Molecular and functional characterization of a cDNA encoding 4-hydroxy-3-methylbut-2-enyl diphosphate reductase from *Dunaliella salina*

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**KEYWORDS**
- Carotenogenesis
- *Dunaliella salina*
- 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase
- Isoprenoid biosynthesis
- Methylerythritol phosphate pathway

**Summary**
In green algae, the final step of the plastidial methylerythritol phosphate (MEP) pathway is catalyzed by 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR; EC: 1.17.1.2), an enzyme proposed to play a key role in the regulation of isoprenoid biosynthesis. Here we report the isolation and functional characterization of a 1959-bp *Dunaliella salina* HDR (*DsHDR*) cDNA encoding a deduced polypeptide of 474 amino acid residues. Phylogenetic analysis implied a cyanobacterial origin for plant and algal HDR genes. Steady-state *DsHDR* transcript levels were higher in *D. salina* cells submitted to nutritional depletion, high salt and/or high light, suggesting that *DsHDR* may respond to the same environmental cues as genes involved in carotenoid biosynthesis.

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**Introduction**
Isoprenoids (also called terpenoids) comprise the largest group of natural products and play essential roles in all organisms. Plants isoprenoids are involved in several biological processes (e.g. photosynthesis, membrane fluidity, growth regulation, cell division, communication and defense responses; Bouvier et al., 2005) and have numerous...
commercial and industrial purposes (e.g. carotenoids applied to human health; Rao and Rao, 2007).

Two distinct pathways are involved in the biosynthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the universal C5 precursors of isoprenoids: the cytosolic mevalonate (MVA) pathway, present in most organisms, but apparently absent in Chlorophyta, and a plastidial pathway known as the methylenotetritol phosphate (MEP) pathway, which occurs in eubacteria, cyanobacteria, apicomplex parasites, algae and higher plants (reviewed in Lichtenthaler et al., 1997; Rohmer, 1999; Schwender et al., 2001; Rodriguez-Concepción and Boronat, 2002; Liu et al., 2005). Cross-talk between these two pathways occurs (Laule et al., 2003; Dudareva et al., 2005) and the complex regulation of this non-mevalonate pathway, which involves eight identified enzymes, still remains unclear (Rohmer, 2003; Hunter, 2007). It has been suggested that several enzymes may regulate the metabolic flux through the MEP pathway, and two rate-determining enzymes have been identified, namely 1-deoxy-d-xylulose 5-phosphate synthase (DXS, EC: 4.1.3.37) and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR, EC: 1.17.1.2). DXS is involved in the initial step, while HDR is responsible for the conversion of 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) to IPP and DMAPP in the final step of the MEP pathway (Lichtenthaler, 1999; Eisenreich et al., 2004).

Recently, selective gene knockout has demonstrated not only that HDR activity is essential in E. coli (Cunningham et al., 2000; Altincicek et al., 2001), but also that this enzyme plays a role in plant isoprenoid biosynthesis (Page et al., 2004). In addition to transcription and post-transcriptional events modulating the MEP pathway (Guevara-Garcia et al., 2005; Rodriguez-Concepción, 2006), evidence of coordinated control between this and other downstream pathways (e.g. carotenoid biosynthetic pathway) has been shown (Lois et al., 2000; Rodriguez-Concepción et al., 2003; Wille et al., 2004). Moreover, over-expression of several MEP pathway enzymes in Arabidopsis thaliana, Mentha piperita and Lycopersicon esculentum resulted in higher accumulation of plastid isoprenoids (Estévez et al., 2001; Mahmoud and Croteau, 2001; Botella-Pavia et al., 2004; Carretero-Paulet et al., 2006).

Plastidial isoprenoid end-products such as carotenoids have been commercially exploited for numerous market applications. An example of these is β-carotene, a carotenoid possessing well-known antioxidant and immunomodulatory activities (Murthy et al., 2005; Raja et al., 2007a) and whose main natural source is the green alga Dunaliella salina (Del Campo et al., 2007; Raja et al., 2007b). This microalga can accumulate more than 10% of algal dry weight of β-carotene under particular abiotic conditions (Ben-Amotz and Avron, 1983; Borowitzka et al., 1990; Cifuentes et al., 1996; Coesel et al., 2008). Therefore, understanding the regulation of isoprenoid biosynthesis in this halophilic organism is important for potential biotechnological and metabolic engineering purposes (León-Bañares et al., 2004).

To date, the enzymes and the regulatory mechanisms involved in MEP pathway in D. salina (Ye et al., 2008) and other green algae remain largely unknown and recent evidence suggests a major role of HDR in controlling the flux of MEP-derived precursors in plants (Botella-Pavia et al., 2004). By means of a subtractive cDNA library generated with cells under abiotic stress (nutrient and salt stress), we were able to isolate a stress-inducible D. salina HDR homolog (DsHDR). In this study, we report the cloning of a full-length DsHDR cDNA encoding the first enzyme of D. salina MEP pathway to be described and characterized. Biotechnological applications of this study are discussed.

Material and methods

Alga growth conditions and analytical methods

Dunaliella salina strain CCAP 19/30 was obtained from the Culture Collection for Algae and Protozoa (Cumbria, UK). The cells were maintained and cultured in modified Walne medium (Walne, 1974) under conditions described previously (9% salinity [w/v] and continuous illumination – 45 μmol m−2 s−1; Ramos et al., 2008) and salinity levels were adjusted with NaCl (9% or 18% w/v). Cell number, total nitrate concentration of the media and total β-carotene content of the cells were determined as described previously (Ramos et al., 2008). Covariance analysis (ANCOVA) and post-hoc comparison test (Fisher’s least significant difference method – LSD) were performed with the computational program STATISTICA v. 6.0 (StatSoft). Differences were considered to be significant at a probability of 5% (p < 0.05).

Carotenogenesis inductive experiments were performed with exponentially growing Dunaliella cultures (2.5 × 10⁶ cells mL⁻¹) and concerned three abiotic stress conditions: high salt, nutrient depletion and high light. Cultures were diluted in equal amounts of either Walne medium
(nutrient-supplemented) or water (nutrient-depleted) at a salinity of 27% (w/v) to obtain a NaCl concentration of 18% (w/v). High light stress was performed with an additional white lamp (150 W Massive N.V/S.A), which increased the light intensity levels from 45 to 500 \( \mu \text{mol m}^{-2} \text{s}^{-1} \).

**Total RNA isolation**

Standard methods (Sambrook et al., 1989) were used unless otherwise indicated. Total RNA was extracted from 5 to 10 \( \times 10^6 \) cells using the TRI REAGENT\textsuperscript{TM} (Sigma) method according to the supplier’s protocol.

**Cloning of DsHDR**

Dot-blot analysis of a \( D. \textit{salina} \) subtractive cDNA library constructed with cells subject to abiotic stress (high salinity and nutrient depletion) allowed the isolation of several cDNA clones differentially expressed under the tested conditions, including a partial one with homology to published plant HDR sequences (data not shown; GenBank accession number EG591728).

Several rapid amplifications of cDNA ends (RACE) in order to reach the 5’ and 3’ ends of this gene (data not shown) were performed using the SMART\textsuperscript{TM} RACE cDNA Amplification Kit (BD Biosciences Clontech). The full-length \( DsHDR \) cDNA was amplified with the sense primer HDR1Fw and the anti-sense primer HDR2Rev (Table 1). The procedure is as follows: 1 cycle of 94 °C, 2 min; 30 cycles of 94 °C – 1 min, 57 °C – 1 min, 68 °C – 3 min; and 1 cycle of 68 °C – 10 min. All RACE products were cloned into pGEM-T Easy vector (Promega) and provided for sequencing.

**Sequencing and bioinformatic analysis**

The nucleotide sequences were determined for both strands using an ABI Prism automated sequencing system (PE Biosystems, Macrogen-Korea). Nucleotide and derived amino acid sequence analysis was performed using DNASTAR (Lasergene) and Geneious Pro 2.5.3 (Biomatters). Multiple alignments of amino acid sequences were performed using the M-Coffee option of the T-Coffee program (Notredame et al., 2000; Moretti et al., 2007). Gaps and poorly aligned sequences were trimmed from alignments using Gblocks version 0.91b (Castresana, 2000; Talavera and Castresana, 2007). The default parameters used by Gblocks (minimum number of sequences for a conserved position, 12; minimum number of sequences for a flanking position, 19; maximum number of contiguous non-conserved positions, 8; minimum length of a block, 10) yielded a final data set of 347 (61% of the original 561 positions). The best-fitting model of evolution was selected by ProtTest v1.2 (Abascal et al., 2005), following the Akaike information criterion. The selected model was used in all phylogenetic reconstructions. Maximum likelihood analyses were carried out with PHYML v2.4.4 (Guindon and Gascuel, 2003) starting from the BIONJ tree, and fixing the proportion of I and G to the value estimated by ProtTest. Bootstrap values were based on 500 replicates. The tree was rooted using the midpoint rooting algorithm in PAUP version 10b (Swofford, 2002). Prediction of putative chloroplast transit peptides was made with the ChloroP 1.1 program (Emanuelsson et al., 1999). The full-length \( DsHDR \) cDNA was deposited in GenBank under the accession number FJ040210.

**RNA gel blot analysis**

Total RNA samples (6 \( \mu \)g) were denaturated and electrophoresed under denaturing conditions on a 1% (w/v) – agarose gel containing formaldehyde (5%) and transferred to a Hybond\textsuperscript{TM}-N nylon membrane (Amersham Biosciences) according to the manufacturer’s instructions. Probe labeling, hybridization, washes and signal detection were performed as described in Ramos et al. (2008). A 459-bp \( DsHDR \) probe used for blot analysis was amplified by PCR with the gene-specific primers HDR3Fw and HDR4Rev (Table 1).

**Genetic complementation assay**

\( DsHDR \) full-length cDNA was digested with \textit{SphI} and \textit{HindIII} at sites introduced via PCR primers (HDF5Fw and HDF6Rev; Table 1), and cloned into a similarly cut pQE-80L expression vector (Qiagen). The pQE-\textit{DsHDR} plasmid was transformed in the \textit{Escherichia coli} mutant strain MG1655 \textit{ara} \textlt{ispH} (McArteer et al., 2001) and selected on LB plates containing 50 mg \textit{mL}^{-1} kanamycin, 50 mg \textit{mL}^{-1} ampicillin, 0.2% glucose (Glc), and 0.5 mM IPTG. As a

**Table 1.** Oligonucleotides used in the present study.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDR1Fw</td>
<td>TGAAGGCCAATCATCCTAGTGATCAAG</td>
</tr>
<tr>
<td>HDR2Rev</td>
<td>AAACGGTGGCAAGTGGATTAGATTAGC</td>
</tr>
<tr>
<td>HDR3Fw</td>
<td>TGAATTTTGCACACAGCTTC</td>
</tr>
<tr>
<td>HDR4Rev</td>
<td>CCGGGTTGTGGATGATTTGGTTGGT</td>
</tr>
<tr>
<td>HDR5Fw</td>
<td>CCGGCTGCGGAGATGATGTTGTCACACAGCTTC</td>
</tr>
<tr>
<td>HDR6Rev</td>
<td>CCAAGCTTTGGTGGATGTTGGGCGAGC</td>
</tr>
</tbody>
</table>

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negative control, the empty pQE-80L was transformed into the previous E. coli mutant strain and plated on LB plates as described above. Cells transformed with the empty pQE-80L were unable to grow on LB medium containing 0.2% glucose.

Results and discussion

Cloning and characterization of DsHDR

A partial DsHDR cDNA clone with a high degree of sequence homology to plant HDR genes was identified upon the screening of a subtractive cDNA library enriched for abiotic stress-induced sequences. This result allowed the design of specific primers for RACE PCR amplification of the full-length DsHDR cDNA (data not shown).

The full-length 1959-bp cDNA clone encoded a deduced protein sequence of 474 amino acids with an estimated molecular weight of 53.2 kDa. The 5′ untranslated region (UTR) consisted of 27-bp upstream of the start codon and a 3′ UTR of 507-bp downstream from the stop codon. DsHDR showed the highest sequence similarity with other Chlorophyta (Chlamydomonas reinhardtii – 67% and Ostreococcus lucimarinus – 64%), plant and cyanobacteria (> 53%) homologs, confirming previous observations that MEP pathway enzymes are highly conserved (Lange et al., 2000).

Multiple sequence alignment of the deduced DsHDR amino acid sequence with other plant and bacterial enzymes is represented in Figure 1. The Viridiplantae (D. salina included) and Synecococcus elongatus sequences contained the four conserved cysteine residues that are known to be essential for the catalytic activity of HDR (Wolff et al., 2003; Gräwert et al., 2004). In E. coli, one cysteine residue (position 272 of the alignment) is not conserved; however, the cysteine residue observed at position 421 may be involved in the [4Fe–4S] coordination. Moreover, two fully conserved histidine residues (positions 156 and 246) suggested to be involved in proton-transfer reactions were also observed (Adam et al., 2002).

The predicted cleavage site of the putative chloroplast transit peptide of DsHDR is indicated in Figure 1. This signal sequence at the N-terminal, absent in bacterial sequences, was identified in other plant HDR proteins, which is consistent with the demonstrated chloroplast subcellular localization of all Arabidopsis MEP pathway enzymes (Rodríguez-Concepción and Boronat, 2002; Hsieh et al., 2008).

Depending on the plant species, single (e.g. A. thaliana) or multiple copies (e.g. Ginkgo biloba) of the HDR gene were identified (Rodríguez-Concepción and Boronat, 2002; Kim et al., 2008). In the particular case of D. salina, DNA gel blot analysis (data not shown) suggested the existence of a single DsHDR copy in its genome since the genomic blot pattern showed one band in all the analyzed samples (high and low stringency conditions).

Phylogenetic analysis

A phylogenetic analysis was performed using the deduced amino acid HDR sequences from bacterial and non-bacterial organisms (Viridiplantae; Figure 2). Initially, from the 561 amino acid positions in the original alignment of the DsHDR fragment, 214 were identified by Gblocks as gapped or poorly aligned and were not considered in subsequent analyses. Thus, the final protein data set contained 61% of the original data set, i.e. 347 amino acids. The RtREV-+I+F model (Dimmic et al., 2002) was selected as best-fitting model among the 96 models tested with a −lnL of 9753.72, gamma shape with 4 rate categories of 0.923 and a proportion of invariable sites of 0.049. This unrooted phylogenetic tree (Figure 2) can be divided into several groups that include Streptophyta (higher plants), Chlorophyta (green algae), Cyanophyta (cyanobacteria) and Eubacteria. Higher plants and green algae HDR are closely related and possibly share a common cyanobacterial ancestry. Evolution of MEP pathway genes in different eukaryotic species has been explained by lateral gene transfer between prokaryotes (Lange et al., 2000). However, it has been suggested that 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and, recently, plant HDR genes may have a cyanobacterial origin (Lange et al., 2000; Guevara-García et al., 2005; Wang et al., 2008). Therefore our data, which included green algae in phylogenetic analyses of HDR sequences for the first time, supports an endosymbiotic origin with a cyanobacterial ancestry for eukaryotic HDR genes.

DsHDR expression profile under carotenogenesis inductive conditions

To examine the expression pattern of DsHDR mRNA levels under different abiotic stresses, conditions known to involve high β-carotene production rates in this microalga (Coesel et al., 2008), two experiments were performed (Figures 3 and 4; Tables 2 and 3). In both experiments, significant differences in β-carotene (µg mL⁻¹) content (Fisher’s LSD test) in cells exposed to
diverse forms of abiotic stress and control conditions were obtained.

Salinity up-shift (9–18% NaCl) and/or nutrient depletion (−N) resulted in an increase, 36 h upon the stress onset, of \( DsHDR \) steady-state mRNA transcript levels as observed in Figure 3. In fact, maximum transcript levels resulted from nutritional stress (9% −N and 18% −N; Figure 3), although the highest accumulation of intracellular β-carotene was observed in cells subjected to both stresses (3.14 \( \mu \)g mL\(^{-1} \), 15.50 pg cell\(^{-1} \); Table 2). Similar results were observed under low light (LL) or high light (HL) and/or nutrient depletion (−N) conditions (Figure 4). The highest \( DsHDR \) steady-state mRNA transcript and β-carotene levels were obtained in \( D. salina \) cells (24h) submitted to nutritional stress (LL−N and HL−N) and both stresses (HL−N; 3.96 \( \mu \)g mL\(^{-1} \), 9.30 pg cell\(^{-1} \); Table 3), respectively.

\( DsHDR \) expression patterns were similar to those observed in the described stress conditions for the downstream carotenoid biosynthetic pathway genes, namely phytoene synthase (PSY), phytoene desaturase (PDS) and lycopene β-cyclase (LCY-β) (Coesel et al., 2008; Ramos et al., 2008). Carotenoid accumulation in this microalga was regulated primarily by nutritional medium limitation, and this factor appears to be important in the metabolic control of MEP biosynthetic pathway as well. Indeed, if cell growth is severely limited by nitrate...
deficiency, gene expression related to carotenoid biosynthesis may be reduced. For instance, Sánchez-Estudillo et al. (2006), using a heterologous probe, have reported that *D. salina* DXS mRNA levels were higher in control cells as compared to nitrate-limited cultures. Nevertheless, additional stress factors known to increment carotenogenic genes transcript levels (high light intensity and high salt) might also regulate MEP genes. In several plants, other factors such as light are known to
upregulate some key MEP pathway genes, including HDR (Botella-Pavía et al., 2004; Kim et al., 2008). However, in the tested time line, which included additional time points (3 and also 48 h; data not shown), light up-shift did not influence steady-state DsHDR transcript levels significantly (as observed in 2-week-old Arabidopsis seedlings; Hsieh and Goodman, 2005). Thus, analysis of recent published data concerning PSY, PDS and LCY-β transcript levels (Coesel et al., 2008; Ramos et al., 2008) and DsHDR current observations suggest the existence of a coordination of both pathways in D. salina under abiotic stress, with particular emphasis on nutrient stress. Interestingly, a similar coordination of MEP with downstream pathways such as the enzymes involved in carotenoid biosynthesis has been observed during tomato fruit ripening and A. thaliana seedling deetiolation (Lois et al., 2000; Botella-Pavía et al., 2004).

**Functional analysis of DsHDR**

A complementation assay with a lethal E. coli mutant defective in the HDR gene (strain MG1655; McArteer et al., 2001) was performed in order to test whether the DsHDR polypeptide showed enzymatic activity similar to that in its E. coli counterpart. The HDR gene is essential for the survival of this E. coli and therefore no growth was observed in the absence of arabinose (Figure 5). Upon transformation with the constructed vector harboring the DsHDR gene (pQE-DsHDR), the lethal phenotype of the mutant strain was rescued and cells were able to grow in medium with glucose (Figure 5D). The opposite was observed for cells transformed with the empty pQE-80L vector (Figure 5C). Therefore, the enzymatic mechanisms involved in the synthesis of the isoprenoid precursors between DsHDR and E. coli HDR might be similar.

**Conclusions**

Since the discovery about a decade ago of the plastidial MEP pathway, numerous studies in plants have led to the characterization of the respective enzymes, in contrast to the scarce data concerning the regulatory mechanisms controlling the synthesis of the different pathway compounds. Carotenoid biosynthesis is dependent on the supply of the MEP-derived precursors and therefore D. salina, which holds a unique capacity of β-carotene accumulation, is a valuable model organism to

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Table 2. Nitrate concentration (mM) in the medium and β-carotene accumulation (µg mL⁻¹; pg cell⁻¹) in D. salina cultures.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Medium</th>
<th>Nitrate concentration (mM)</th>
<th>β-Carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(µg mL⁻¹)</td>
</tr>
<tr>
<td>0</td>
<td>LL+N</td>
<td>0.32 ± 0.01</td>
<td>1.02 ± 0.16</td>
</tr>
<tr>
<td>24</td>
<td>LL+N</td>
<td>0.53 ± 0.02</td>
<td>0.74 ± 0.09</td>
</tr>
<tr>
<td>24</td>
<td>LL-N</td>
<td>0.06 ± 0.03</td>
<td>1.15 ± 0.11</td>
</tr>
<tr>
<td>24</td>
<td>HL+N</td>
<td>0.17 ± 0.05</td>
<td>3.10 ± 0.30</td>
</tr>
<tr>
<td>24</td>
<td>HL-N</td>
<td>0.12 ± 0.02</td>
<td>3.96 ± 0.55</td>
</tr>
</tbody>
</table>

Cells pre-adapted to low light conditions (LL) and 9% salinity were transferred to a nutrient-supplemented medium (+N) or nutrient-depleted medium (−N). Data are the means of 3 replicates ± SD.

Table 3. Nitrate concentration (mM) in the medium and β-carotene accumulation (µg mL⁻¹; pg cell⁻¹) in D. salina cultures.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Medium</th>
<th>Nitrate concentration (mM)</th>
<th>β-Carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(µg mL⁻¹)</td>
</tr>
<tr>
<td>0</td>
<td>9%+N</td>
<td>0.44 ± 0.02</td>
<td>2.17 ± 0.12</td>
</tr>
<tr>
<td>36</td>
<td>9%+N</td>
<td>1.01 ± 0.04</td>
<td>1.57 ± 0.04</td>
</tr>
<tr>
<td>36</td>
<td>18%+N</td>
<td>1.57 ± 0.04</td>
<td>1.59 ± 0.10</td>
</tr>
<tr>
<td>36</td>
<td>9%−N</td>
<td>0.00 ± 0.00</td>
<td>1.76 ± 0.13</td>
</tr>
<tr>
<td>36</td>
<td>18%−N</td>
<td>0.00 ± 0.00</td>
<td>3.14 ± 0.29</td>
</tr>
</tbody>
</table>

Cells pre-adapted to 9% salinity were transferred to a nutrient-supplemented medium at 9% (9%+N) or 18% (18%+N) salinity and to nutrient-depleted medium at both salinities (9%−N, 18%−N). Data are the means of 3 replicates ± SD.
better comprehend the complex regulation of isoprenoid biosynthesis (Ben-Amotz et al., 1982; Ye et al., 2008). DsHDR seems to be regulated at the transcriptional level in response to environmental changes, but further research should also examine post-transcriptional regulation analysis of the MEP pathway. Understanding \textit{D. salina} metabolic flux through the MEP pathway, since several enzymes could act as control points, might also provide the basis for metabolism engineering for massive accumulation of \(\beta\)-carotene and other carotenoids in this alga. Indeed, the biotechnological potential of \textit{D. salina} has recently been enhanced by the development of DNA transformation methods (reviewed by Coll, 2006; Leo\'n et al., 2007) and the current genome sequencing efforts (Liolios et al., 2006). The combination of genomics and gene expression data will facilitate future prospects of turning this microalga into a highly valuable cell factory system (Lamers et al., 2008).

Acknowledgments

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References


Coesel SN, Baumgartner AC, Teles LM, Ramos AA, Henriques NM, Cancela L, et al. Nutrient limitation is the main regulatory factor for carotenoid

Figure 5. Complementation of \textit{E. coli hdr} mutant (MG1655 ara-::ispH) with \textit{D. salina HDR}. The \textit{E. coli} mutant strain was not able to grow on LB plates containing 0.2% glucose (Glc) as expected (A). This strain was transformed with the empty expression plasmid pQE-80L (B, C) and the plasmid containing \textit{D. salina HDR} (pQE-DsHDR; D). After transformation, only the \textit{E. coli} transformants containing the pQE-DsHDR were able to grow in the presence of Glc and absence of arabinose (Ara; D).


Dunaliella HDR 977