Supporting Information

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SI Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions. pAC184-derived plasmids for carotenoid production, i.e., pAC-ZEAX*ipi* (zeaxanthin), pAC-CANTH*ipi* (canthaxanthin), pAC-BETA*ipi* (β-carotene), and pAC-LYC (lycopene) (1) were kind gifts from Francis Cunningham (University of Maryland, College Park, MD) and Juergen Polle (City University of New York, Brooklyn, NY). Nineteen different ATP-binding cassette (ABC) transporters were cloned into pET19b from PCR products using the restriction enzymes Nde1 and BamH1 or Xho1. All 19 transporters were sequence verified to confirm their identities. *Escherichia coli* Top10 cells were used to host single plasmids for transformation and plasmid preparation purposes, whereas BL21 (DE3) cells were used for double transformations, production of carotenoids from pAC184-derived plasmids, and the expression of transporters from pET19b-derived plasmids. All transformations were performed using the heat-shock method. Cultures were grown in Luria-Bertani (LB) medium containing 25 μg/mL chloramphenicol (for pAC plasmids) and 50 μg/mL carbenicillin (for pET plasmids); 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce expression of the transporter from pET plasmids.

Two-Phase Culture System. To detect the secreted carotenoids, a two-phase culture system was adopted for growing *E. coli* (2–6). Double transformant colonies (containing pAC184- and pET19b-derived plasmids) were picked into fresh LB medium containing the two antibiotics and grown overnight at 37 °C, with aeration at 200 rpm. Glycerol stocks were made from these overnight cultures using 25% (vol/vol) sterile glycerol. For secretion experiments, overnight cultures of the double transformants were set up from glycerol stocks in LB medium containing the two antibiotics growing at 37 °C, with aeration at 200 rpm. For transporter screens (Figs. S3 and S4), 0.5 mL overnight cultures were used to inoculate 5 mL LB medium containing the two antibiotics and 1 mM IPTG and were gently overlayed with 1 mL decane (>99% purity; Fisher/Acros Organics). For thorough analyses of transporter-mediated carotenoid secretion (Figs. 3 and 5), 1 mL overnight cultures were used to inoculate 10 mL LB medium with the antibiotics and 1 mM IPTG and were gently overlayed with 5 mL decane. Two-phase cultures were incubated at 30 °C, with aeration at 250 rpm under mild-absent light conditions for 24–144 h, depending on the carotenoid and experiment.

Carotenoid Secretion from Washed Cells in Buffer. Overnight cultures (0.5 mL) of double transformants were inoculated into 5 mL LB medium with the two antibiotics and aerobically grown at 37 °C, 200 rpm for, for 2–3 h until the OD_{595} reached 0.5–0.6 (log phase); 1 mM IPTG was added to all cultures that were further grown aerobically at 37 °C, 200 rpm, for 4 h. Cells were collected by centrifugation at 3,000 × *g*, 10 min, and 4 °C, and washed with 10 mL of ice cold 50 mM KPi (potassium phosphate) buffer containing 5 mM $MgSO_4$. After two more similar washes, the cells were resuspended in 5 mL of the wash buffer supplemented with 0.5% (wt/vol) sterile glucose and were gently overlayed with 2 mL decane. These two-phase cell suspensions were incubated at 30 °C, with aeration at 250 rpm for 24–96 h, depending on the carotenoid.

Analytical Measurements. At any given time point, aliquots were drawn from the two-phase cultures sufficient to make three different spectrophotometric measurements; secreted carotenoid, produced carotenoid, and cell viability. Secreted carotenoid amounts were measured in the decane phase, after removing any membrane debris by centrifugation at 16,000 × *g* for 1 min. Pure decane (stored in the absence of light to avoid noisy spectra) was used to blank the spectrophotometer (Beckman DU 800), and the concentrations of carotenoids in the samples were extrapolated from standard curves generated from analytical standard carotenoids dissolved in decane (zeaxanthin, Sigma Aldrich; canthaxanthin, Santa Cruz Biotech; β-carotene, MP Biomedicals; lycopene, Toronto Research Chemicals). Some carotenoid solutions needed to be heated with stirring to allow the carotenoids to dissolve completely. All spectrophotometric measurements involving organic solvents were made in quartz microcuvettes with a path length of 1 cm. The molar extinction coefficients (ε) in decane were calculated as the averages of all data points used to derive the respective standard curves, using the formula $A = \varepsilon lc$, where A is absorbance; ε is molar extinction coefficient; l is path length; and c is concentration. εs of carotenoids are known to be highly solvent dependent (7). In the absence of published ε in decane, we compared our calculated ε to the published values obtained in other solvents (8). The extent of the agreement depended on the hydrophobicity of the carotenoid; lycopene ($\varepsilon_{decane} - 159 \times 10^3\ M^{-1}cm^{-1}$; $\varepsilon_{hexane} - 186 \times 10^3\ M^{-1}cm^{-1}$) and β-carotene ($\varepsilon_{decane} - 132 \times 10^3\ M^{-1}cm^{-1}$; $\varepsilon_{hexane} - 139 \times 10^3\ M^{-1}cm^{-1}$) agreed the best, canthaxanthin ($\varepsilon_{decane} - 987 \times 10^2\ M^{-1}cm^{-1}$; $\varepsilon_{petroleum\ ether} - 124 \times 10^3\ M^{-1}cm^{-1}$) agreed moderately, and zeaxanthin's coefficient ($\varepsilon_{decane} - 298 \times 10^1\ M^{-1}cm^{-1}$; $\varepsilon_{petroleum\ ether} - 134 \times 10^3\ M^{-1}cm^{-1}$) was found to be considerably lower in decane. These comparisons suggested low solubility of the polar carotenoids zeaxanthin and canthaxanthin in decane, and therefore their absolute quantities must be interpreted with caution. Produced carotenoid was measured from the aqueous fractions of the two-phase cultures. Cells were collected from a 200-μL aliquot by centrifugation at 16,000 × *g* for 1 min, and the supernatant was removed carefully using a pipette. Cell pellets were treated with 200 μL acetone and were incubated at 55 °C for 15 min with vigorous intermittent vortices to extract the intracellular carotenoid content. The cell debris was then removed by centrifugation at 16,000 × *g* for 2 min, and absorbance of the supernatant was measured, after blanking the spectrophotometer with acetone (certain pure carotenoid standards were insoluble in acetone at the required concentrations to generate $\varepsilon_{acetone}$, so ε_{decane} was used instead, for uniformity with secreted concentrations). Finally, cell viability was assessed from the absorbance of 200 μL of the aqueous culture phase, using a 595-nm filter on a plate reader (Beckman DTX 880). Cell number was calculated using the relation OD_{595} of $1 = 8 \times 10^{11}$ cells/L and was used to calculate the produced carotenoid amounts, presented as milligram per cell × 10^{-12}.

Spheroplast Formation. For lycopene secretion experiments, spheroplasts were made out of healthy BL21 (DE3) cells harboring pAC184- and pET19b-derived plasmids, using a method described before (2, 9, 10). Overnight cultures of the double transformants were collected by centrifugation at 3,000 × *g*, 15 min, for 4 °C. The supernatant was removed, and the cell pellets were washed once with 10 mM KPi buffer, pH 7.0. After collecting the cells again by centrifugation, the pellets were resuspended in 10 mL of a 0.5 M sucrose solution in 10 mM KPi buffer, pH 7.0. Lysozyme (chicken egg white) was then added to a final concentration of 50 μg/mL, and the samples were incubated in a 37 °C water bath for 2 h. After the incubation, 10 mL KPi buffer, pH 7.0, was added to dilute the samples 1:1. Subsequently, 10 mM

K-EDTA, pH 7.0, was added, and the samples were incubated at room temperature for 15 min. The spheroplasts were collected by centrifugation at 500 × *g*, 15 min, for 4 °C. The supernatant was gently removed, and the spheroplasts were resuspended in 10 mL LB medium containing the two antibiotics and 1 mM IPTG and were gently overlayed with 5 mL decane. These two-phase spheroplast cultures were grown at 30 °C, with aeration at 250 rpm under mild-absent light conditions.

ATP Hydrolysis (ATPase) Measurements. ATPase activity measurements on purified *E. coli* MsbA (EcoMsbA) were performed using a colorimetric method described elsewhere (11, 12); 0.5 μg of detergent-purified EcoMsbA (13) was used for each measurement, and 100 mM potassium-Hepes buffer, pH 7.0, containing 5 mM $MgSO_4$ was used as the assay buffer. EcoMsbA diluted in the assay buffer was mixed with a range of concentrations of the substrates to be tested (or equal volumes of their solvents as controls) and incubated on ice for 15 min. The substrates were β-carotene (predominantly trans; MP Biomedicals) (prepared by first making a 1 mM stock in methanol and then diluting 50 μL of it in 1 mL water to produce a 50 μM working stock in 1:20 methanol:water) and spearmint oil (100%, Now Essential Oils) [prepared fresh as a working stock of 0.1% (vol/vol) in anhydrous DMSO]. After incubation on ice, 2.5 mM ATP (dissolved in the assay buffer) was added to each reaction simultaneously using a multichannel pipette. The reactions were incubated at 30 °C for 5 min, followed by protein denaturation at 80 °C for 15 s. A malachite green-ammonium molybdate solution, freshly activated using 1:100 10% Triton X-100, was added to each reaction and incubated for 5 min at room temperature with mild shaking. The absorbance was read on a plate reader (Beckman DTX 880) using a 595-nm filter, and the concentration of Pi was extrapolated from a Pi standard curve. ATPase values are presented as nmoles of Pi liberated from hydrolyzed ATP per minute per milligram of transporter.

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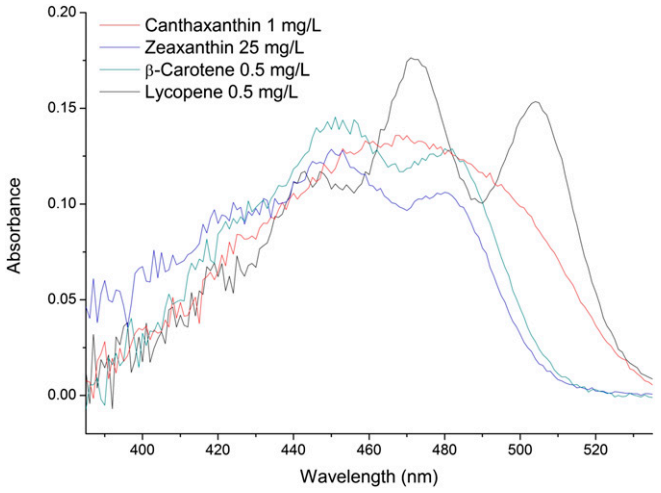

Fig. S1. The absorbance spectra of all carotenoids used in this study. Analytical standard carotenoids, zeaxanthin, canthaxanthin, β-carotene, and lycopene, were dissolved in decane and tested for absorbance in the visible region of the spectrum at the indicated concentrations, blanked against decane. Each carotenoid revealed a characteristic absorbance spectrum between 380 and 540 nm with the following absorbance maxima (λ_{max}): zeaxanthin, 450 nm; canthaxanthin, 460 nm; β-carotene, 453 nm; lycopene, 474 nm. The molar extinction coefficients (ε) obtained in decane at the λ_{max} were as follows: zeaxanthin, 298×10^1 $M^{-1}cm^{-1}$; canthaxanthin, 987×10^2 $M^{-1}cm^{-1}$; β-carotene, 132×10^3 $M^{-1}cm^{-1}$; lycopene, 159×10^3 $M^{-1}cm^{-1}$ (see *SI Materials and Methods* for further details).

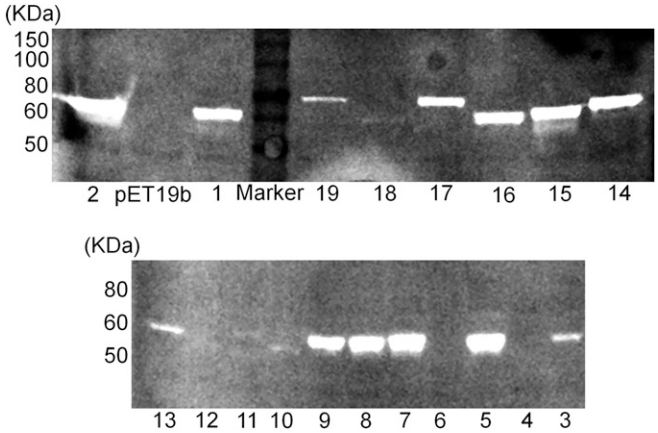

Fig. S2. The expression of 19 ABC transporters used in this study. *E. coli* BL21 (DE3) cells harboring pac184-based plasmid for producing the carotenoid and pET19b-derived plasmid for expressing the ABC transporter were alkaline lysed and loaded onto a reducing 10% SDS/PAGE gel. Western blot analysis using the anti-6xHis primary antibody (Santa Cruz Biotech) showed that apart from transporter numbers 4, 6, and 12, the expression of the rest was detectable, and the proteins migrated close to their predicted sizes (for transporter names and sequences 1–19; Table S1). Cells harboring pET19b empty vector control were devoid of a positive signal.

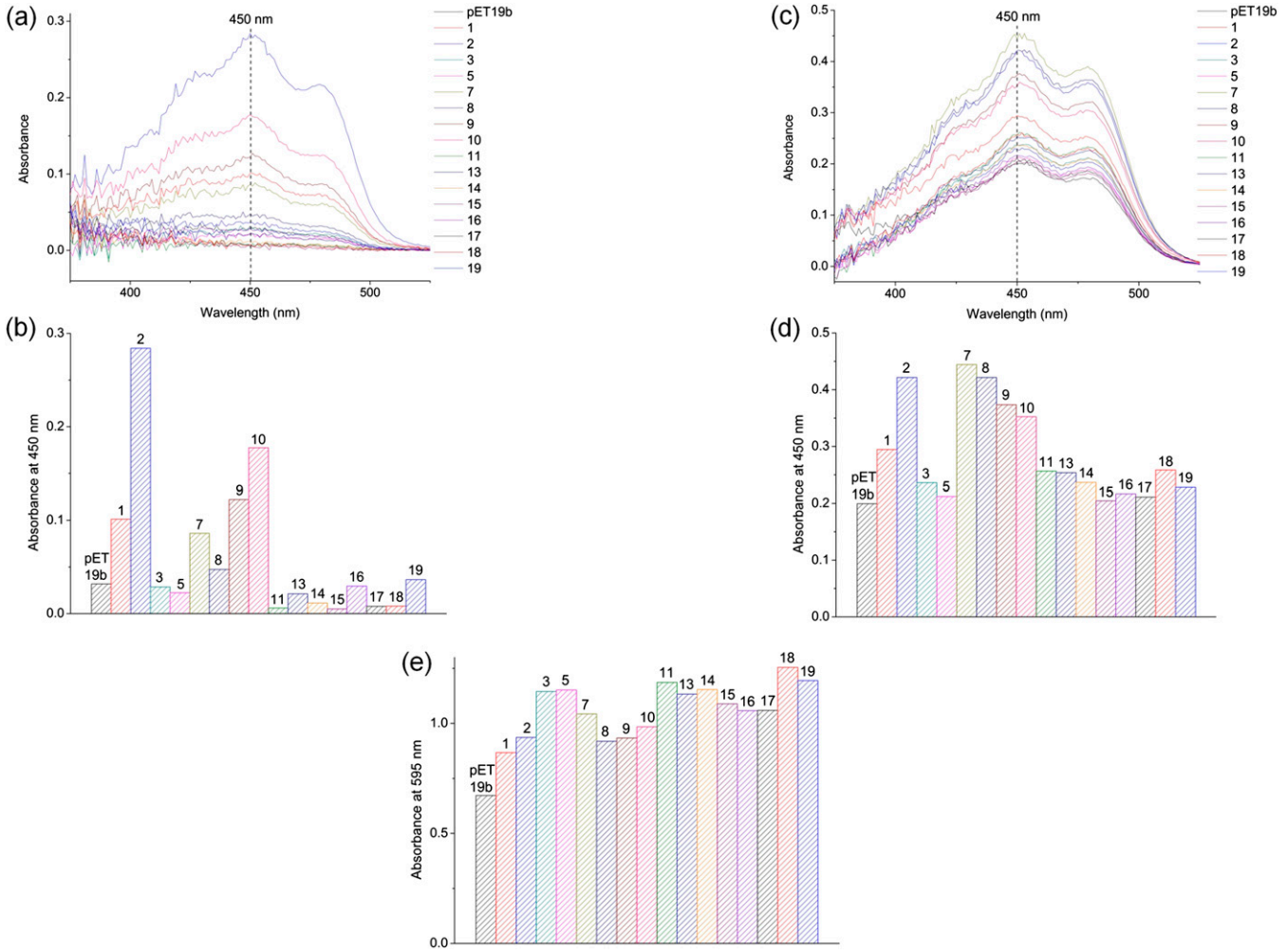

Fig. S3. Transporter secretion screen for zeaxanthin. The constitutive production of zeaxanthin in *E. coli* was achieved through the use of the plasmid pAC-ZEAX*ipi* (1). Double transformant cells producing zeaxanthin and expressing the panel of transporters were grown in two-phase culture systems as described in the legend to Fig. 3, with the following exceptions: 1 mL decane was overlayed over 5 mL cell culture, and the incubation period was 24 h. The relative amounts of zeaxanthin in the samples were estimated by comparing the absorbance values in the visible region of the spectrum (λ_{max} – 450 nm). The raw absorbance values are presented here. (*A* and *B*) Secreted zeaxanthin was measured in the decane fractions of the samples. Debris was removed using centrifugation at 16,000 × *g* for 1 min before making absorbance measurements. (*C* and *D*) Produced intracellular zeaxanthin amounts were measured using 1-mL cell pellets that were treated with acetone for 15 min at 55 °C, after which cell debris was removed by centrifugation as above. (*E*) Cell viability was assessed by measuring the absorbance cultures at 595 nm. Transporter number 2, i.e., *Salmonella enterica* ser. *typhimurium* MsbA, containing mutation I89T (abbreviated StMsbA*), was the best hit in this screen for zeaxanthin secretion.

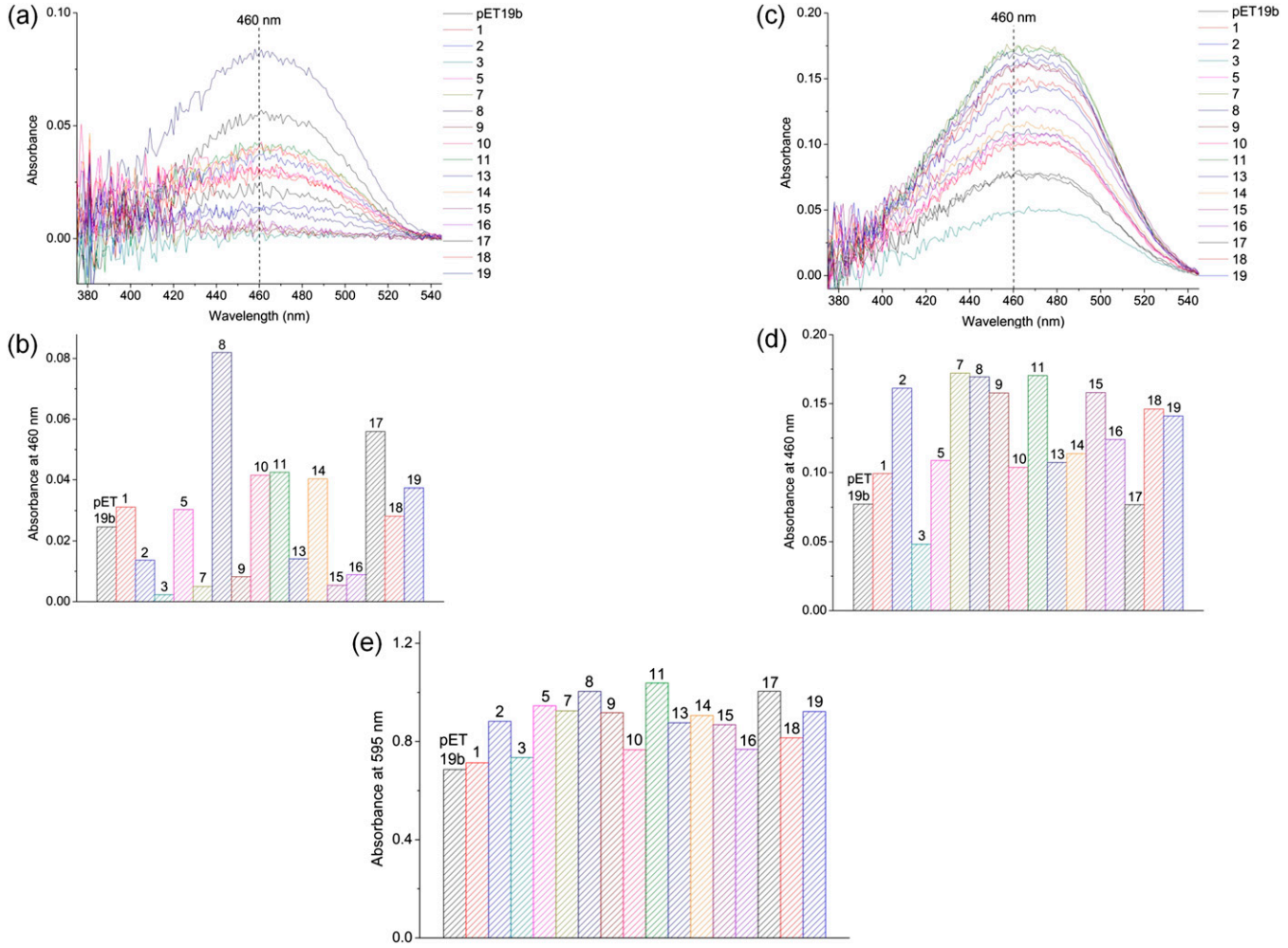

Fig. S4. Transporter secretion screen for canthaxanthin. The constitutive production of canthaxanthin in *E. coli* was achieved through the use of the plasmid pAC-CANTH*ipi* (1). The screen was performed in an identical manner described in the legend to Fig. S3, except the incubation period was 48 h. As suggested by the relative peak absorbance values at λ_{max} – 460 nm pertaining to (*A* and *B*) secreted and (*C* and *D*) produced canthaxanthin and (*E*) the viability of cell cultures, transporter number 8, i.e., *E. coli* MsbA (abbreviated EcoMsbA), was the best hit for canthaxanthin secretion.

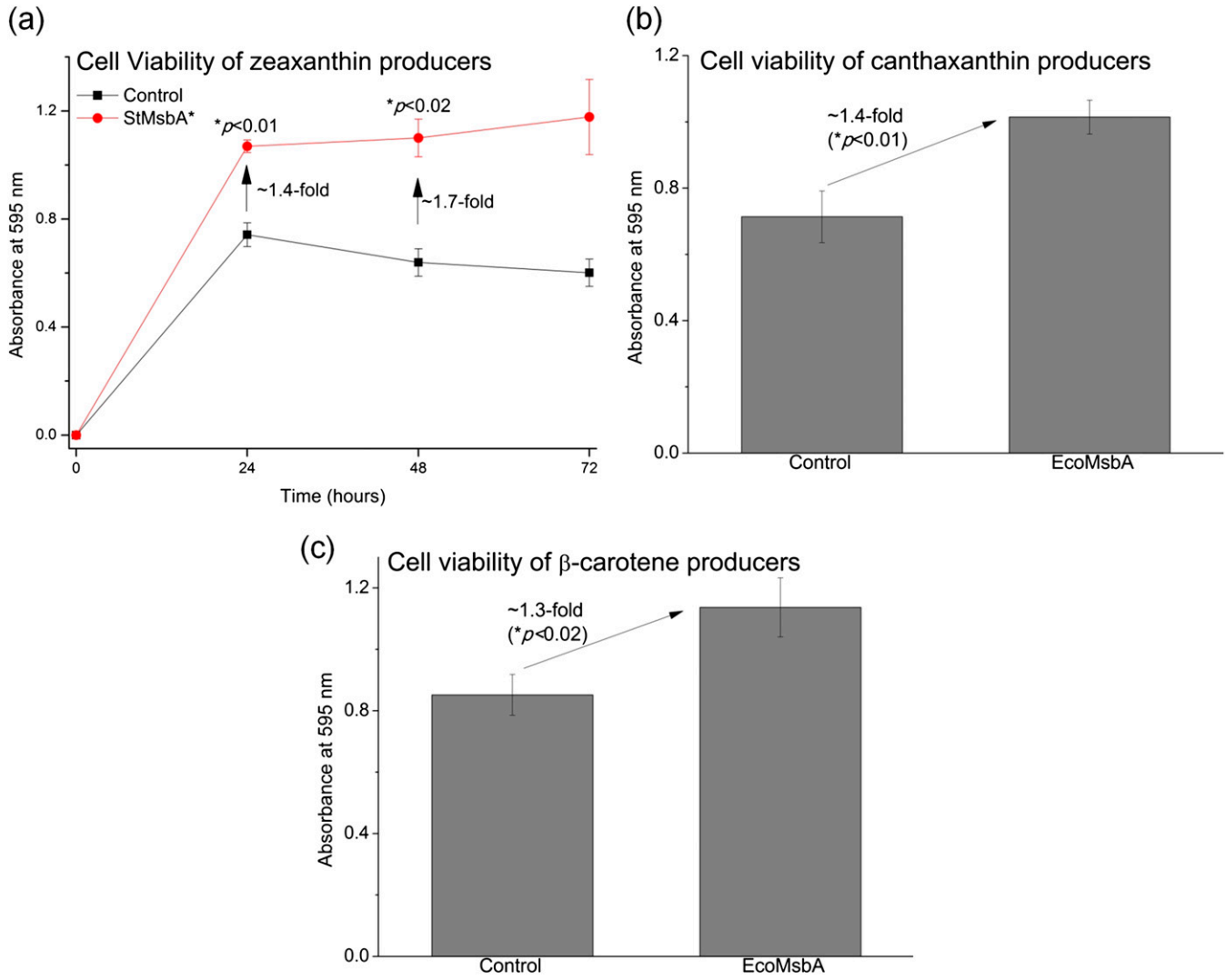

Fig. S5. Transporter expression facilitates cell viability. In double transformant *E. coli* BL21 (DE3) cells overexpressing StMsbA* or EcoMsbA, and producing (*A*) zeaxanthin ($n = 3$ biological repeats ± SEM), (*B*) canthaxanthin ($n = 4$ biological repeats ± SEM), or (*C*) β-carotene ($n = 3$ biological repeats ± SEM), the cell viability measured through absorbance at 595 nm was found to be significantly higher compared with the carotenoid-producing cells containing the non–transporter-expressing empty vector pET19b (*P* from two-tailed, paired Student *t* tests). The time points for measurements were 24–72 h for zeaxanthin-, 96 h for canthaxanthin-, and 144 h for β-carotene–producing cells.

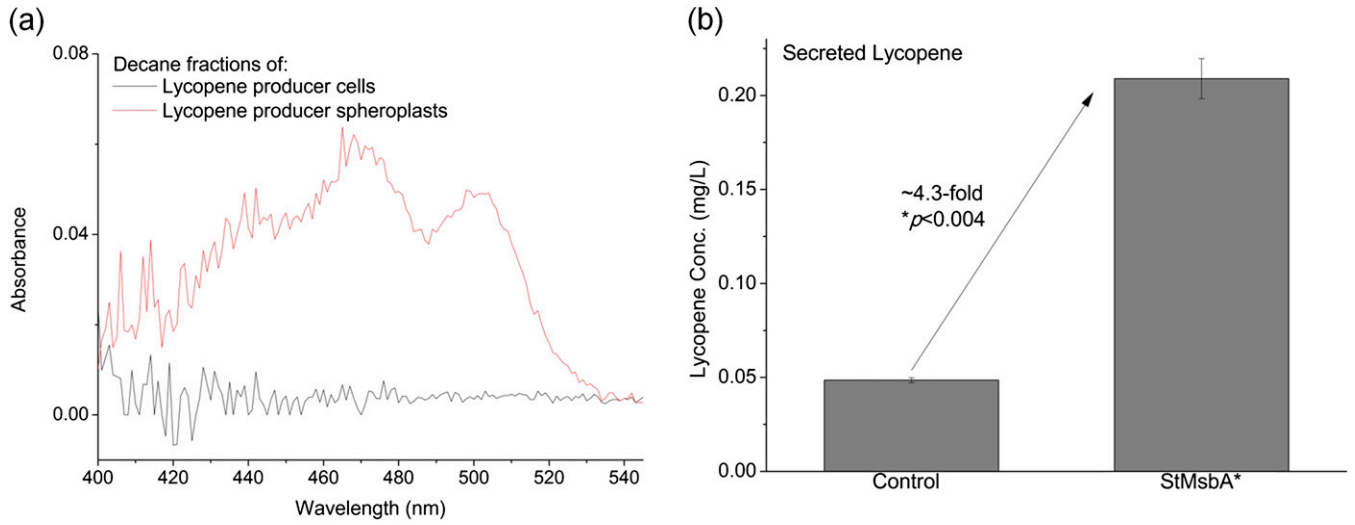

Fig. S6. Lycopene secretion from spheroplasts. The production of lycopene in *E. coli* was achieved using the plasmid pAC-LYC (1). (*A*) Lycopene-producing *E. coli* BL21 (DE3) cells expressing StMsbA* were grown in two-phase culture systems with decane. However, as observed by others (2), we were unable to achieve any extraction of lycopene into the decane phase unless we used *E. coli* spheroplasts instead of whole cells. Spheroplasts were made using a well-established osmolytic method (2, 9, 10). However, the time taken for lycopene to be detected in the decane phase varied between 24 and 72 h, probably due to the variability in spheroplast formation and the proportion of revertants. (*B*) StMsbA*-mediated secretion, as extrapolated from λ_{max} – 474 nm, was found to result in a significant ~4.3-fold increase in lycopene in the decane phase compared with the nonexpressing control ($P < 0.004$; two-tailed paired Student *t* test, $n = 3$ biological repeats ± SEM). Unlike healthy whole cells, spheroplasts did not actively divide during the incubation, as monitored at OD_{595}.

Table S1. Nineteen ABC transporters bearing homology with MsbA were selected to screen for the secretion of isoprenoid compounds in this study

No.	ABC transporter	NCBI accession no.	Mutations
1	MsbA–*Vibrio cholerae*	ZP_01948624	I45V, E133K.
2	MsbA–*Salmonella enterica* ser. *Typhymurium*	YP_005396412.1	I89T.
3	MsbA–*Shewanella* sp. MR-1/MR-4	NP_718380.1/YP_734550.1*	None.
4	MsbA–*Helicobacter pylori*	ZP_14917409	Δ(M1-H54), G147R, S354N, Q391K, A491V, H506N, I549T.
5	MsbA–*Pasteurella multocida*	NP_245798	Q352P.
6	MsbA–*Pseudomonas aeruginosa*	ZP_14648619	G155S, S193L, G232S, A316E, P330S, E413G, C436S.
7	MsbA–*Klebsiella pneumoniae*	YP_001334607	None.
8	MsbA–*Escherichia coli*	ZP_11901836	None.
9	MsbA–*Haemophilus influenzae*	NP_438233	V474G.
10	CydC–*Haemophilus influenzae*	NP_439314	None.
11	CydD–*Haemophilus influenzae*	YP_248818	None.
12	Putative ABC–*Haemophilus influenzae*	NP_438823	N53D.
13	Putative ABC–*Haemophilus influenzae*	NP_439210	None.
14	Putative ABC–*Bacillus subtilis*	ZP_06874379	C6R.
15	Putative ABC–*Aggregatibacter actinomycetemcomitans*	YP_004949254	F109S.
16	MsbA–*Salmonella enterica* ser. *Paratyphi A*	YP_151042.1	None.
17	Putative ABC–*Enterococcus faecalis*	ZP_05559270	E480D.
18	Putative ABC–*Streptococcus pneumoniae*	YP_006063394	L49S, Y416C.
19	Putative ABC–*Chlorobium tepidum*	NP_661163	T450A, D524G.

Among the 19 transporters, some had mutations that were created for the purpose of solving X-ray crystal structures.

*The protein sequence of this transporter has similar (~97%) identity with two sequences in the NCBI database: MsbA–*Shewanella oneidensis* MR-1 (NP_718380.1) and *Shewanella* sp. MR-4 (YP_734550.1). Because this transporter was not found to be a positive hit for biofuel secretion, we did not investigate its identity any further. The N- and C-terminal sequences of the transporter are given for the reference of the reader: N terminus, MTASPKDEMWTVFKRL-LAYLKPMKGMFLLSVAGLIVYGLVDAAFISFIGPFIDKGFSSSTPAISNGIALPTNQGFHADNQVLLIAPIVVILMFSLRGFANFVSTYGISYMSARLIMDMRQQVFQHYLSLPVSYMDK-ENTGNLISKVTFDTEQIARASGSALISIVRDGVTVIGMLGLMFYNSWKLSLCILVIGPIMGLVMTIVSRRFRKVSKQIQTAMGDVSAATEQMIKGHKNVLAFGGQETETARFAKINDRNR-HQNMKLAIAQAISQPLIMVIGSFAL; and C terminus, PESDTGTYTVKRAKGNLRFDNVSFSYEGQERRALDKIDFEVSQGQTLALVGRSGSGKSTIASLVTRFYTGLASGDILLDDVSI-YDYSLKSLRSQVALVSQQVTLFNDTIANSIAYAYPGEVTREQIIEAATLAHAMEFIEQLPDGLDTQVGENGVLLSGGQRQRIAIARAMLRDAPVLILDEATSALDTESEKAIQQGLDN-LRQNRTSVVIAHRLSTIESADQILVVDQGRIVERGTHKSLLELGGMYAKLYQMQFGS.