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Use of a Reporter Gene To Follow High-Pressure Signal Transduction in the Deep-Sea Bacterium *Photobacterium* sp. Strain SS9

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***Photobacterium* sp. strain SS9 is a deep-sea bacterium which modulates the abundances of several outer membrane proteins as a function of hydrostatic pressure. These proteins include the product of the previously cloned *ompH* gene (D. H. Bartlett, M. Wright, A. A. Yayanos, and M. Silverman. Nature (London) 342:572–574, 1989). Subsequent to conjugal plasmid delivery it was possible to cross an *ompH*::*lacZ* transcriptional fusion into the genome of SS9, replacing the wild-type *ompH* gene, generating strain EC10. EC10 is not impaired in growth at high pressure, indicating that under the growth conditions employed, OmpH is not required for baroadaptation. β-Galactosidase production in EC10 is induced by high pressure to approximately the same extent that OmpH production is in the parental strain, SS9. Therefore, OmpH abundance appears to be primarily regulated at the transcriptional level. EC10 was used for the isolation of *ompH* regulatory mutants. Derivatives of EC10 which produce reduced levels of β-galactosidase at both low and high pressure and which appeared to possess mutations outside the *ompH*::*lacZ* locus were obtained. All of these regulatory mutants displayed alterations in the high-pressure repression of a second outer membrane protein, designated OmpL, and two of the mutants were also deficient in the high-pressure induction of a third outer membrane protein, designated OmpI. The most dramatic phenotype was present in mutant EC1002, whose growth was extremely barosensitive. EC1002 is the first pressure-sensitive mutant ever isolated. Prolonged incubation of EC1002 at high pressure led to the accumulation of cells with wild-type growth characteristics at high pressure. These cells are suggested to possess suppressor mutations, as they remain deficient in β-galactosidase production and maintain their high-pressure-adapted phenotype for many generations in the absence of high-pressure selection.**

Changes in the physical and chemical environments of gram-negative bacteria are frequently reflected by changes in the abundances of their outer membrane (OM) proteins (14, 23, 24). In many cases these proteins facilitate nutrient and waste-product permeation, although with various degrees of specificity. The environmental cues which have been observed to influence OM protein levels include osmolarity; temperature; pH; redox potential; and the availability of phosphate, iron, divalent cations, nucleosides, and particular saccharides (8, 14, 23, 24). Because many of these OM proteins are among the most abundant proteins in the cell, they provide readily identifiable biochemical signatures of the environment, as well as of certain aspects of physiological status.

Photobacterium sp. strain SS9 is a moderately barophilic, deep-sea bacterium which has previously been reported to modulate the synthesis of one of its OM proteins, designated OmpH, in response to an unusual stimulus, elevated hydrostatic pressure (3). The *ompH* gene has been cloned, and its nucleotide sequence has been determined (2). The deduced OmpH amino acid sequence possesses statistically significant similarity to porin P2 from *Haemophilus influenzae* (19) and thus may form a channel in the OM. Although high pressure increases both OmpH protein and *ompH* transcript abundances in an analogous fashion (3), it is not known whether pressure is modulating the rate of transcription or the half-life of the *ompH* message.

We are seeking to develop *Photobacterium* sp. strain SS9 as a model system in which to follow hydrostatic pressure signal transduction as well as to determine factors necessary for baroadaptation. In this report the first genetic manipulations of *Photobacterium* sp. strain SS9 are presented, including the isolation of an *ompH* mutant and several *ompH* regulatory mutants. Although under the culture conditions employed *ompH* mutants were not observed to possess defects in baroadaptation, one of the *ompH* regulatory mutants was dramatically sensitive to elevated pressure. This mutant represents the first pressure-sensitive mutant isolated from any bacterial species.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown at 37°C in LB medium (19), and *Photobacterium* sp. strain SS9 was routinely cultured at 15°C in 2216 marine medium (28 g/liter; Difco Laboratories). The antibiotics kanamycin (50 μg/ml for *E. coli*, 200 μg/ml for *Photobacterium* strains) and rifampin (100 μg/ml) were added to the media when required. Antibiotics were obtained from Sigma Chemical Co. For solid media, agar (Difco Laboratories) was added at 17 g/liter. When desired, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was added at 40 mg/liter. Stock solutions of X-Gal were prepared at 40 mg/ml in *N*,*N*-dimethylformamide.

High-pressure growth studies. To examine the high-pressure growth of the *Photobacterium* strains, each strain was cultured to stationary phase in 2216 medium at 1 atm (1 atm = 101.29 kPa), 15°C. Stationary-phase cultures were diluted into 80 ml of 2216 medium buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (100 mM, pH 7.5; Sig-

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TABLE 1. Strains, plasmids, and phage used in this study

Strain, plasmid, or phage	Relevant genotype or characteristic(s)	Reference or source
Strains		
Photobacterium sp.		
SS9	Facultatively barophilic deep-sea *Photobacterium* isolate	9
SS9-R	Rifampin-resistant SS9 derivative	This work
DB110	Lac$^-$ SS9-R derivative	This work
EC10	*ompH*::*lacZ* transcriptional fusion strain derived from DB110	This work
EC1001	*ompH*::*lacZ* structural mutant	This work
EC1002	*ompH*::*lacZ* regulatory mutant	This work
EC1006	*ompH*::*lacZ* regulatory mutant	This work
EC1011	*ompH*::*lacZ* regulatory mutant	This work
EC1020	*ompH*::*lacZ* regulatory mutant	This work
E. coli		
DH5α	*recA* strain used for maintaining plasmids	13
ED8654	Strain in which pRK2073 is maintained	22
Plasmids		
pRK2073	Helper plasmid which carries the *tra* genes necessary for conjugal transfer	4
pKT231	Broad host range, Km^r	1
pMC903	Contains *lacZ* reporter gene	7
pDB501	*ompH* structural gene and upstream sequence	3
pEC7	*ompH*::*lacZ* transcriptional fusion in pKT231, Km^r	This work
pEC9	*ompH* structural gene and upstream sequence in pKT231, Km^r	This work
Phage M13mp19	Cloning and sequencing vector	30

ma Chemical Co.) to a final optical density at 595 nm of approximately 0.001. Each 80-ml volume of diluted culture was used to fill 16 to 18 sterile 4.5-ml polyethylene transfer pipets (Samco). Transfer pipets were then heat sealed with a hand-held sealing clamp (Nalgene). Half of the pipets were incubated at 272 atm of hydrostatic pressure, 9°C, and the other half were incubated at 1 atm, 9°C. At the appropriate times, one transfer pipet from each pressure condition was removed to monitor the optical densities of the two cultures. Cells were incubated at elevated hydrostatic pressure in stainless steel pressure vessels equipped with quick-connect fittings for rapid decompression and recompression as described by Yayanos and Van Boxtel (31).

Mutagenesis. SS9-R is a spontaneous rifampin-resistant derivative of wild-type strain SS9. ICR 191 (Sigma Chemical Co.) and ethylmethane sulfonate (EMS) (Eastman Kodak Co.) mutagenesis experiments were performed on the basis of the protocols of Miller (19). The β-galactosidase-deficient derivative of SS9-R, designated DB110, was obtained following chemical mutagenesis with ICR 191. A total of 10^5 cells of SS9-R were inoculated into 1 ml of 2216 medium containing 2 μg of mutagen per ml. This ICR 191 concentration was experimentally determined to result in little growth inhibition. Once the cells had grown to stationary phase, approximately 10^8 cells per ml, serial dilutions of the cells were plated onto solid 2216 medium containing X-Gal. This treatment resulted in the identification of one β-galactosidase-deficient mutant out of 10,000 colonies screened. SS9-R and DB110 retained wild-type characteristics with regard to both growth at low and high pressure and OmpH pressure inducibility (data not shown). For EMS mutagenesis, strain EC10 was cultured in 2216 broth to approximately 10^8 cells per ml. The cells were washed and resuspended in half the original volume in minimal marine salts medium (32 g of sea salts [Sigma Chemical Co.] per liter, 10 mM Tris [pH 7.5], 1 g of NH_4Cl per liter, 0.2 g of $MgCl_2 \cdot 6H_2O$ per liter, 0.4 ml of 10 μM $FeCl_3$ per liter). Twenty microliters of EMS was added to 2 ml of cells, and the culture was aerated at 15°C for 16 h. The cells were diluted 1/10 in 2216 medium, grown for 24 h at 15°C, and then serially diluted in 2216 broth and plated onto 2216–X-Gal agar. Under these conditions the frequency of generating colonies deficient in β-galactosidase production was 0.27%.

DNA manipulations and plasmid constructions. DNA preparations, agarose gel electrophoresis, restriction endonuclease digestions, ligations, nick translations, and Southern transfers and hybridizations were performed as previously described (25). Restriction endonucleases and T4 DNA ligase were obtained from GIBCO BRL, and *E. coli* DNA polymerase I was obtained from NEN Research Products. Southern transfers were made onto the nylon membrane Nytran (Schleicher & Schuell). Probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; ICN Biomedicals) by nick translation. Hybridization and washes (0.1% sodium dodecyl sulfate [SDS]–6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) were performed at 65°C. Autoradiography was performed with Kodak X-Omat AR film.

The construction of the *ompH*::*lacZ* fusion plasmid, pEC7, was accomplished in three steps. First, a cytosine residue located 10 bp downstream of the *ompH* translational start was deleted by oligonucleotide-directed in vitro mutagenesis of an *ompH*-containing M13mp19 subclone (29). This was accomplished by the method of Kunkel (16) with the Muta-gene M13 in vitro mutagenesis kit (Bio-Rad Laboratories) and the oligonucleotide 5′-TGCTACAAGATCTTTTTCAT-3′. After mutagenesis, the resulting mutant *ompH* gene was subcloned as a 2.3-kb *Bam*HI-*Eco*RI fragment onto the kanamycin-resistant broad-host-range plasmid pKT231 (1). Finally, the 7.6-kb *Bam*HI-*Bgl*II *lacZ* cassette from plasmid pMC903 (7) was cloned into the *Bgl*II restriction site of the mutant *ompH* gene in the appropriate orientation for the *ompH* promoter to direct

lacZ expression. The resulting plasmid, bearing the *ompH*::*lacZ* transcriptional gene fusion, was designated pEC7.

Plasmid pEC9, containing the wild-type *ompH* gene, was constructed by partial *Eco*RI digestion of the *ompH*-containing M13mp19 subclone and insertion of a 2.4-kb *Eco*RI fragment, which contains the entire *ompH* gene and 750 bp of upstream sequence, into the unique *Eco*RI site of pKT231. The fragment was oriented so that *ompH* transcription proceeded in a direction opposite to that of the transcription of the kanamycin- and streptomycin-resistance genes on the vector.

Bacterial conjugations. Plasmids were transferred from *E. coli* to *Photobacterium* strains through triparental matings similar to that described by Ditta et al. (11). Stationary-phase cultures of the *E. coli* plasmid-bearing strain, the transfer function donor strain ED8654(pRK2073), and the SS9 recipient strain were each washed with 2216 marine medium and resuspended to a density of 10^9 cells per ml. Portions (10^8 cells) of each of the three strains were then spotted together onto a polycarbonate filter (pore size, 0.4 μm; diameter, 25 mm [Poretics Corporation]), placed on a nonselective 2216 agar plate, and mated at room temperature for 12 to 15 h. Mated cells were resuspended off the filter in 2216 medium and plated onto 2216 agar containing rifampin and kanamycin and incubated at 15°C to allow for growth of the SS9 exconjugants. The frequency of conjugation was approximately 10^{-5} exconjugants per recipient cell under these conditions.

Construction of an SS9 *ompH*::*lacZ* transcriptional fusion strain. In order to integrate the *ompH*::*lacZ* fusion present on pEC7 into the SS9 genome, pEC7 was mobilized by conjugation from *E. coli* into strain DB110. Exconjugants were repeatedly subcultured in the absence of antibiotic selection for the plasmid. After each round of subculturing, cells were plated onto marine medium both with and without added kanamycin to determine the proportion of cells which still retained pEC7. By the third round, only 10% of the cells were still kanamycin resistant, indicating that most of the bacterial population had lost the plasmid. One hundred Lac^+ colonies from the third round of culturing were screened for resistance to kanamycin, and four were found to be sensitive to the antibiotic. The Lac^+ Kan^s phenotype of the four clones suggested that the wild-type *ompH* gene in these clones had been replaced with the *ompH*::*lacZ* gene fusion by homologous recombination. This was confirmed by Southern blot analysis with pEC7 as a hybridization probe. Furthermore, Western blot (immunoblot) analyses with antisera directed against OmpH or against *E. coli* β-galactosidase confirmed that EC10 produces no detectable OmpH but does produce β-galactosidase.

Isolation of OM proteins. OM proteins were enriched for in detergent extractions from the *Photobacterium* strains as previously described (5). By this method, integral membrane proteins, including many outer membrane proteins, partition into a Triton X-114 detergent phase. Proteins are precipitated out of the detergent phase by adding 1 volume of distilled water and 2 volumes of 100% ethanol and mixing and precipitating for 12 h at −20°C. Proteins are pelleted by centrifugation in a microcentrifuge at 16,000 × *g*, 4°C for 20 min. Triton X-114 detergent was obtained from Sigma Chemical Co.

SDS-PAGE and Western blotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in the manner of Laemmli (17). Whole-cell proteins or OM proteins were solubilized in SDS sample buffer and separated by electrophoresis through a 12.5% polyacrylamide gel. Proteins were either stained with Coomassie brilliant blue dye (Sigma Chemical Co.) or subjected to Western blot analysis. Proteins to be immunoblotted were transferred onto Magnagraph membrane (Micron Separations Inc.) in a Trans-Blot cell (Bio-Rad Laboratories) with half-strength Towbin buffer (27) (12.5 mM Tris and 96 mM glycine, pH 8.3) lacking methanol. Blots were blocked and reacted with primary and secondary antibodies in Tris-buffered saline containing 1% gelatin (Difco Laboratories), 0.2% NaN_3, and 0.1% Tween 20 at room temperature. The chemiluminescent compound Lumi-Phos 530 (Boehringer Mannheim) served as the substrate for the alkaline phosphatase-conjugated goat anti-rabbit secondary antibody to visualize the immunopositive proteins. Both the *E. coli* β-galactosidase primary antibody and the alkaline phosphatase-conjugated goat anti-rabbit secondary antibody were purchased from Organon Teknika. The preparation of polyclonal antisera directed against denatured OmpH has previously been described (3). Autoradiography was performed with Kodak X-Omat AR film.

Quantification of OmpH abundance. Whole-cell extracts of DB110 were subjected to SDS-PAGE and immunoblotted as described above, with the following modifications. Proteins were transferred onto nitrocellulose (Kodak) with half-strength Towbin buffer containing 20% methanol, and OmpH antisera were detected with ^{125}I-labelled protein A (DuPont Corp.). After incubation with labelled protein A, the blot was exposed to X-ray film to identify the location of the OmpH bands on the membrane. The OmpH bands were excised, and the radioactivity associated with the OmpH on each was determined in a gamma counter.

Quantification of β-galactosidase activity. The β-galactosidase activity in whole-cell extracts was assayed as described by Miller (19).

RESULTS

High-pressure-inducible β-galactosidase production in EC10. Measurement of *ompH* gene expression has previously been accomplished directly by Northern (RNA) blot analysis or indirectly by immunological identification of OmpH or by Coomassie brilliant blue R staining of proteins separated by SDS-PAGE (3). To facilitate the monitoring of *ompH* promoter activity and to further assess the regulation of *ompH* transcript abundance by high pressure, an SS9 *ompH*::*lacZ* transcriptional fusion strain was constructed. In this strain, designated EC10, *lacZ* expression, directed by the *ompH* promoter, can be observed directly by colorimetric methods. Additionally, strain EC10 was used to assess the phenotype of an *ompH* mutant.

Because EC10 *ompH* promoter activity results in the synthesis of mRNA lacking most of the wild-type *ompH* transcript sequence, those features of the *ompH* message which could lead to pressure-modifiable differences in stability are likely to be missing in the *ompH*::*lacZ* fusion transcript. Therefore, quantitating high-pressure effects on β-galactosidase production in EC10 allows high-pressure induction of *ompH* transcription initiation to be distinguished from high-pressure enhancement of *ompH* mRNA stability.

Changes in OmpH abundance in response to increasing hydrostatic pressures were followed in strain DB110. SDS-PAGE analysis of DB110 OM proteins isolated from cells grown at 1 to 408 atm hydrostatic pressure is shown in Fig. 1. OmpH (indicated by the open arrow) abundance increased as pressure increased from 1 to 272 atm (the high-pressure optima for growth) and decreased as the pressure increased further to 408 atm. The extent of the OmpH induction in DB110 was masked somewhat in this type of analysis because of the presence of an OM protein which comigrated with OmpH. These OM protein profiles also revealed that OmpH was only one of several OM proteins in SS9 which were

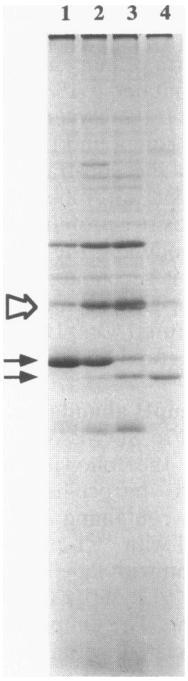

FIG. 1. OM protein composition of strain DB110 as a function of increasing hydrostatic pressure. OM proteins were extracted from equivalent amounts of total cellular proteins from DB110 cultured at 9°C at 1, 136, 272, and 408 atm of hydrostatic pressure (lanes 1 to 4, respectively) and subjected to SDS-PAGE. Cells from each culture were harvested in mid-log growth. The open arrow points to OmpH, and closed arrows point to additional pressure-regulated proteins whose production is suggested to be coordinately regulated with OmpH.

regulated, either induced or repressed, by changes in growth pressures.

Quantification of the OmpH induction seen in DB110 as growth pressures were increased from 1 to 408 atm was done by Western blot analysis. The amount of ^{125}I-labelled protein A bound to OmpH increased from 1,306 to 3,902 to 9,137 cpm as pressure increased from 1 to 136 to 272 atm. At 408 atm of pressure, OmpH abundance decreased to 3,057 cpm. These data indicated a sevenfold increase in OmpH production at 272 atm.

To determine whether the pressure regulation of OmpH synthesis seen in DB110 is due to regulation of *ompH* transcription initiation, the high-pressure induction of β-galactosidase was examined in EC10. The units of β-galactosidase activity, measured at 1, 136, 272, and 408 atm, were 298, 2,228, 2,372, and 1,751, respectively. Thus, an eightfold induction in β-galactosidase activity was observed in EC10 at 272 atm, an induction which closely mirrored the sevenfold induction observed for OmpH abundance in DB110 at the same pressure.

Generation and isolation of *ompH* regulatory mutants. The previous experiments demonstrated that OmpH production in SS9 is primarily controlled by the rate of *ompH* transcription and that therefore *ompH* regulatory transcription factors are likely to exist. The approach taken in beginning to define these factors was to isolate *ompH* regulatory mutants. Because of the convenience of assaying β-galactosidase levels compared with OmpH levels, isolation of regulatory mutants was attempted in the *ompH*::*lacZ* fusion strain EC10 rather than in wild-type SS9.

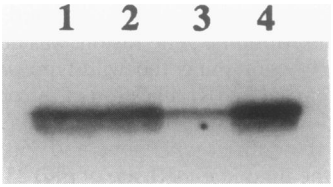

FIG. 2. Western blot analysis of plasmid-directed OmpH synthesis in various SS9 mutants. Whole-cell extracts from pEC9-containing EC10 (lanes 1 and 4), EC1001 (lane 2), and EC1020 (lane 3) were prepared after growth to stationary phase at 15°C. Following SDS-PAGE and electroblotting, proteins were reacted with OmpH antisera and detected by chemiluminescent methods.

A collection of mutants derived from strain EC10 was generated by using the chemical mutagen EMS and screened on marine medium containing X-Gal to identify mutants deficient in β-galactosidase activity. All screening was performed at 1 atm. *ompH* promoter-directed *lacZ* expression in EC10 is sufficient even at 1 atm to readily differentiate between basal and reduced levels of β-galactosidase. Our hypothesis was that regulatory mutants impaired in expressing *ompH* at 1 atm would also be impaired in its expression at high pressure and that the pressure regulation results from the activity of an existing regulatory protein(s), not from the production of a new regulatory protein at high pressure. By analogy, the regulatory protein OmpR is required for *ompF* and *ompC* expression under all conditions (23, 24). Among the EC10-derived mutants, a range of diminished β-galactosidase levels was observed. Only clones which appeared to lack β-galactosidase activity and which were phenotypically Lac$^-$ were saved for further study.

To distinguish Lac$^-$ clones having *ompH*::*lacZ* reporter gene mutations from those with mutations in genes whose products are required for *ompH* gene expression, a plasmid bearing the *ompH* gene and potential upstream regulatory sequences, designated pEC9, was conjugated into 40 Lac$^-$ mutants. OmpH abundance in the exconjugants was determined by Western blot analysis. Reporter gene mutants were predicted to produce large quantities of OmpH protein, as these mutants would still retain *trans*-acting regulatory elements needed for *ompH* expression from pEC9, while *ompH* regulatory mutants were predicted to produce little OmpH because of a lack of one of these essential transcription factors. This strategy did indeed separate the Lac$^-$ EC10 mutants into two classes as predicted.

A Western blot that distinguishes a representative reporter gene mutant, EC1001, from a representative regulatory mutant, EC1020, is shown in Fig. 2. Whole-cell proteins from exconjugants of EC10, EC1001, and EC1020 containing pEC9 were immunoblotted and reacted with OmpH antisera. EC1001 (lane 2) displayed a level of OmpH synthesis from pEC9 equivalent to that seen in the parent strain, EC10 (lanes 1 and 4), whereas the Lac$^-$ mutant EC1020 (lane 3) displayed a reduced level of OmpH compared with both EC10 and EC1001. Of the Lac$^-$ clones analyzed in this manner, only 4 of 40 tested strains, EC1002, EC1006, EC1011, and EC1020, were determined to bear a mutation in an *ompH* regulatory locus, with the majority of Lac$^-$ clones appearing to have arisen from mutations in the *ompH*::*lacZ* reporter gene.

Characterization of EC10 *ompH*::*lacZ* regulatory mutants. Because the OM protein profiles of DB110 cultured at 1, 136, 272, and 408 atm of hydrostatic pressure revealed that the abundances of several other OM proteins, in addition to OmpH, are pressure regulatable (Fig. 1, filled arrows), the OM

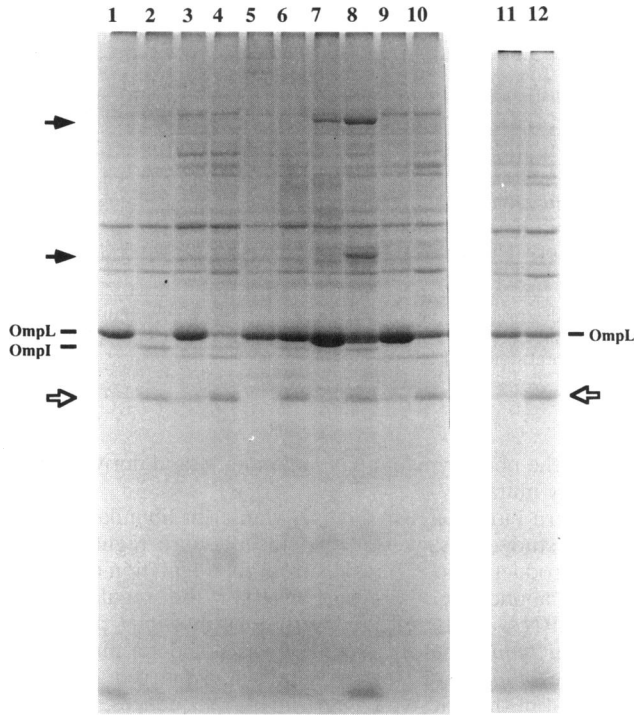

FIG. 3. OM protein profiles of EC10 and EC10-derived *ompH* regulatory mutants. The OM proteins expressed in EC10, EC1001, EC1006, EC1011, EC1020, and EC1002 from 1-atm cultures (lanes 1, 3, 5, 7, 9, and 11) and 272-atm cultures (lanes 2, 4, 6, 8, 10, and 12) were analyzed by SDS-PAGE. All cells were grown at 9°C and collected in mid-log growth. OM proteins were isolated from equivalent amounts of total cellular proteins from each strain. EC1002 OM proteins (lanes 11 and 12) were electrophoresed separately (see text for details). OmpL and OmpI are indicated by black bars. The open arrows identify a pressure-regulated protein whose pressure inducibility is not affected in the *ompH* regulatory mutants. Filled arrows point to proteins unique to EC1011.

protein profiles of the EC10 *ompH* regulatory mutants were examined. It was speculated that OmpH and one or more of these other OM proteins might be coordinately regulated and thus simultaneously affected in the *ompH* regulatory mutants. Precedence for the coordinate regulation of OM proteins comes from the OmpF-OmpC system in *E. coli* (24, 26). In this system, the abundances of the porin proteins OmpF and OmpC are inversely proportional and change in response to alterations in environmental cues such as osmolarity, nutrient availability, and temperature.

EC10; EC1001, the *ompH::lacZ* reporter gene mutant; and the four *ompH* regulatory mutants, EC1002, EC1006, EC1011, and EC1020, were each grown at 1 and 272 atm, 9°C. OM proteins were isolated from an equivalent amount of mid-log-phase cells from each culture and analyzed by SDS-PAGE. These OM protein profiles are shown in Fig. 3. The OM proteins of EC1002 (lanes 11 and 12) were electrophoresed separately because of the length of time required to obtain high-pressure protein samples of EC1002 (see the description below). Except for the absence of OmpH, the pattern of high-pressure induction and repression of OM proteins in EC10 was found to be the same as that in its parental strain DB110. Conversely, the high-pressure repression of one OM protein, designated OmpL, and the high-pressure induction of another OM protein, designated OmpI, were altered in some or all of the four regulatory mutants. The repression of OmpL synthesis at 272 atm in both EC10 (Fig. 3, lanes 1 and 2) and EC1001 (lanes 3 and 4) was pronounced; however, in the *ompH* regulatory mutants this repression was diminished (lanes 5 to 12). The high-pressure induction of OmpI was unaffected in the regulatory mutants EC1006 and EC1011 compared with EC10. However, in EC1002 and EC1020 no induction of OmpI was detected at 272 atm. EC1011 was unique among the SS9 mutants because it produced additional OM proteins not seen in the regulatory mutants EC1001 and EC10 (Fig. 3, lanes 7 and 8 [proteins indicated by filled arrows]).

Finally, the growth of the *ompH* mutant, EC10, and the *ompH* regulatory mutants was analyzed under conditions of elevated hydrostatic pressure. Despite the strong high-pressure induction of OmpH in DB110, growth of the *ompH* mutant, EC10, was similar to that of its parental strain, DB110, at both high and low pressure under the culture conditions employed. However, all of the *ompH* regulatory mutants displayed reduced growth rates and final cell densities compared with strain EC10. Furthermore, one of the four regulatory mutants, EC1020, was identified as being moderately pressure sensitive, and another regulatory mutant, EC1002, was identified as being markedly pressure sensitive. A set of growth curves for EC10 and EC1002 grown in rich media at 9°C at both 1 and 272 atm of hydrostatic pressure is shown in Fig. 4. Like wild-type SS9, EC10 showed better growth at 272 atm than at 1 atm. But EC1002 displayed extremely pressure-sensitive growth, with no significant growth at high pressure even after growth at 1 atm had plateaued. These results and those of the other experiments characterizing the *ompH* mutant, EC10, and the *ompH* regulatory mutants are summarized in Table 2.

Although the pressure sensitivity of EC1002 was evident up to 60 h into the growth experiment, extended culturing of EC1002 at 272 atm resulted in rapid growth, with the final cell density of this high-pressure culture matching that of the 1-atm culture. These results were observed during each of six separate incubations of EC1002 at low and high pressures. To determine whether the basis of the growth of EC1002 at high pressure was physiological or genetic, the growth of EC1002 cells adapted to high pressure, i.e., cells which had eventually grown at high pressure, was reexamined at high and low pressures. High-pressure-adapted and unadapted EC1002 cells were grown at 1 atm for 48 h in rich media at 15°C (11 cell generations). Cells from these two cultures were subcultured and grown at both 1 and 272 atm, 9°C. Figure 5 gives the growth curves generated from these experiments. The high-pressure-adapted EC1002 cells displayed no pressure sensitivity, and, in fact, the relative growth of these cells at 1 and 272 atm was comparable to that of EC10 at 1 and 272 atm (Fig. 4A). Conversely, the unadapted EC1002 cells showed the same pressure sensitivity as seen previously (Fig. 4B). These results suggest that during the high-pressure cultivation of EC1002, those EC1002 cells that eventually arise may have acquired mutations resulting in the return to barophilic growth characteristics. Because these EC1002 derivatives do not regain β-galactosidase activity, the results imply that high-pressure growth of EC1002 results from selection for cells bearing suppressor mutations.

DISCUSSION

We have reported here the development of the first genetic system in a deep-sea barophilic bacterium, *Photobacterium* sp. strain SS9, and discussed the use of this system in the initial characterization of the regulatory mechanisms controlling

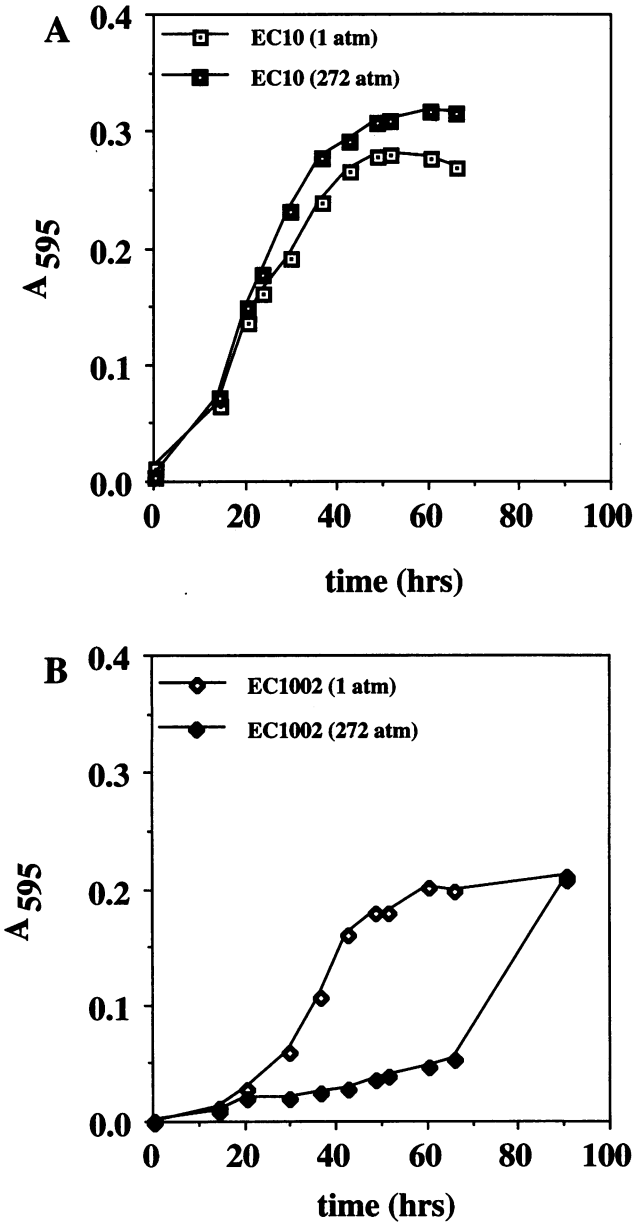

FIG. 4. Growth of EC10 (A) and the pressure-sensitive *ompH* regulatory mutant EC1002 (B) at both 1 and 272 atm, 9°C.

ompH gene expression in response to changes in hydrostatic pressure. Plasmid delivery into SS9 was accomplished via conjugation. Our system requires the use of very high concentrations of antibiotic (200 μg of kanamycin per ml) for plasmid selection and rifampin counterselection with an SS9 rifampin-resistant mutant as the recipient strain. In addition to the IncQ plasmids utilized in this study, kanamycin- or streptomycin-resistant IncP and IncW plasmids have also been successfully transferred (7a).

The ability to transfer plasmids into *Photobacterium* sp. strain SS9 was exploited in the construction of strain EC10, in which the *ompH* gene was replaced by homologous recombination with an introduced *ompH*::*lacZ* transcriptional fusion. EC10 has been used to monitor *ompH* promoter activity,

TABLE 2. Characteristics of EC10 and EC10-derived *ompH* regulatory mutants

Characteristic	Result[a] for strain:					
	SS9	EC10	EC1002	EC1006	EC1011	EC1020
Growth at 1 atm	+	+	+/−	+/−	+/−	+/−
Growth at 272 atm	+	+	−	+	+	+/−
OmpL repression at 272 atm	+	+	+/−	−	+/−	+/−
OmpI induction at 272 atm	+	+	−	+	+	−
Induction of additional OM proteins at 272 atm	−	−	−	−	+	−

[a] +, positive result; −, negative result; +/−, reduced growth or regulation relative to that of SS9, except for growth at 272 atm, which is relative to growth of the identical strain at 1 atm.

examine the phenotype of an *ompH* mutant, and derive *ompH* regulatory mutants.

Northern blot analysis of *ompH* transcript abundance in a previous study (3) suggested that the pressure regulation of OmpH production in SS9 results from the regulation of *ompH* message abundance. However, whether the regulation of *ompH* mRNA levels reflected variations in *ompH* promoter activity or *ompH* message stability was not determined. Because the *ompH*::*lacZ* fusion transcript expressed in EC10 lacks most of the wild-type *ompH* mRNA sequences, different β-galactosidase levels in EC10 grown at different pressures are most simply interpreted as being reflective of differential rates of *ompH* transcription. In fact, the magnitude of β-galactosidase pressure induction in EC10 appears to be approximately the same as OmpH pressure induction in the EC10 parental strain, DB110, particularly when the inductions are compared at 272 atm. From these data we conclude that *ompH* message abundance is indeed principally regulated by the rate of *ompH* transcription.

Despite the fact that OmpH is one of the most abundant OM proteins produced by *Photobacterium* sp. strain SS9 at its high-pressure optima, the *ompH* mutant was not impaired in growth at high pressure. Indeed, we have yet to determine the growth conditions, e.g., specific types of nutrient limitation, temperatures, or osmolarities, under which the growth of strain EC10 is markedly reduced compared with that of its parental strain, DB110. DNA sequence analysis of the *ompH* gene suggests that OmpH functions as a porin protein (2). It is well established that among the multiple porins gram-negative bacteria produce, overlapping transport specificities exist which can compensate for one another (23). Therefore, it is possible that if a high-pressure adaptive property exists for OmpH, it can be compensated for under our growth conditions by another protein. The identification of additional high-pressure-regulated OM proteins in SS9 and the isolation of an *ompH* regulatory mutant deficient in pressure regulation of multiple OM proteins which is pressure sensitive are certainly consistent with this hypothesis.

The approach taken to identify regulatory processes affecting *ompH* gene expression was to isolate *ompH* regulatory mutants. To this end EC10 was chemically mutagenized, and mutants defective in the production of β-galactosidase were identified on indicator media at 1 atm. Because of the difficulty of screening Lac^+ and Lac^- colonies at high pressure, regulatory mutants were sought at 1 atm with the supposition that elevated pressure would not induce the synthesis of a new *ompH* regulatory factor but would rather influence the activity of a preexisting one. The phenotypes of the isolated regulatory mutants support this hypothesis. However, we cannot rule out

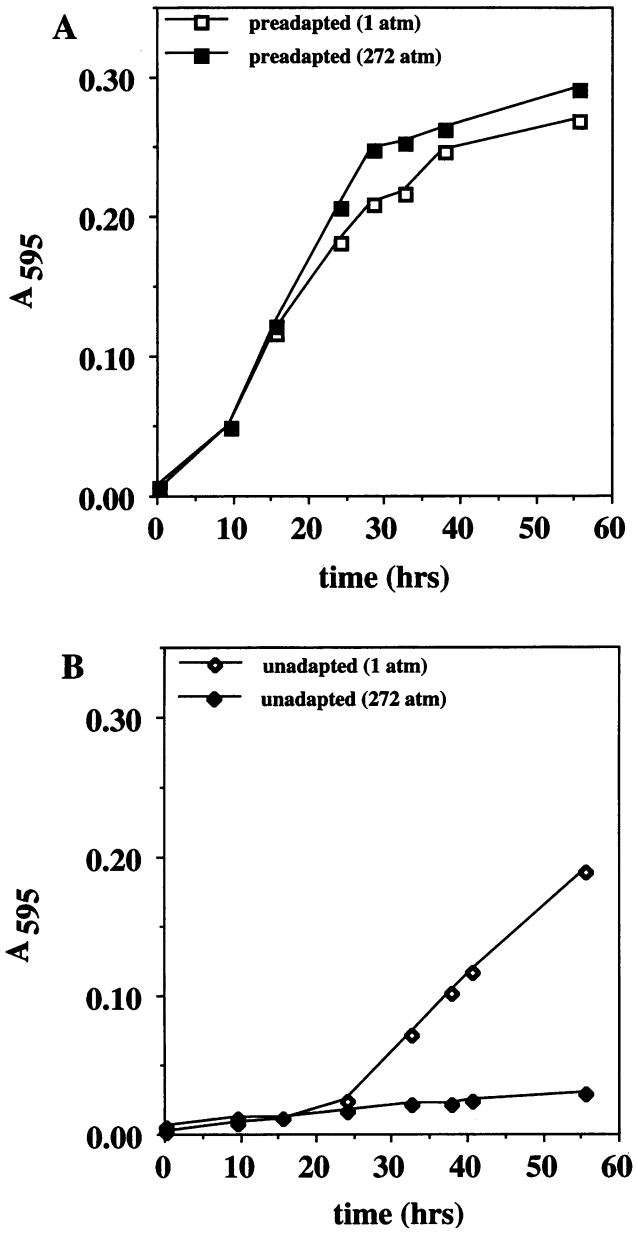

FIG. 5. Growth of high-pressure-adapted EC1002 (A) and high-pressure-unadapted EC1002 (B) at 1 and 272 atm, 9°C. (See text for details.)

the possibility that new regulatory mutants could be found by screening at high pressure.

By introducing an *ompH*-containing plasmid into the Lac$^-$ mutants and screening for diminished OmpH synthesis, four *ompH* regulatory mutants were identified. All of these mutants possess additional OM protein alterations. This suggests that *ompH* expression is coupled with that of other OM protein-encoding genes. For example, during the course of this study we have found two additional pressure-regulated OM proteins, designated OmpL, whose abundance decreases with pressure inversely to that of OmpH, and OmpI, whose abundance increases with pressure until at 408 atm it is the predominant OM protein. The repression of OmpL at high pressure was diminished in all four regulatory mutants, and the induction of OmpI at high pressure was diminished in two of four regulatory mutants. EC1011 was unique among the ompH regulatory mutants in its synthesis of additional OM proteins not seen in EC10. It is important to note that this phenotype is not necessarily a direct consequence of the *ompH*-regulatory gene mutation present in EC1011. Slowly growing porin mutants frequently give rise to strains bearing additional mutations which lead to the production of new OM proteins (12, 15).

Coordinate regulation of the *E. coli* OM porin proteins OmpF and OmpC is well documented. In this system, the abundances of OmpF and OmpC vary inversely with each other in response to environmental changes in osmolarity, temperature, and redox potential (8, 21). Regulation of *ompF* and *ompC* gene expression is complex and involves a two-component signalling system, with the OmpR component of this system acting as a transcriptional activator, as well as other factors less well understood (26). OmpF synthesis can also be regulated posttranscriptionally by the *micF* antisense RNA (20). The diverse phenotypes of the *ompH* regulatory mutants are also suggestive of intricate control processes. Clearly, it will be necessary to obtain multiple members of all regulatory mutant classes before it will be possible to better assess the nature and complexity of *ompH* regulation. In this regard it is noteworthy that we have recently succeeded in isolating *ompH* regulatory mutants, using the transposable element mini-Mu (7a). Because transposon-derived mutants possess a linked marker associated with the mutant phenotype, mini-Mu mutants in SS9 will greatly expedite the isolation and subsequent characterization of *ompH* regulatory loci.

Among the *ompH* regulatory mutants, EC1002 has the most dramatic property, which is growth sensitivity at high pressure. So while an *ompH* mutation does not result in pressure-sensitive growth, the specific mutation in EC1002 which affects both *ompH* gene expression and presumably the expression of additional genes does result in a pressure-sensitive characteristic. This represents the first such conditional mutant which has been isolated for any organism and underscores the power of the genetic approach in assessing high-pressure adaptation. To date, no properties of deep-sea bacteria have been demonstrated to be required for baroadaptation, although there are certain features, such as the production of polyunsaturated fatty acids, which have been correlated with baroadaptation (9, 29). Future characterization of pressure-sensitive mutants such as EC1002 offers the opportunity for identifying functions necessary for adaptation to high pressure. In this regard, it is noteworthy that the high-pressure induction of OmpI is greatly reduced in EC1002. OmpI function, perhaps in conjunction with OmpH, could be required for high-pressure growth.

The putative suppressor mutations which EC1002 readily accumulates at high pressure indicate the selective pressure which elevated hydrostatic pressure places on this strain. Characterization of these strains could reveal additional functions important to high-pressure growth. Mutants which possess enhanced barotolerance have previously been isolated from strains of the barosensitive bacterium *Enterococcus faecalis* (6, 18). These mutants were found to display derepression of several enzymes controlled by catabolite repression, and it was speculated that such a mutation might result in greater barotolerance due to increased ATP production and/or acid neutralization ability. It is important to note that at this time we cannot rule out the possibility that the stably maintained high-pressure-adapted forms of EC1002 result from a delayed physiological adaptation. The *E. coli* CreC protein catabolite control of the phosphate regulon is heritable over many cell generations in the absence of inducer via a mechanism proposed to involve protein phosphorylation and protein-protein

interactions (28). Conclusive demonstration for the high-pressure selection of suppressor mutations within EC1002 will require characterization of a locus from these strains conferring high-pressure-adapted growth to EC1002.

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