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An Engineered Microbial Platform for Direct Biofuel Production from Brown Macroalgae

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An Engineered Microbial Platform for Direct Biofuel Production from Brown Macroalgae

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Prospecting macroalgae (seaweeds) as feedstocks for bioconversion into biofuels and commodity chemical compounds is limited primarily by the availability of tractable microorganisms that can metabolize alginate polysaccharides. Here, we present the discovery of a 36–kilo–base pair DNA fragment from *Vibrio splendidus* encoding enzymes for alginate transport and metabolism. The genomic integration of this ensemble, together with an engineered system for extracellular alginate depolymerization, generated a microbial platform that can simultaneously degrade, uptake, and metabolize alginate. When further engineered for ethanol synthesis, this platform enables bioethanol production directly from macroalgae via a consolidated process, achieving a titer of 4.7% volume/volume and a yield of 0.281 weight ethanol/weight dry macroalgae (equivalent to ~80% of the maximum theoretical yield from the sugar composition in macroalgae).

Volatile energy costs and pressure to conserve fossil fuel resources have ignited efforts to produce biofuels and renewable commodity chemical compounds via microbial fermentation of biomass. Pursuant to these goals, microbial engineering aims to increase product yields and bioconversion efficiencies. Equally critical, development of scalable and diverse feedstocks will empower sustainable use of this technology and drive the widespread adoption of renewable bio-economies. At present, corn and sugarcane are vetted industrial feedstocks, but "food versus fuel" concerns may preclude their long-term use. Inedible lignocellulosic plant materials are preferable feedstocks, but current microbial technologies for fermentation of the simple sugars in lignocellulose have yet to overcome the cost of the complex processes needed to release these sugars from recalcitrant polysaccharides (*1*). Therefore, distinct strategies are required to develop scalable and sustainable nonlignocellulosic biomass resources such as marine macroalgae (seaweeds) for use as next-generation feedstocks.

Brown macroalgae exhibit several key features of an ideal feedstock for production of biofuels and renewable commodity chemical compounds. Requiring no arable land, fertilizer, or fresh water resources, cultivation of these crops circumvents economic concerns associated with land management and avoids adverse impacts on food supplies. Macroalgae are already grown for human consumption, but not as a staple crop. Large-scale cultivation is practiced in several countries, yielding 15 million metric tons per year (*2*); in these countries, macroalgae are also used as animal feeds, agricultural fertilizers, and sources of polymers. Because brown macroalgae does not contain lignin, sugars can be released by simple operations such as milling or crushing. This bio-architectural feature gives macroalgae a distinct advantage over lignocellulosic biomass, facilitates higher yields, and averts the need for energy-intensive pretreatment and hydrolysis processes before fermentation. An analysis prepared for the U.S. Department of Energy (DOE) reports a macroalgae productivity of 59 dry metric tons/ha/year and an ideal ethanol yield from macroalgae of 0.254 weight (wt) ethanol/wt dry macroalgae (*2*). Based on these numbers, an optimum bioethanol productivity of 19,000 liters/ha/year is estimated. This value is approximately two times higher than the ethanol productivity from sugarcane and

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5 times higher than the ethanol productivity from corn (*3*).

The most abundant sugars in brown macroalgae are alginate, mannitol, and glucan (glucose polymers in the form of laminarin or cellulose). Ethanol production from glucan and mannitol yields approximately 0.08 to 0.12 wt ethanol/wt dry macroalgae (*2*). However, the full potential of ethanol production from macroalgae cannot currently be realized because of the inability of industrial microbes to metabolize the alginate component. For example, fermentation of glucan in *Saccharina latissima* by *Saccharomyces cerevisiae* produced ~0.45% volume/volume (v/v) ethanol (*4*). Compared with glucose, the catabolism of mannitol generates excess reducing equivalents, causing an unbalanced reduction-oxidation (redox) environment under fermentative conditions. Hence, ethanol production from mannitol is feasible only in the presence of electron shunts (such as micro-aerobic conditions). Semifermentative conditions enabled ethanol production from mannitol by *Zymobacter palmae* with a yield of 0.38 wt ethanol/wt mannitol (*5*). Additionally, this strain cometabolized glucan and mannitol, producing ~1.6% v/v from these sugar fractions in *Saccharina hyperborea* under micro-aerobic conditions (*5*, *6*). In contrast to mannitol, each mole unit of alginate fermented to ethanol consumes two reducing equivalents. Therefore, the catabolic pathway of alginate provides both an additional source of sugars and a counterbalance to the excess-reducing equivalents produced by mannitol catabolism, enabling ethanol fermentation from all three sugar components in macroalgae simultaneously.

Alginate is a linear block copolymer of two uronic acids, β-D-mannuronate (M) and α-L-guluronate (G), arranged in varying sequences, with uniform regions of M (poly-M) or G (poly-G), and/or a mixture of M and G (poly-MG) (*7*). To date, several microorganisms that can metabolize alginate have been characterized. In these microorganisms, a class of enzymes called alginate lyases (Alys) catalyze the depolymerization of alginate into oligomers via an endolytic β-elimination reaction (*8*). Oligomers are further degraded into unsaturated monomers by an exolytic enzyme known as oligoalginate lyase (Oal). These monomers spontaneously rearrange into 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) (*9*). Subsequently, DEH reductase (DehR) reduces DEH into 2-keto-3-deoxy-gluconate (KDG), a common metabolite that is fed into the Entner-Doudoroff (ED) pathway. KDG eventually yields pyruvate and glyceraldehyde-3-phosphate via the activities of KDG kinase (KdgK) and KDG-6-phosphate aldolase (Eda) (Fig. 1A) (*10*, *11*).

A recent report described the engineering of a homoethanol pathway in the alginate-metabolizing microbe *Sphingomonas* sp. A1 (*12*). However, the lack of robustness under standard industrial fermentation conditions, genetic and metabolic intractability, and scarcity of tools for genetic manipulation of such native organisms are likely to prevent their rapid engineering for production optimization or minimization of by-products (*13*). Here, we sought to circumvent the limitations of native strains and unlock the potential of all major sugar components in brown macroalgae for industrial fermentation by engineering the capacity for alginate degradation, uptake, and metabolism (Fig. 1) into the well-characterized microorganism *Escherichia coli*. We chose this microorganism as a prototype because of its natural ability to metabolize mannitol and glucose. Furthermore, *E. coli* has also been used as a host to produce a wide array of native and heterologous metabolites, including alcohols (*14*–*17*), polyketides (*18*, *19*), plant natural products (*20*–*23*), fatty acid ethyl esters (*24*), alkanes (*25*), 1,3-propanediol (*26*), and 1,4-butanediol (*27*).

Engineering a secretable Aly enzyme for alginate degradation in *E. coli*. We first engineered a secretable Aly system to enable efficient and rapid degradation of alginate by our microbial platform. The objective was to couple this

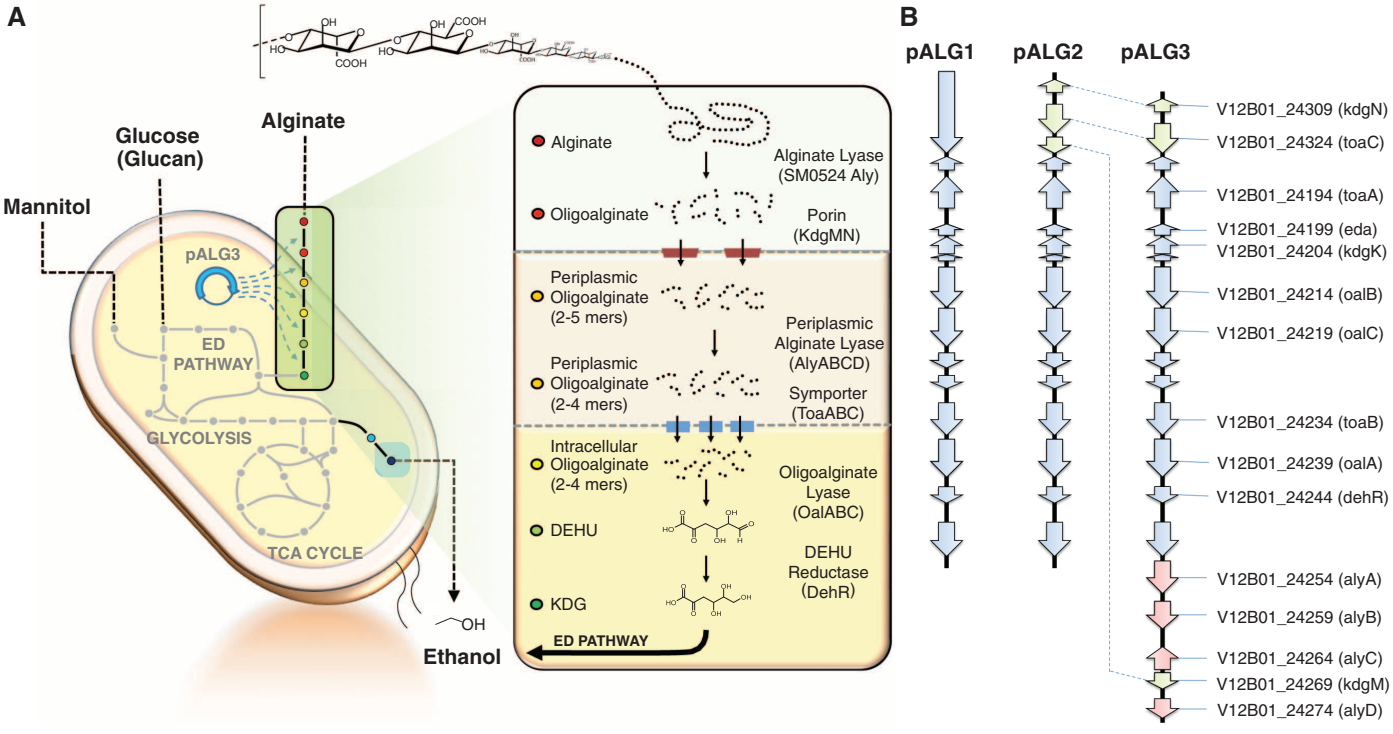

Fig. 1. Design of the microbial platform for production of biofuels and renewable commodity chemical compounds from macroalgae. (**A**) Schematic representation of the engineered *E. coli* platform for alginate degradation, uptake, and metabolism. Alginate polymer is first degraded into oligomers by an alginate lyase. The oligomers are then translocated through the outer-membrane porins (KdgMN) into the periplasmic fraction, where those oligomers with degree of polymerization (DP) > 3 are degraded into di-, tri-, and tetramers by periplasmic alginate lyases (AlyABCD). These subsequent oligomers are transported into the cytosol via oligoalginate transporters (ToaABC). Oligoalginate lyases (OalABC) then degrade oligomers into monomer units (DEH). DEH is converted by DEH reductase (DehR) to KDG, which enters the ED pathway. This platform can be used to synthesize fuels and chemicals such as ethanol. (**B**) The genetic organization of the synthetic pathway (pALG1, pALG2, and pALG3) containing genes discovered in *V. splendidus* 12B01. Blue, original ORFs found from fosmid library generation (pALG1); pink and green, auxiliary genes that improved *E. coli* growth on alginate (added to pALG2 and pALG3, respectively).

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engineered system with the alginate uptake and metabolic pathway, enabling consolidated bioprocessing (CBP) for production of biofuels and renewable commodity chemical compounds directly from macroalgae without thermal and chemical pretreatment or enzymatic saccharification before fermentation. Commonly proposed as a cost-effective strategy, CBP combines enzyme production, feedstock degradation and metabolism, and product formation in a single-process step, thus eliminating the costs of production and purification of feedstock-degrading enzymes (*1*, *28*). Considering that some energy and carbon flux must necessarily be diverted toward Aly synthesis, a single enzyme with a low molecular weight and high, bifunctional (both M- and G-polymer–specific) activity was desirable. The Aly derived from *Pseudoalteromonas sp.* SM0524 (SM0524 Aly) was an attractive candidate because it is a small bifunctional enzyme (32 kD) that mainly produces dimers, trimers, and tetramers from alginate substrates (*29*). In addition, SM0524 Aly is secreted by its natural producer and is thus functional in the extracellular milieu. Therefore, we chose this enzyme as a model Aly.

Next, we identified a system to be engineered for secretion of SM0524 Aly. Numerous surface-display and medium secretion systems have been developed in *E. coli* for various applications. However, to avoid compromising downstream product synthesis and to ensure the overall efficiency of the CBP process, we established a number of specific criteria: (i) The system must not require coexpression of complex and multicomponent secretion machinery such as Out (*30*, *31*); (ii) secretion must occur constitutively without reliance on further treatment (*32*); (iii) the enzyme must be secreted into the medium (detached from the cell surface) because faster diffusion and better access to the substrate can provide more efficient degradation; (iv) to ensure economical usage of cellular resources, the major fraction of the enzyme expressed must be deployed into the medium; and (v) secretion of the enzyme must begin at the early stages of fermentation, enabling rapid substrate degradation for subsequent metabolism and chemical production by the fermenting cell.

We found antigen 43 (Ag43) to be an excellent candidate that could potentially satisfy all of these criteria. It is an autotransporter protein native to most *E. coli* strains that contains within itself all the information required for membrane localization and extracellular secretion (*33*). Ag43 is produced as a pre-protein composed of an N-terminal signal peptide that directs membrane translocation, a succeeding passenger or α domain, and a C-terminal carrier or β domain (Fig. 2A) (*34*). Maturation of the pre-protein into Ag43α and Ag43β subunits arises from removal of the 52–amino acid signal peptide by a signal peptidase and an internal cleavage between amino acids D551 and P552, presumably through autocatalysis. (Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.) This cleavage mechanism is supported by the presence of an aspartyl protease active site within Ag43α (*35*). Ag43α is naturally secreted outside the cell yet remains bound to the cell surface via noncovalent interaction with Ag43β, which forms an integral outer-membrane protein (*35*). This indicates the presence of residues within Ag43α responsible for such binding interaction.

Although Ag43 has been previously engineered to display epitopes and protein domains on the cell surface of bacteria (*36*), we wanted our system to release Aly beyond the cell surface and into the surrounding medium. To this end, we implemented two important design specifications in engineering our Ag43-mediated Aly secretion system (Fig. 2A). First, to prevent surface attachment we removed a substantial portion of Ag43α, from amino acid A52 to N455. We replaced this region of the passenger domain with a truncated SM0524. This truncated SM0524 (tSM0524 Aly), containing the catalytic domain for alginate degradation, begins at amino acid D168 of the full-length lyase (GenBank no. ACB87607). Second, we added the aspartyl protease active site, previously removed from the region between A52 and N455, back at a location C-terminal to tSM0524 Aly and immediately

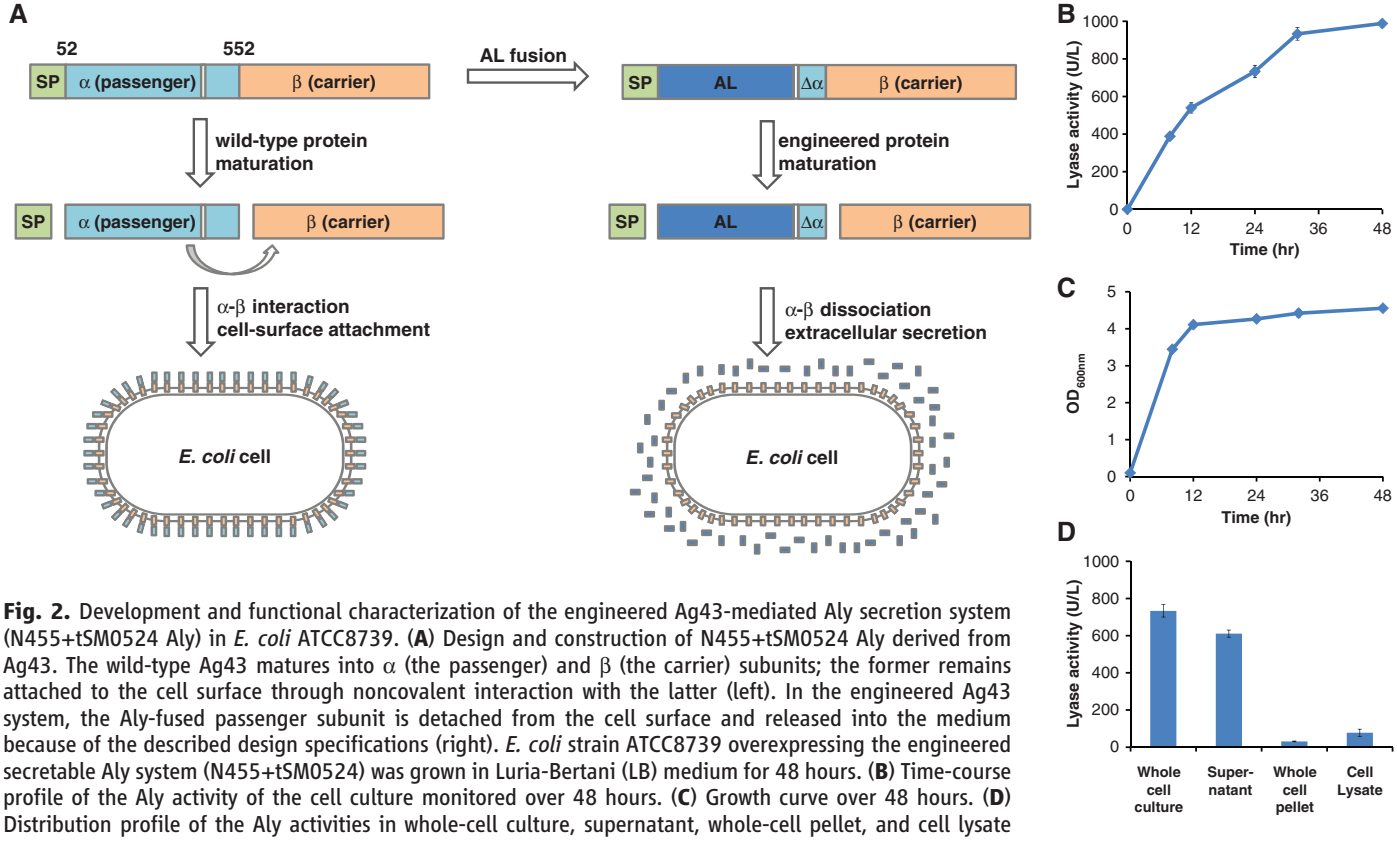

Fig. 2. Development and functional characterization of the engineered Ag43-mediated Aly secretion system (N455+tSM0524 Aly) in *E. coli* ATCC8739. (**A**) Design and construction of N455+tSM0524 Aly derived from Ag43. The wild-type Ag43 matures into α (the passenger) and β (the carrier) subunits; the former remains attached to the cell surface through noncovalent interaction with the latter (left). In the engineered Ag43 system, the Aly-fused passenger subunit is detached from the cell surface and released into the medium because of the described design specifications (right). *E. coli* strain ATCC8739 overexpressing the engineered secretable Aly system (N455+tSM0524) was grown in Luria-Bertani (LB) medium for 48 hours. (**B**) Time-course profile of the Aly activity of the cell culture monitored over 48 hours. (**C**) Growth curve over 48 hours. (**D**) Distribution profile of the Aly activities in whole-cell culture, supernatant, whole-cell pellet, and cell lysate samples prepared from the 24-hour cell culture. SD of the triplicate measurements is shown in error bars.

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N-terminal to N455. We expected that with these two design specifications, the passenger fusion protein consisting of the Aly would be released to the medium from the carrier. Using a single-copy plasmid vector, we expressed this engineered Aly secretion construct (N455+tSM0524 Aly) in an *E. coli* strain (ATCC8739) under the control of a constitutive promoter ($P_{D/E20}$) derived from bacteriophage T5.

We examined the functional performance of our Aly secretion system by applying samples of interest to an alginate solution and monitoring their alginate-degrading activities. A 48-hour time-course of the cell culture medium showed Aly activities as high as 1000 U/liter. Forty percent of the Aly activity was detected within the first 10 hours of cell growth, indicating that tSM0524 Aly can be rapidly secreted for effective substrate degradation in the early stages of fermentation (Fig. 2B). Analysis of the whole-cell culture, supernatant, whole-cell pellet, and cell lysate prepared from the cell culture growing at 24 hours revealed that the Aly activity in the supernatant was significantly higher than the activities in the cell pellet or lysate and similar to that in the whole-cell culture. This result indicated that tSM0524 Aly was efficiently secreted into the medium, with little remaining on the cell surface or inside the cell (Fig. 2C).

Table 1. ORFs in pALG1, pALG2, and pALG3 and their annotated functions. Dashes substitute for the gene name when the function is not established.

Locus tag	Gene size (bp)	Annotated function based on conserved domain	Assigned gene name
V12B01_24189	762	Solute-binding protein	—
V12B01_24194	1764	Sodium/solute symporter	*toaA*
V12B01_24199	624	KDGPA/eda	*eda*
V12B01_24204	930	KDGK	*kdgK*
V12B01_24209	333	Unknown	—
V12B01_24214	2205	HeparinaseII/III–like protein and alginate lyase	*oalB*
V12B01_24219	2151	HeparinaseII/III–like protein and alginate lyase	*oalC*
V12B01_24224	822	Fumaryl-acetoacetate hydrolase, 4-hydroxyphenylacetate decarboxylase	—
V12B01_24229	714	GntR transcriptional repressor	—
V12B01_24234	1776	Sodium/solute symporter	*toaB*
V12B01_24239	2076	HeparinaseII/III–like protein	*oalA*
V12B01_24244	879	NADPH/NADH dehydrogenase	*dehR*
V12B01_24249	1869	Signaling protein/chemoreceptor	—
V12B01_24269	858	Oligogalacturonate-specific porin	*kdgM*
V12B01_24309	723	Oligogalacturonate-specific porin	*kdgN*
V12B01_24324	1656	Sodium/solute symporter	*toaC*
V12B01_24254	1740	Alginate lyase	*alyA*
V12B01_24259	1566	Alginate lyase	*alyB*
V12B01_24264	1230	Alginate lyase	*alyC*
V12B01_24274	1038	Alginate lyase	*alyD*

Engineering the metabolic pathway for transport and metabolism of alginate oligomers in *E. coli*. We engineered functional alginate transport and metabolic systems into our host strain via expression of genes from a heterologous DNA fragment (Fig. 1A). The enzymes responsible for alginate metabolism in several bacteria have been described, but the only known alginate transport system is found in *Sphingomonas* sp. A1. This transport system comprises an unusual outer-membrane protein complex and inner-membrane adenosine 5′-triphosphate (ATP)–binding cassette (ABC) transporter, which together can directly incorporate alginate polymer into the cytosol (*37*). Functional expression of such a large and complicated alginate transport system in *E. coli* is daunting and has never been demonstrated. However, other systems such as the *Erwinia chrysanthemi* oligopectin ABC transporter TogMNAB and symporter TogT (*38*) are much simpler. Considering the similarities between pectin and alginate, as well as the widespread occurrence of the Tog system in pectinolytic

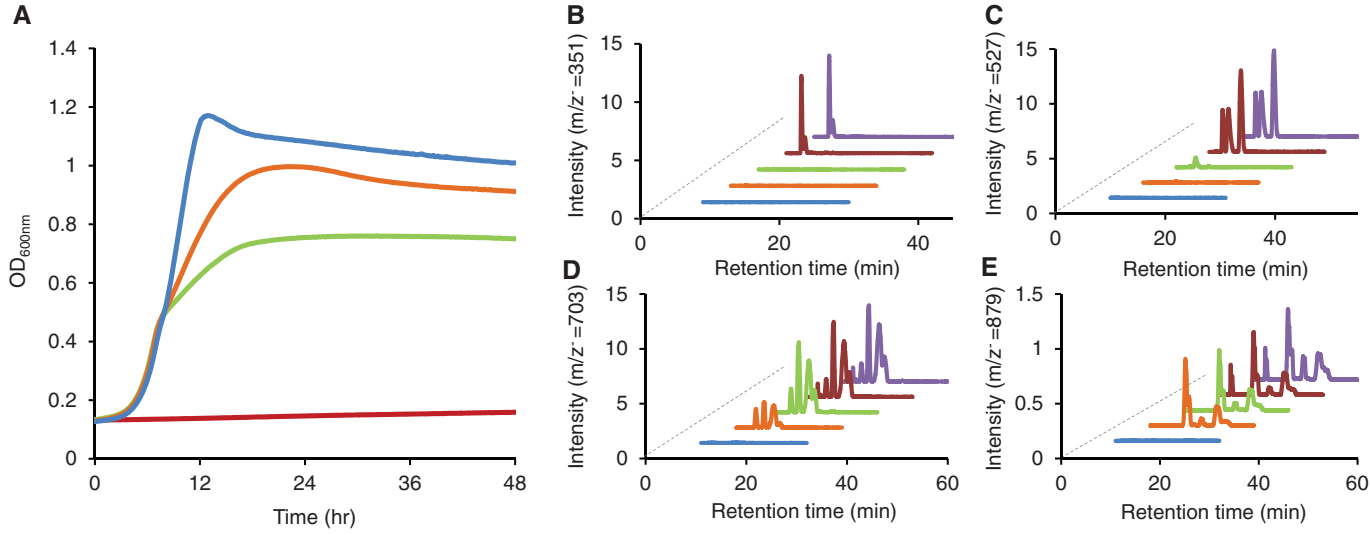

Fig. 3. *E. coli* growth on alginate predegraded by SM0524 Aly after sequential incorporation of auxiliary transporters and Alys into pALG1. (**A**) Growth phenotypes of *E. coli* strain ATCC8739 harboring pBeloBAC11 (red), pALG1 (green), pALG2 (orange), and pALG3 (blue) on M9 salt medium containing 1% alginate predegraded with SM0524 Aly. An average of three independent growth curves is shown in each case. SD of these triplicate measurements was smaller than 2%. The mass spectrometric analyses of the growth medium for residual (**B**) dimer [mass/charge ratio (m/z^-) = 351], (**C**) trimer (m/z^- = 527), (**D**) tetramer (m/z^- = 703), and (**E**) pentamer (m/z^- = 879) after a 48-hour culture. Starting medium (purple), negative control pBeloBAC11 (red), pALG1 (green), pALG2 (orange), and pALG3 (blue).

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bacteria, we postulated that a gene cluster encoding similar transport machineries and metabolic enzymes might be found within the genome of some alginolytic bacteria.

In searching the genomes of bacteria available in the National Center for Biotechnology Information database, we recognized a 30–kilo–base pair (kbp) fragment that might contain all necessary genes for alginate degradation, transport, and metabolism within the genome of *Vibrio splendidus* 12B01 (V12B01_24199 to V12B01_24274 in the Gene Identification AAMR00000000). However, direct cloning of a 30-kbp DNA fragment is technically challenging, and reconstruction of the pathway with the targeted genes was even more difficult because the function and regulation of these genes had not been characterized. We overcame these challenges by creating a fosmid library of random DNA fragments using the genomic DNA of this microbe. *E. coli* transformants harboring this library were then screened for growth on minimal-salt medium containing alginate oligomers. We found that colonies that grew on this carbon source contained a fosmid with the insertion of a ~40-kbp genomic fragment. The fosmid, designated pALG1, consisted of intact open reading frames (ORFs) V12B01_24189 to V12B01_24249 (Fig. 1B). This fragment contains the same genes putatively responsible for alginate metabolism located in our original search (Table 1). No other fragments were found in positive transformants, suggesting a lack of alginate metabolism variation in *V. splendidus*. pALG1 comprised several intact ORFs. Assignment of protein function showed that the ORFs in pALG1 encode for enzymes exclusive to *V. splendidus*—with the exception of KDGK and KDGPA, the enzymes in the ED pathway, which are also present in *E. coli* (Table 1).

To probe the function and necessity of each gene in conferring this growth phenotype, we individually deleted each corresponding ORF (predicted to be responsible for alginate metabolism) in pALG1. Growth was assessed on alginate predegraded by SM0524 Aly. As shown in fig. S1, individual deletion of the ORFs in pALG1 encoding for V12B01_24189, V12B01_24224, V12B01_24229, V12B01_24234 (a putative sodium/solute symporter we termed ToaB), and V12B01_24239 (a putative heparinaseII/III-like protein) (fig. S1, C and G to J) did not affect *E. coli* growth, indicating that they are not essential in conferring alginate oligomer metabolism. The rest of the ORFs, when individually deleted, reduced the ability of the *E. coli* host to grow on predegraded alginate. Deletion of ORF V12B01_24194, which encodes for another putative sodium/solute symporter (which we termed ToaA), completely abolished the growth phenotype of our strain (fig. S1D). We have thus identified an essential transport system of alginate oligomers that has not been previously described.

Deletion of three ORFs that encode for putative heparinaseII/III–like protein/alginate lyases impaired growth of the *E. coli* host (figs. S1, E, F, and J, and S2). On the basis of the model pathway for alginate metabolism, we speculated that these three enzymes function as oligoalginate lyases (Oal), similar to the recently characterized A1-IV from *Sphingomonas* sp. A1, and Atu3025 from *A. tumefaciens* C58 (40 to 45% primary sequence identity) (*39*, *40*). We designated the enzymes encoded by the V12B01_24239, V12B01_24214, and V12B01_24219 ORFs OalA, OalB, and OalC, respectively. Deletion of ORFs encoding for OalB and OalC resulted in limited impact on degradation of oligomers in vitro, but deletion of the ORF encoding for OalA dramatically reduced degradation of alginate oligomers (fig. S3). It is not clear why the OalA deletion had minimal impact on the growth of *E. coli*, whereas deletion of OalB and OalC delayed cell growth on oligoalginate (fig. S2). However, taken together these results suggest that degradation of the various alginate oligomers proceeded most effectively when all three putative Oal enzymes were present (figs. S1 to S3).

We also found that deletion in pALG1 of ORF V12B01_24244, which encodes for a putative NADPH/NADH–dependent alcohol dehydrogenase (NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; NADH, reduced form of nicotinamide adenine dinucleotide), seriously impaired the growth of our strain (fig. S1K). This enzyme exhibits ~40% DNA sequence identity with the recently characterized NADPH-dependent uronate reductase from *Sphingomonas* sp. strain A1 that catalyzes conversion of DEH into KDG (*11*). However, biochemical assays demonstrated a preference of this gene's enzyme product for NADH as a cofactor rather than NADPH (fig. S4). These results describe a distinct NADH-dependent DehR. Altogether, we identified the minimum genetic prerequisites for alginate oligomer utilization including a symporter (ToaA) and an NADH-dependent DehR.

Expression of auxiliary genes to improve alginate utilization. We next explored other ORFs (Table 1) in the flanking regions of the isolated 36-kbp fragment from *V. splendidus* 12B01 that may be involved in alginate metabolism. Because they are similar to the outer membrane oligopectin porins in *Erwinia* sp. (*41*), we hypothesized that ORFs V12B01_24269 and V12B01_24309 encode for transport systems (termed KdgM and KdgN, respectively) important for channeling alginate oligomers through the outer membrane. We also recognized that ORF V12B01_24324 exhibited high sequence identity to ToaA (64%) and ToaB (88%); hence, we named it ToaC. To investigate the effect of expressing these putative auxiliary transporters, we cloned the ORFs into pALG1 to yield pALG2 (Fig. 1B). We also recognized other ORFs (V12B01_24254, V12B01_24259, V12B01_24264, and V12B01_24274) with a high degree of similarity to the previously discovered Alys. These ORFs encode for enzymes we designated AlyA, AlyB, AlyC, and AlyD, respectively. To test the effect of expressing these additional putative Alys, we cloned the corresponding genes along with genes encoding KdgMN and ToaC into pALG1 to create pALG3 (Fig. 1B). We tested the growth of *E. coli* harboring these new constructs on predegraded alginate (Fig. 3A) and showed that the final cell-culture density of strains harboring pALG2 and pALG3 increased as compared with those expressing pALG1 (Fig. 3A). Compared with *E. coli* strains expressing pALG1, *E. coli* strains expressing pALG2 greatly reduced populations of alginate tetramers in the medium, and only traces of alginate trimers remained (Fig. 3, B to D). With expression of AlyABCD, *E. coli*

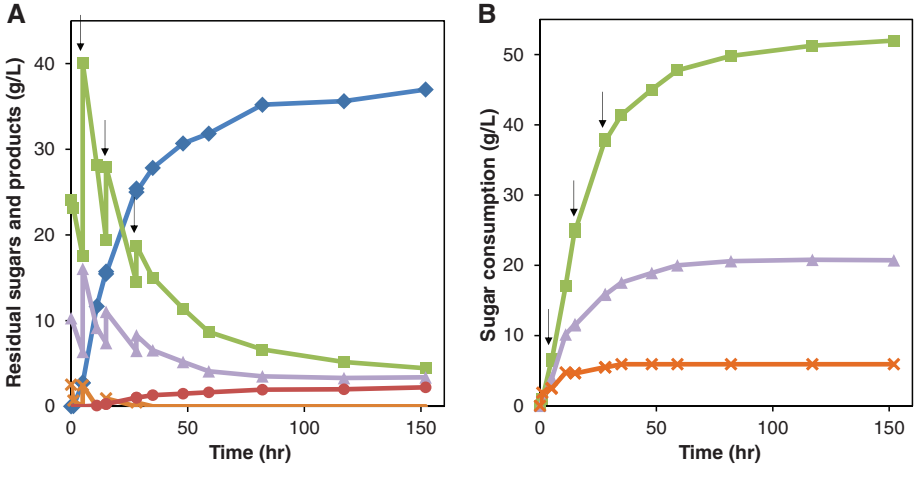

Fig. 4. Fed batch fermentation of macroalgae *S. japonica* to ethanol using BAL1611. (**A**) Ethanol production and carbohydrate consumption profiles. (**B**) Total carbohydrate consumption. Ethanol (blue), mannitol (green), alginate (purple), glucose (orange), and succinate (red). The starting medium contains 50 g of dry milled macroalgae. The arrows denote the time points when dry milled macroalgae (50, 20, and 10 g at 5, 15, and 28 hours, respectively) was added to the fermentation to make the final working volume of 1 liter. These data are representative of nine independent fermentation runs, ranging in final ethanol titer from 35 to 41 g/liter (average, 37.8 g/liter; SD = 2.4 g/liter) in the same timeframe.

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harboring pALG3 consumed all oligomer populations. These auxiliary genetic components thus help enable complete alginate oligomer utilization (Fig. 3, B to E).

Ethanol fermentation from macroalgae using the engineered *E. coli*. After installation of the alginate metabolism pathway genes from pALG3 into the *E. coli* chromosome, we evaluated the performance of this platform for producing ethanol directly from macroalgae. To engineer an efficient ethanol-production phenotype (*17*), we introduced a heterologous homoethanol pathway consisting of *Zymomonas mobilis* pyruvate decarboxylase (Pdc) and alcohol dehydrogenase B (AdhB). To divert carbon flux away from fermentative by-products, we deleted *pflB-focA*, *frdABCD*, and *ldhA* from the genome of the host strain. We first confirmed the ability of the resultant strain (BAL1366) to secrete alginate lyase (fig. S5A) and grow on alginate without an enzymatic saccharification step (fig. S5B). We then incorporated the engineered secretable alginate lyase system, N455+tSM0524 Aly, into the chromosome of this strain. The resulting strain (BAL1611) was tested for its ability to ferment a 5% sugar mixture consisting of alginate, mannitol, and glucose at a ratio of 5:8:1, simulating a typical sugar ratio in brown macroalgae (fig. S6). To explore the engineered strain's fermentation performance at different temperatures, we tested BAL1611 at 25°, 30°, and 37°C (fig. S6). We found that higher ethanol titers were achieved (up to approximately 20 g/liter or 2.4% v/v) when the operating temperature was maintained at 25° to 30°C. This temperature range was therefore chosen for subsequent laboratory-scale fermentation experiments.

We used *Saccharina japonica* (kombu), a common and widely available brown macroalgae, as a model fermentation substrate. Using our microbial platform, we demonstrated macroalgae fermentation producing ethanol at a final titer of ~4.7% v/v, which is comparable with the recently reported benchmark ethanol titer derived from lignocellulosic biomass fermentation using *S. cerevisiae* (*42*). This ethanol titer corresponds to a bioconversion value of ~0.281 wt ethanol/wt biomass with yield of ~0.41 wt ethanol/wt total sugars (alginate, mannitol, and glucan). This fermentation achieved over 80% of the maximum theoretical yield. Additionally, 83% of this titer was achieved within the first 48 hours of fermentation, corresponding to an overall rate of 0.64 g/liter/hour (Fig. 4A), with a sugar consumption ratio of approximately 2.5 mol mannitol/mol alginate (Fig. 4B).

The common fermentation by-product lactate was present at a final concentration of approximately 2 g/liter, a relatively small amount. This lactate formation may be due to a function of the methylglyoxylate pathway (*43*). Acetate, succinate, and formate concentrations were extremely low and could not be accurately quantified. These results indicate that carbon flux from pyruvate was efficiently channeled toward ethanol synthesis. Moreover, although *E. coli* can naturally assimilate mannitol and glucose, our engineered strain substantially increased the rate of mannitol fermentation and the resulting ethanol titer relative to the native process (fig. S7). These results support our hypothesis that the alginate pathway plays an important role in balancing the intracellular redox environment by consuming the excess-reducing equivalents generated from mannitol catabolism. Overall, by facilitating simultaneous alginate degradation and assimilation, we demonstrated that our tractable, engineered microbial platform can efficiently ferment all sugars in macroalgae into ethanol.

Catabolism of alginate, mannitol, and glucose in our engineered microbe generates the common metabolic intermediate pyruvate, from which a plethora of fuels and commodity chemical compounds could be synthesized. Hence, our CBP-enabling microbial platform for conversion of marine biomass would be complementary to and synergistic with microbial platforms that will be developed for lignocellulosic feedstocks, contributing toward a broad effort to realize production of renewable fuels and commodity chemical compounds from sustainable biomass resources.

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Materials and Methods
Figs. S1 to S7
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