrap data sets (4) were generated from each individual pairwise alignment and used to produce neighbor-joining trees. This process was repeated 1,000 times to calculate the bootstrap proportions for the internal branches. A software script pairdist.py was written in the Python programming language (www.python.org). Sequences used and the corresponding accession numbers are shown in Table S1.

**Constructions of Strains.** See Table S2. Streptococcal mutant strains are isogenic to *S. mutans* wild-type NG8 (5). The Δ*yidC2* mutant (AH398) was generated previously (6). The *yidC2* C-terminal deletion strain *yidC2*ΔC (AH410) was generated by PCR ligation mutagenesis. Sequence encoding the N-terminal 254-aa residues of YidC2 and the upstream promoter region was amplified using NG8 genomic DNA as template and primers AH39F 5′-*CCCGGG*AAATAAATGCCAACCTTCAATCA-3′ (SmaI site underlined) and AH44R 5′-TTA*TCTAGA*TGGTTTAATGATATGGTTTGTGAT-3′ (XbaI site underlined) with a stop codon (bold) at the 5′ end of the reverse primer. The product was cloned into pCR2.1 (Invitrogen). Genomic DNA from the Δ*yidC2* mutant AH398 was used as template to amplify the erythromycin marker and sequence downstream of *yidC2* using primers AH17F 5′-CGG*GAATTC*AAGCTTAGGAGTGATTACATGAACAAAAATATA-3′ (HindIII site underlined) and AH27R 5′- GTTGGTGCCGCCGGAGCTGA-3′. The amplified product was digested with HindIII. Plasmid containing the *yidC2* C-terminal deletion was digested with HindIII-SmaI and the appropriate fragment gel purified. The HindIII digested fragments were ligated and served as template for second-round PCR amplification using primers AH39F and AH27R. The 2.8-kb PCR amplified fragment was introduced to NG8 by natural transformation. Transformants were selected on THYE plates supplemented with 10-μg/ml erythromycin.

**Expression of Oxa1 and Cox18 in *S. mutans*.** To ensure proper membrane targeting of Oxa1 and Cox18 in the *S. mutans* background, chimeric constructs were generated to append the N-terminal targeting domains of YidC2 or YidC1 onto the yeast homologs. DNA 572-bp upstream or 513-bp upstream and encoding the N-terminal 50- or 52-aa residues of either YidC1 or YidC2, respectively, was amplified by PCR with NG8 genomic DNA as a template using primers AH39F 5′-CCCGGGAAATAAATGCCAACCTTCAATCA-3′ (SmaI site underlined) and AH39R 5′-GGTACCGATAACACTTCCCATTGGGGC-3′ (KpnI site underlined) for SmYidC2, and AH 40F 5′-CCCGGGTGACTTAATTGAGGAAGATGTCT-3′ (SmaI site underlined) and AH40R 5′-GGTACCGATGGATAAAAAGCGGATGGTC-3′ (KpnI site underlined) for SmYidC1. Products were cloned into pCR2.1 to generate pRC1 and pRC2. Plasmids harboring *OXA1* or *COX18* (7, 8) were digested with KpnI-SmaI and DNA fragments encoding residues 43–402 of Oxa1 or 35–316 of Cox18 were gel-purified and ligated to pRC1 or pRC2 previously digested with KpnI-EcoRV. The *yidC2-OXA1* and *yidC1-COX18* chimeric genes were excised with SmaI and ligated to similarly digested *E. coli-S. mutans* shuttle vector pDL289 (9) to generate pAH374 and pAH376. Plasmids were introduced into *S. mutans* NG8 by natural transformation and *yidC2* was deleted subsequently, as previously described (6). The lack of genetic competence of *S. mutans* upon deletion of *yidC2* dictates this order.

**Expression of Fusion Proteins in Yeast.** For expression of YidC1, YidC2, and YidC2ΔC in yeast, the sequence encoding the promoter and the N-terminal 119-aa residues of Oxa1 was amplified by PCR using the primers *GS01* (5′-GGGCTCGAGGCATCGGACATTTTCCCG-3′) and *GS02* (5′-GGGCGCGGCCGCCTCCAAGACGTGTTG-3′) and cloned into the XhoI/NotI sites of pRS426 (10, 11). Into the NotI and SacI sites of the resulting plasmid, the following PCR products were cloned independently: the sequence corresponding to amino acids 47–271 of YidC1 was amplified by PCR using the primers *SmYidC1-F* (5′-GCGGCGGCCGCCTTTTTATCCATCAATGGCAGGATTGG-3′) and *SmYidC1-R* (5′-GCGGAGCTCTTATTTTCTCTTTTTATGTGCTTTCTT TTTAGC-3′); the sequence corresponding to amino acids 46–310 of YidC2 using the primers *SmYidC2-F* (5′-GCGGCGGCCGCAATGGGAAGTGTTATCCAATATTTAGC-3′) and *SmYidC2-R* (5′-GCGGAGCTCTTATTGCTTATGGTGACGCTGTTTACC-3′); and the sequence corresponding to amino acids 46–257 of YidC2 was amplified with the primer *SmYidC2-F* and *SmYidC2ΔC-R* (5′-GCGGAGCTCTTACCGCAATTTTGGTTTAATG-3′).

The sequence encoding the promoter and the N-terminal 317 aa of Oxa1 was amplified by PCR from yeast genomic DNA using the primers 5′-CCGCTCGAGCTGCTTAACAGTATTCGCGACC-3′ and 5′- TCTAGATCTCAAAATCATTGTCT GTAGGACG-3′ and cloned into the sites XhoI/XbaI of pBluescript. The region encoding amino acids 240 to 271 of YidC1 was amplified using the primers 5′-GCGTCTAGAAAAATTATTGCAGAACGCCAGCG-3′ and 5′-GGGGAGCTCTTATTTTCTCTTTTTATGTGCTTTCTTTTTAGC -3′. The sequence encoding amino acids 258 to 310 of YidC2 was amplified using the primers 5′-GTCTAGAAAGCAAATTGATGAAGAATTTAAGAAAAACC-3′ and 5′-CCCGAGCTCTTATTGCTTATGGTGACGCTGTTTACC-3′. The sequence encoding amino acids 1 to 86 of Hht1 was amplified by PCR from yeast genomic DNA using the primers 5′-GGGTCTAGAATGGCCAGAACAAAGCAAACAGC-3′ and 5′-GGCGAGCTCTTATTGAAATCTCAAGTCGGTCTTG-3′. The 3 resulting PCR products were digested with XbaI and SacI and cloned into the pBluescript vector downstream of the *OXA1* promoter and the sequence encoding the N-terminal 317 aa of Oxa1. The resulting inserts were released with XhoI and SacI and cloned into the yeast expression vector pRS426. Via the same strategy, C-terminal extensions were added to a mitochondrially targeted version of *E. coli* YidC (11). The sequences encoding $YidC1^{240–271}$, $YidC2^{258–310}$, and $Hht1^{1–86}$ were amplified using the same primers as above, digested with XbaI and SacI, and cloned into a pBluescript plasmid containing the sequence for mitochondrially targeted YidC (11). The inserts were released by digestion with XhoI and SacI and cloned into pRS426.

**Isolation of Membranes from *S. mutans*.** One hundred milliliters of cells were grown in BHI broth supplemented with 20 mM DL-threonine for 16 h at 37 °C, then subcultured into 1 L of medium and grown to late exponential phase ($O.D._{600}$ = 0.5 to

0.7). Cells were harvested and suspended in 5-ml buffer A (10 mM Tris-HCl [pH 6.8], 10 mM Mg acetate, 25% sucrose), followed by incubation in lysozyme (0.3–0.4 mg/ml) and 2,000 U mutanolysin (Sigma Chemical Co.) at 37 °C with gentle shaking for ≈2 h to form protoplasts. Protoplast formation was monitored by Gram staining. The protoplasts were centrifuged at 3,000 × *g* for 10 min, washed twice with Buffer A, and lysed by osmotic shock by suspension in 50 mM Tris (pH 7.5), 10 mM $MgSO_4$, supplemented with 0.8 M NaCl. To the lysate was added 100 μl of protease inhibitor mixture (Sigma, cat# P8465), 10 μg/ml of DNase (Ambion), and 50 μg RNase (Roche), and the mixture incubated at room temperature for 45 min with swirling. Unlysed protoplasts and cell debris were removed by centrifugation at 3,000 × *g* for 10 min and membranes pelleted by centrifugation at 100,000 × *g* 1.5 h at 4 °C. Membrane pellets were suspended in 500 μl of solubilization buffer (50 mM NaCl, 50 mM imidazole, 2 mM 6-aminohexanoic acid, 1 mM EDTA, pH 7.0) using a mini-teflon homogenizer. Protein concentration was determined using the bicinchoninic acid assay.

**Streptococcal Competence Assay.** Overnight cultures were diluted 20-fold in prewarmed THYE supplemented with 5% heat inactivated horse serum and incubated at 37 °C until an $OD_{600}$ of ≈0.2 was reached. One microgram of synthetic competence stimulating peptide (12) was added and the cultures were incubated for an additional 20 min. Following the incubation period, 1 μg of pDC123 ($Cm^r$) (13) was added and transformants were incubated for 30 min, followed by the addition of 0.5 ml fresh THYE supplemented with 5% heat inactivated horse serum and the incubation continued for another 2 h. The cultures were plated in duplicate on THYE plates with or without the appropriate antibiotics and incubated at 37 °C with 5% $CO_2$ for 48 h. The transformation efficiency was expressed as the percentage of transformants of the mutant or complemented strain relative to the NG8 wild type from 2 independent experiments.

**Isolation of Mitochondrial Ribosomes.** Translation products were radiolabeled in isolated mitochondria for 15 min at 25 °C. Ten percent of the reaction was removed and directly applied to the gel. The residual extract was lysed in 1% Triton X-100, 5 mM $MgCl_2$, 350 mM KCl, 2 mM PMSF, 1x EDTA-free protease inhibitor mix (Roche), 20 mM Tris pH 7.5 for 20 min at 4 °C. The extracts were cleared by centrifugation for 5 min at 18,000 × *g*, loaded on a layer of 1.2 M sucrose in lysis buffer, and centrifuged for 60 min at 190,000 × *g*. Proteins of the total, the ribosome-containing pellet and the supernatant were analyzed by autoradiography and Western blotting.


1. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680.
2. Felsenstein J (1989) PHYLIP - Phylogeny Inference Package. *Cladistics* 5:164–166.
3. Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. *CABIOS* 8:275–282.
4. Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783–791.
5. Knox KW, Hardy LN, Wicken AJ (1986) Comparative studies on the protein profiles and hydrophobicity of strains of *Streptococcus mutans* serotype c. *J Gen Microbiol* 132(9):2541–2548.
6. Hasona A, et al. (2005) Streptococcal viability and diminished stress tolerance in mutants lacking the signal recognition particle pathway or YidC2. *Proc Natl Acad Sci USA* 102(48):17466–17471.
7. Herrmann JM, Neupert W, Stuart RA (1997) Insertion into the mitochondrial inner membrane of a polytopic protein, the nuclear encoded Oxa1p. *EMBO J* 16:2217–2226.
8. Funes S, Nargang FE, Neupert W, Herrmann JM (2004) The Oxa2 protein of *Neurospora crassa* plays a critical role in the biogenesis of cytochrome oxidase and defines a ubiquitous subbranch of the Oxa1/YidC/Alb3 protein family. *Mol Biol Cell* 15:1853–1861.
9. Buckley ND, Lee LN, LeBlanc DJ (1995) Use of a novel mobilizable vector to inactivate the *scrA* gene of *Streptococcus sobrinus* by allelic replacement. *J Bacteriol* 177(17):5028–5034.
10. Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene* 110(1):119–122.
11. Preuss M, Ott M, Funes S, Luirink J, Herrmann JM (2005) Evolution of mitochondrial Oxa proteins from bacterial YidC: Inherited and acquired functions of a conserved insertion machinery. *J Biol Chem* 280:13004–13011.
12. Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG (2001) Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *J Bacteriol* 183:897–908.
13. Chaffin DO, Rubens CE (1998) Blue/white screening of recombinant plasmids in Gram-positive bacteria by interruption of alkaline phosphatase gene (phoZ) expression. *Gene* 219(1–2):91–99.

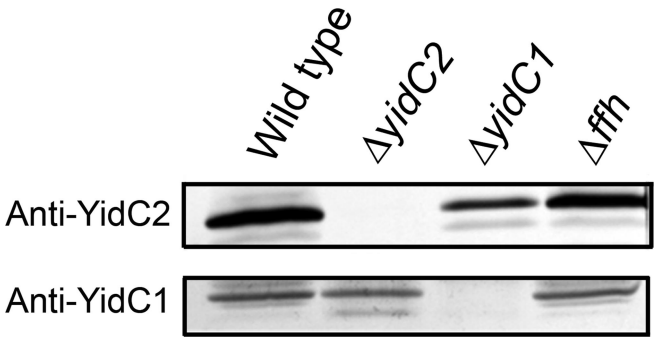


**Fig. S1.** YidC1 and YidC2 are expressed and inserted into the *S. mutans* membrane in the absence of the SRP (Δ*ffh*). Western immunoblots of membrane proteins from *S. mutans* wild-type, and Δ*yidC2*, Δ*yidC1*, and Δ*ffh* mutants reacted with anti-YidC2 or anti-YidC1 C-terminal peptide-specific antiserum (1:1,000 dilution) [Dong Y, et al. (2008) Functional overlap but lack of complete cross-complementation of *Streptococcus mutans* and *Escherichia coli* YidC orthologs. *J Bacteriol* 190:2458–2469]. For each sample 80 $\mu$g of membrane proteins were separated using a 10% polyacrylamide gel and electroblotted onto nitrocelluose membranes.

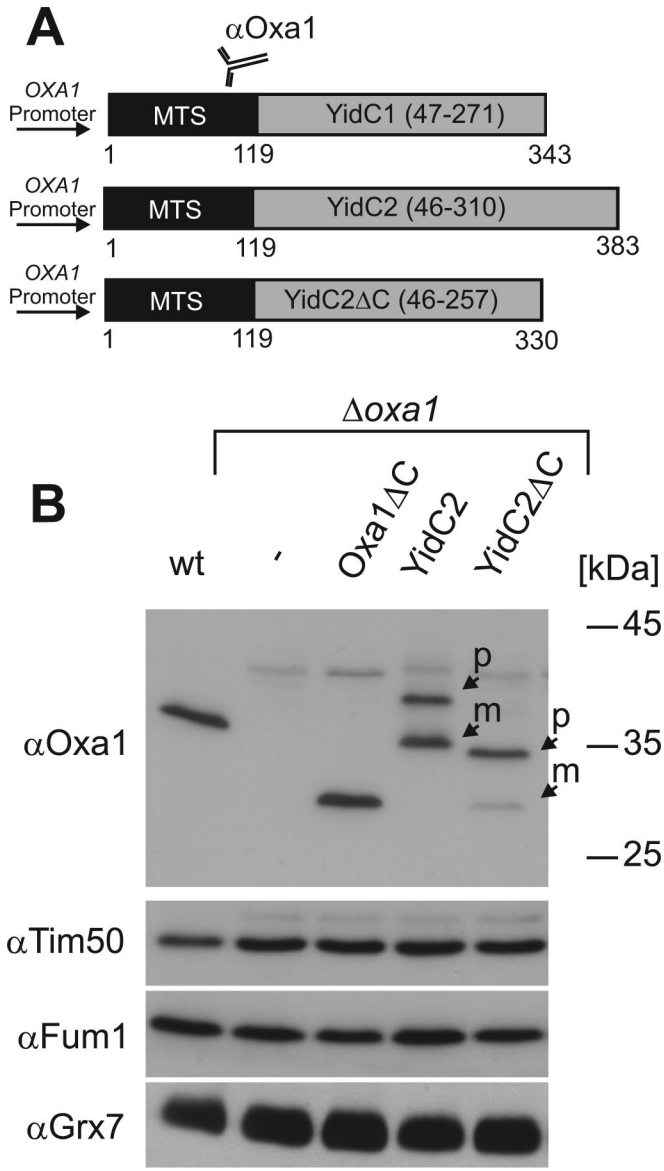


**Fig. S2.** Expression of *S. mutans* YidC1, YidC2, and YidC2ΔC in yeast mitochondria. (*A*) Schematic representation of chimeric *S. mutans* YidC1 and YidC2 fusions constructed for expression in yeast mitochondria. The numbers indicate the amino acid residues of the fusion protein. Black boxes depict the N-terminal region of yeast Oxa1 containing the mitochondrial targeting signal (MTS) and the location of the epitope recognized by the Oxa1-specific antibody (αOxa1). The YidC2ΔC construct lacks the 53 C-terminal amino acid residues of YidC2. (*B*) Oxa1-deficient yeast cells (Δ*oxa1*) were transformed with plasmids expressing the indicated fusion proteins. Equal amounts of cell extracts of these mutants, as well as of a wild-type (wt) control, were analyzed by Western blotting using an antibody that recognizes an N-terminal epitope of Oxa1. For the loading control, the membrane was probed with antibodies against the mitochondrial proteins Tim50 and fumarase (Fum1) as well as the nonmitochondrial protein Grx7. Note that migration of Oxa1, Oxa1ΔC, YidC2, and YidC2ΔC roughly matched their predicted masses of 39.6, 31.9, 37.5 and 31.7 kDa, respectively. Upon expression of the YidC fusion proteins, 2 forms were detected in the yeast cells, a larger precursor form and a smaller mature form. The accumulation of the precursor forms suggests a potential effect on import efficiency of the YidC variants into the mitochondria, presumably because of the highly hydrophobic moment of these proteins. Although the levels of YidC2ΔC were lower than those of YidC2, the mature mitochondria-located form of YidC2ΔC was clearly detectable in the cells.

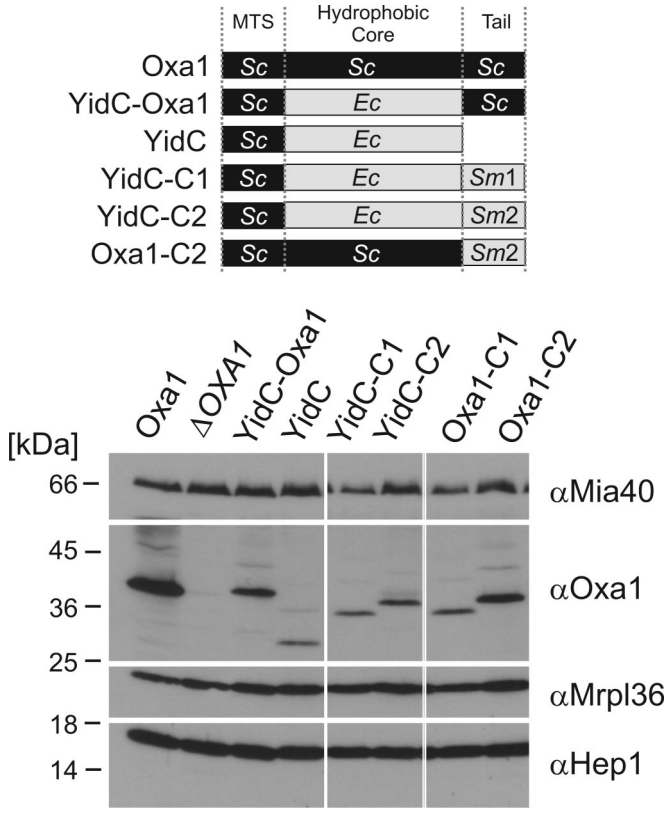


**Fig. S3.** Expression of fusion proteins in yeast. The fusion proteins indicated were expressed in Oxa1-deficient yeast cells. Equal amounts of cell extracts were analyzed by Western blotting, as described for Fig. S2. Note that all fusion proteins were stably expressed.

**Table S1. Accession numbers of the sequences used for the analysis shown on Fig. 1**

| Organism | Protein | Accession Number |
|---|---|---|
| *Anopheles gambiae* | Oxa1 | XP_315429 |
| *Anopheles gambiae* | Oxa2 | XP_001688695 |
| *Arabidopsis thaliana* | Alb3 | NP_001118405 |
| *Arabidopsis thaliana* | Alb4 | NP_173858.2 |
| *Bacillus subtilis* | SpoIIIJ | NP_391984 |
| *Bacillus subtilis* | YqjG | NP_390269 |
| *Clostridium acetobutylicum* | SpoIIIJ | AAK81656 |
| *Drosophila melanogaster* | Oxa1 | NP_648417 |
| *Drosophila melanogaster* | Oxa2 | NP_648286 |
| *Escherichia coli* | YidC | NP_418161 |
| *Haemophilus influenzae* | YidC | ZP_00156859 |
| *Homo sapiens* | Oxa2 | NP_776188 |
| *Homo sapiens* | Oxa1 | NP_005006 |
| *Lactobacillus plantarum* | YidC1 | NP_786873 |
| *Lactobacillus plantarum* | YidC2 | NP_785152 |
| *Lactococcus lactis* | YidC2 | YP_808581 |
| *Lactococcus lactis* | YidC1 | YP_808173 |
| *Listeria welshimeri* | YidC2 | YP_849592 |
| *Listeria welshimeri* | YidC1 | YP_850974 |
| *Mus musculus* | Oxa1 | NP_081212 |
| *Mus musculus* | Oxa2 | NP_001028482 |
| *Neurospora crassa* | Oxa1 | XP_961714 |
| *Neurospora crassa* | Oxa2 | XP_963619 |
| *Oryza sativa* | Alb3 | NP_001042038 |
| *Oryza sativa* | Alb4 | AAS07376 |
| *Pisum sativum* | Alb3 | CAA73179.1 |
| *Pseudomonas putida* | YidC | NP_742176 |
| *Rattus norvegicus* | Oxa2 | NP_001099470 |
| *Rattus norvegicus* | Oxa1 | XP_001078039 |
| *Saccharomyces cerevisiae* | Cox18 | NP_011576 |
| *Saccharomyces cerevisiae* | Oxa1 | NP_011081 |
| *Schizosaccharomyces pombe* | Oxa1–1 | NP_593559 |
| *Schizosaccharomyces pombe* | Oxa1–2 | NP_596177 |
| *Schizosaccharomyces pombe* | Oxa2 | NP_588329 |
| *Staphylococcus haemolyticus* | YidC2 | YP_252859 |
| *Streptococcus mutans* | YidC1 | NP_720790 |
| *Streptococcus mutans* | YidC2 | NP_722054 |
| *Vibrio cholerae* | YidC | NP_062588 |

**Table S2. Strains used in this study**

| Description (strain name) | Genotype | Source or reference |
|---|---|---|
| *Streptococcus mutans* | | |
| Wild-type (NG8) | | (1) |
| Δ*yidC2* | *yidC2*Δ::ERM | (2) |
| Δ*yidC2* OXA1 | *yidC2*Δ::ERM<br>pDL289 (Kan$^r$):YidC2- YidC2 $^{1–52}$–OXA1 $^{43–402}$ | Present study |
| Δ*yidC2* OXA/COX18 | *yidC2*Δ::ERM<br>pDL289 (Kan$^r$):YidC1-YidC1 $^{1–50}$–COX18 $^{35–316}$ | Present study |
| *yidC2*ΔC | *yidC2* $^{1–254}$ΔC:ERM | Present study |
| *Saccharomyces cerevisiae* | | |
| Wild-type (W303–1A) | MAT *a*, *ade2 ura3 leu2 his3 trp1* [*rho*$^+$] | (3) |
| Δ*oxa1* | MAT *a*, *ade2 ura3 leu2 his3 oxa1*Δ::*HIS3 trp1* [*rho*$^+$] | (4) |
| Δ*oxa1* YidC1 | MAT *a*, *ade2 ura3 leu2 his3 oxa1*Δ::*HIS3 pRS426(URA3, 2 μm):OXA1-OXA1* $^{1–119}$*-YidC1*$^{47–271}$ *trp1* [*rho*$^+$] | Present study |
| Δ*oxa1* YidC2 | MAT *a*, *ade2 ura3 leu2 his3 oxa1*Δ::*HIS3 pRS426 (URA3, 2 μm):OXA1-OXA1* $^{1–119}$*-YidC2*$^{46–310}$ *trp1* [*rho*$^+$] | Present study |
| Δ*oxa1* YidC2ΔC | MAT *a*, *ade2 ura3 leu2 his3 oxa1*Δ::*HIS3 pRS426 (URA3, 2 μm):OXA1-OXA1* $^{1–119}$*-YidC2*$^{46–257}$ *trp1* [*rho*$^+$] | Present study |
| Oxa1ΔC | MAT *a*, *ade2 ura3 leu2 his3 trp1 oxa1*Δ*318−402*::*HIS3* [*rho*$^+$] | (5) |


1. Knox KW, Hardy LN, Wicken AJ (1986) Comparative studies on the protein profiles and hydrophobicity of strains of *Streptococcus mutans* serotype c. *J Gen Microbiol* 132:2541–2548.
2. Hasona A, et al. (2005) Streptococcal viability and diminished stress tolerance in mutants lacking the signal recognition particle pathway or YidC2. *Proc Natl Acad Sci USA* 102:17466–17471.
3. Thomas BJ, Rothstein R (1989) Elevated recombination rates in transcriptionally active DNA. *Cell* 56:619–630.
4. Hell K, Neupert W, Stuart RA (2001) Oxa1p acts as a general membrane insertion machinery for proteins encoded by mitochondrial DNA. *EMBO J* 20:1281–1288.
5. Szyrach G, Ott M, Bonnefoy N, Neupert W, Herrmann JM (2003) Ribosome binding to the Oxa1 complex facilitates cotranslational protein insertion in mitochondria. *EMBO J* 22:6448–6457.