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Porin from the halophilic species *Ectothiorhodospira vacuolata*: cloning, structure of the gene and comparison with other porins

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Abstract

The gene coding for the anion-specific porin of the halophilic eubacterium *Ectothiorhodospira* (*Ect.*) *vacuolata* was cloned and sequenced, the first such gene so analyzed from a purple sulfur bacterium. It encodes a precursor protein consisting of 374 amino acid (aa)-residues including a signal peptide of 22-aa residues. Comparison with aa sequences of porins from several other members of the *Proteobacteria* revealed little homology. Only two regions showed local homology with the previously sequenced porins of *Neisseria* species, *Comamonas acidovorans*, *Bordetella pertussis*, *Alcaligenes eutrophus*, and *Burkholderia cepacia*. Genomic Southern blot hybridization studies were carried out with a probe derived from the 5′ end of the gene coding for the porin of *Ect. vacuolata*. Two related species, *Ect. haloalkaliphila* and *Ect. shaposhnikovii*, exhibited a clear signal, while the extremely halophilic bacterium *Halorhodospira* (*Hlr.*) *halophila* (formerly *Ect. halophila*) did not show any cross-hybridization even at low stringency. This result is in good accordance with a recently proposed reassignment within the family Ectothiorhodospiraceae, which included the separation of the extremely halophilic species into the new genus *Halorhodospira*. © 1997 Elsevier Science B.V.

Keywords: Ectothiorhodospiraceae; *Halorhodospira*; PCR; primary structure

1. Introduction

Ectothiorhodospiraceae are Gram-negative halo-alkaliphilic purple sulfur bacteria which belong to the γ subdivision of the *Proteobacteria* (Imhoff, 1984; Stackebrandt et al., 1988). They prefer or require saline and alkaline growth conditions. The deposition of elementary sulfur during sulfide oxidation outside the cell characterizes the family (Imhoff, 1989). Eubacteria such as Ectothiorhodospiraceae equalize the osmotic pressure of the surrounding medium with high concentrations of organic compatible solutes in the cytoplasm and possess normal salt sensitive enzymes (Trüper and Galinski, 1990; Imhoff, 1993).

Ect. vacuolata possesses a typical Gram-negative cell wall structure (Imhoff et al., 1981; Meißner et al., 1988). The outer membrane contains a major polypeptide (37 kDa), which was shown to be the porin (Wolf et al., 1996). This type of outer membrane protein was also found in mitochondria and chloroplasts (Fluegge and Benz, 1984; Benz, 1994; Nikaido, 1994, Rauch and Moran, 1994). Porins are highly polar, lack hydrophobic segments, and have a predominantly β-pleated structure (Cowan and Rosenbusch, 1994). Some porins are also of medical interest. Recent publications emphasize the role of the neisserial porin (PorB) in the process of infection and persistency within the target cells, and the contribution of mammalian porins (VDAC) to channels affected in cystic fibrosis and encephalomyopathy (Reymann et al., 1995; Rudel et al., 1996; Heiden et al., 1996).

The porin of *Ect. vacuolata* has mainly β-sheet

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Abbreviations: aa, amino acid(s); bp, base pair(s); *Ect.*, *Ectothiorhodospira*; *Hlr.*, *Halorhodospira*; kDa, kilodalton; LDAO, *N*,*N*-dimethyldodecylamine *N*-oxide; MALDI, matrix-assisted laser desorption/ionisation; N-T29 and N-T31, PCR fragments; ORF, open reading frame; PCR, polymerase chain reaction(s); TFA, trifluoroacetic acid.

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secondary structure and possesses a putative binding site for anions (Wolf et al., 1996). In this communication we describe the cloning, sequencing and gene structure of the *Ect. vacuolata* porin, comparing it with available primary structures of outer membrane proteins. Moreover, hybridization studies with genomic DNA of the two closely related species *Ect. haloalkaliphila* and *Ect. shaposhnikovii*, and with that of the extremely halophilic bacterium *Halorhodospira halophila* were carried out. The last species (formerly *Ect. halophila*) was formerly supposed to be also related to *Ectothiorhodospira.* However, it was reassigned to the new genus *Halorhodospira* (Thiemann and Imhoff, 1996; Imhoff and Süling, 1996). The data presented herein are in agreement with this separation.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Ect. vacuolata $\beta1^T$ (DSM 2111), *Ect. haloalkaliphila* $51/7^T$ (BN 9903, ATCC 51 935), *Ect. shaposhnikovii* $N1^T$ (DSM 243), and *Halorhodospira halophila* 51/1 (BN 9624) were grown photoheterotrophically in screw-capped 1-l bottles at 37°C and 10 000 lux in the medium described by Imhoff (1988). The Ph was 9.0, and the total salt content was 5% (w/v) for *Ect. vacuolata*, *Ect. haloalkaliphila* and *Ect. shaposhnikovii*, and 25% (w/v) for *Hlr. halophila*.

2.2. Isolation of the porin

Purification of the porin was carried out according to Wolf et al. (1996): the porin was isolated from the cell envelope by extraction with *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO) after treatment with TritonX-100. The purification was carried out by anion exchange chromatography and subsequent molecular sieve chromatography.

2.3. Peptide cleavage

The porin of *Ect. vacuolata* (100–150 μg), dissolved in 250 μl LDAO-buffer [0.027% (w/v) LDAO, 20 mM Tris/HCl, 20 mM $MgCl_2$, 3 mM NaN_3, pH 8.0], was denaturated by incubating it at 95°C for 10 min. Proteolytic cleavage was carried out by adding 24 μg/ml trypsin (Promega, Heidelberg, Germany) in the same buffer at 37°C for 7.5 h. The digestion was stopped by heating at 95°C for 10 min.

2.4. Peptide separation

Peptides were separated by reverse-phase chromatography on a LiChrospher 100RP-18 column (Merck, Darmstadt, Germany) using a 60 min gradient of 0.1% trifluoroacetic acid (TFA) to 0.09% TFA-60% acetonitrile.

2.5. Amino acid sequence analysis and PCR primer

Selected fractions were dried in a vacuum centrifuge. The peptides dissolved in 0.09% TFA-60% acetonitrile were N-terminally sequenced by Edman degradation in a pulsed-liquid gas-phase protein sequencer, model 477A equipped with a PTH-amino acid analyzer, type 120A (Applied Biosystems Inc., Weiterstadt, Germany). As primers for PCR four degenerate oligonucleotides were synthesized, based on the N-terminal amino acid (aa) sequence and three selected peptides.

2.6. Isolation of DNA and hybridization technique

Chromosomal DNA of the *Ect.* species was isolated by standard methods (Sambrook et al., 1989). A rapid small scale plasmid extraction was performed by the method of Birnboim (1983). Southern hybridizations with oligonucleotides probes were performed as described by Sambrook et al. (1989).

2.7. Cloning

For the cloning and the construction of a genomic library, *Escherichia coli* NM522 [supE, thi, Δ(lac-proAB), Δ(hsd5($r_{k-}m_{k-}$), F′, proAB, lacIqZΔM15)] and the plasmids pTZ18R (Pharmacia, Freiburg, Germany) and pGEM7Zf(−) (Promega, Heidelberg, Germany) were used. *E. coli* NM522 was grown in LB broth and 60 μg/ml ampicillin. For PCR, chromosomal DNA of *Ect. vacuolata* was used as template and peptide derived oligonucleotides as primers. PCR conditions were varied according to McPherson et al. (1992). PCR-amplified fragments were separated on low gelling temperature agarose gels (1%, Type VII, Sigma, Deisenhofen, Germany). Size fractions <1000 base pairs (bp) were eluted from the gel by phenol/chloroform extraction. Ends of the PCR fragments were blunted by standard methods (Sambrook et al., 1989). The fragments were then cloned into the dephosphorylated *Sma*I site of the plasmids. Recombinant clones were sequenced by the method of Sanger et al. (1977) using the Pharmacia (Freiburg, Germany) DNA-sequencing kit. To create a subgenomic gene library genomic DNA of *Ect. vacuolata* was digested either with *Bam*HI or *Xma*I. The fragments were separated by agarose (1% as above) gel electrophoresis and blotted to a Hybond-N+ Nylonmembrane (Amersham, Braunschweig, Germany). After hybridization with a labelled gene probe of *Ect. vacuolata*, DNA at the location of the detected fragment was eluted from the gel and cloned with plasmids. In situ colony screening of the gene

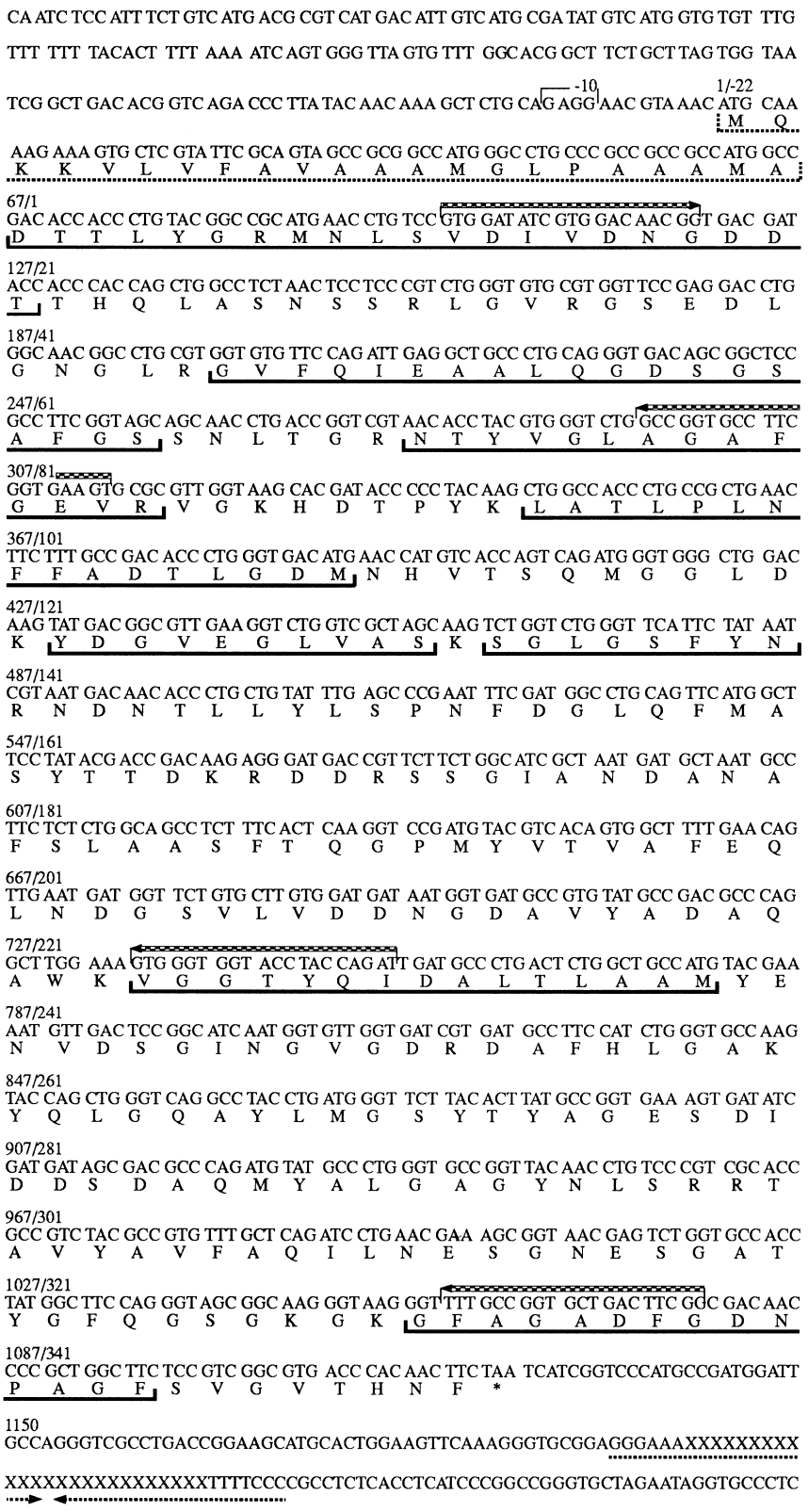

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the porin of *Ectothiorhodospira vacuolata* β1. The upper line shows the nucleotide sequence, the deduced amino acid sequence is shown below. The dotted underlined amino acid residues (−22 to −1) represent a typical bacterial signal sequence. The underlined peptide sequences have been determined by amino acid sequence analysis. The patterned arrows mark the PCR primers. The probable ribosome-binding region is located at nucleotide positions −13 to L-10. The translation stop codon is marked by an asterisk.The putative rho-independent terminator region, which could not be sequenced completely, is indicated by dotted arrows.

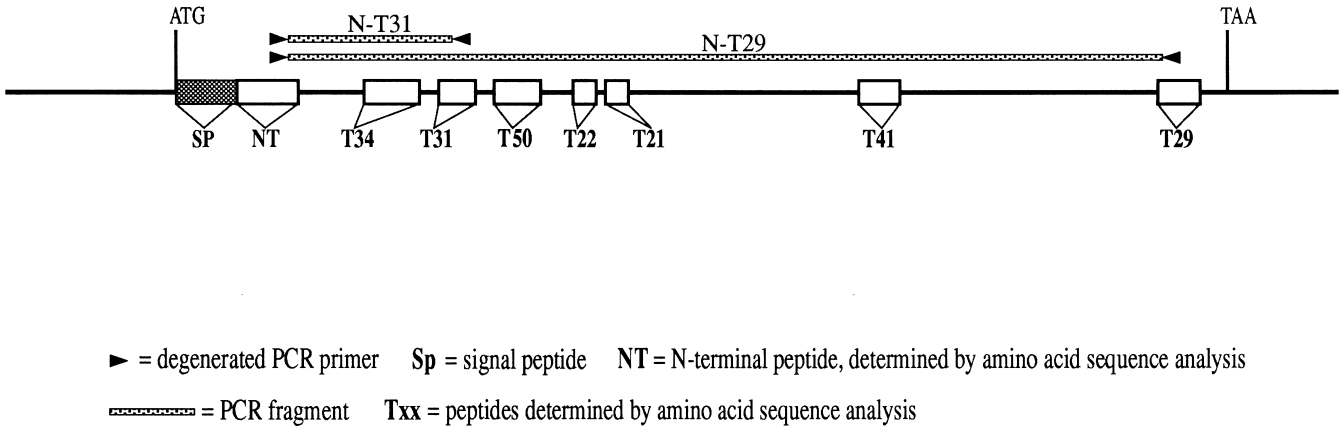

Fig. 2. General view of the porin gene structure of *Ectothiorhodospira vacuolata*. PCR-fragments: *N-T31* (214 bp) and *N-T29* (980 bp).

banks was based on the method described by Grunstein and Hogness (1975).

2.8. Sequence homology search

(1) The protein sequence including the signal peptide, derived from the structural gene, was used in a TBLASTN search in the non-redundant NCBIDNA data bank, in a BLASTP search in the merged non-redundant protein data bank, and in a FASTA search in the SWISSPROT data bank. Sequences with probabilities for chance hits $\leq 5 \times 10^{-3}$ were considered significantly similar to the porin sequence. (2) Hybridization studies with *Bam*HI-digested genomic DNA of *Ect. haloalkaliphila* 51/7, *Ect. shaposhnikovii* N1 and *Hlr. halophila* 51/1 were realized by Southern hybridisation. A DNA probe, derived from the 5′-part of the gene of *Ect. vacuolata*, was used. It was labelled with [α-^{32}P]-ATP by nick translation (Nick Translation Kit, Boehringer, Mannheim, Germany).

3. Results

3.1. Cloning strategy and sequencing of the porin gene

The porin of *Ect. vacuolata* was purified to homogeneity by molecular sieve chromatography and treated with trypsin as described in Section 2: Materials and methods. The fragments were separated by reverse-phase chromatography and sequenced by Edman degradation. The procedure yielded 8 N-terminal peptide sequences of 8- to 21-aa residues, which represented about 30% of the expected aa sequence including the N-terminus previously obtained by sequencing the undigested protein (Fig. 1, underlined aa residues; Fig. 2, white rectangles). In PCR one forward primer derived from the N-terminal peptide was used with three reverse primers derived from the tryptic peptides Nos. 29, 31 and 41 (*tn*, 5′-GTIGAYATIGTIGAYAAYGG; *t29*, 5′-CCRAAR-TCIGCICC IGCRAA; *t31*, 5′-ACYTCICCRAAIG-CICCIGC; *t41*, 5′-ATYTGRTAIGTICCICCIAC; I, Inosine; R, A or G; Y, C or T). PCR yielded fragments of various sizes. PCR fragments with sizes below the expected gene length (<1000 bp) were sequenced. The sequences obtained were compared with the known protein information. This revealed one gene fragment of 214 bp (*N-T31*) flanked by the N-terminal and the *t31* PCR primer and including the genetic information for the tryptic peptide T34 (Figs. 1 and 2). Southern hybridization of PCR fragments with radio labelled *N-T31* revealed another, larger PCR amplified gene fragment (*N-T29*) flanked by the N-terminal and the *t29* PCR primer. This fragment covered 980 bp (Fig. 2). PCR fragments obtained using the third combination *tN*/*t41* primer never showed hybridization with the *N-T31* probe. The *N-T29* fragment was sequenced in both directions. The resulting sequences were used to synthesize internal oligonucleotides that could be used for the sequencing of genomic gene libraries of *Ect. vacuolata* (see below). The screening of both a *Bam*HI and a *Xma*I subgenomic library with a labelled *N-T31* PCR fragment established three positive clones. All these contained the whole gene, two on a *Bam*HI (4.3-kb) fragment and the remaining one on a *Xma*I (2.5-kb) fragment. Both strands were sequenced.

3.2. Nucleotide sequence analysis

The nucleotide sequence shown in Fig. 1 contains one open reading frame. It consists of 1122 bp encoding a protein of 374 aa. The other forward reading frames show various stop signals. In contrast to meningococcal porin genes (Butcher et al., 1991), the porin gene of *Ect. vacuolata* does not show any long open reading frames (ORFs) in its complementary strand. The G+C content in the structural gene is 57%, which is less than the published 62% of the whole genome (Imhoff, 1989).

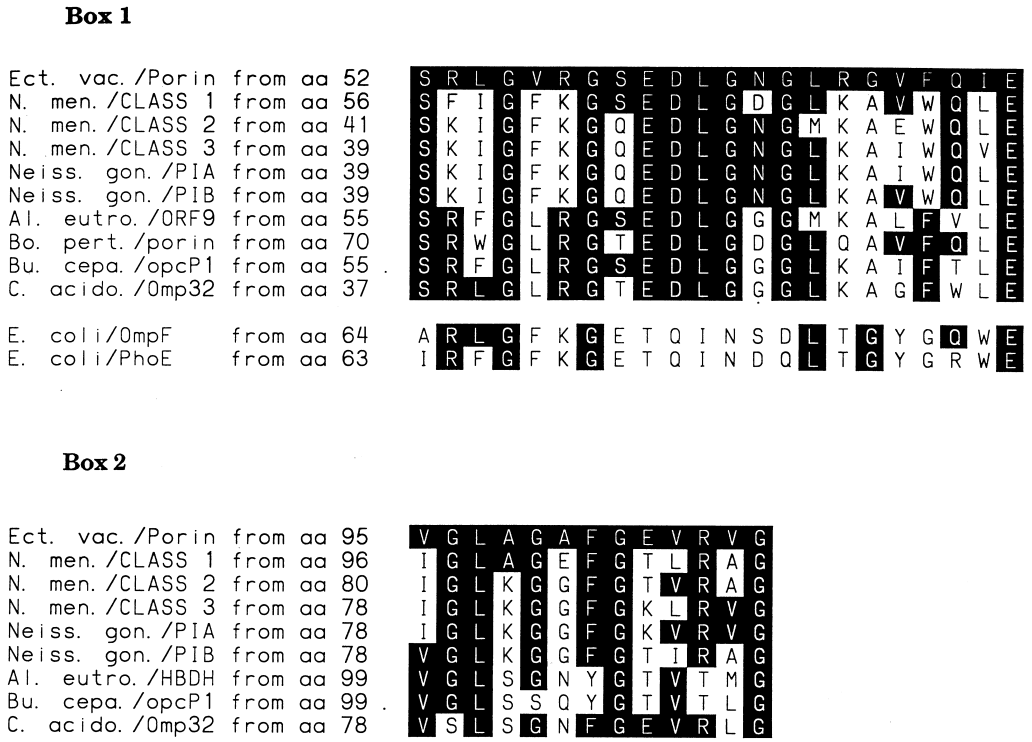

Fig. 3. Alignment of the regions of the porin amino acid sequence from *Ectothiorhodospira vacuolata* (line 1) that show significant homologies to other proteins. Amino acid residues identical to the porin sequence of *Ectothiorhodospira* vacuolata are shaded and those which are homologous are boxed. Ect. vac. = *Ectothiorhodospira vacuolata*; N. men. = *Neisseria meningitidis*; Neiss. gon. = *Neisseria gonorrhoeae*; C. acido. = *Comamonas acidovorans*; Al. eutro. = *Alcaligenes eutrophus*; Bo. pert. = *Bordetella pertussis*; Bu. cepa. = *Burkholderia cepacia*. Omp32, OmpF, CLASS 1, CLASS 2, CLASS 3, PIA, PIB, opcP1, PhoE = outer membrane proteins (porins); ORF9 = Open reading frame, which probably encodes a porin.

There is a possible ribosome binding site at position -13 to -10 from the translation start codon (Shine and Dalgarno, 1974; Watson et al., 1991). No obvious promoter consensus sequence in the -10 and -35 region upstream of the transcriptional start site was to be found; downstream of position 1,237 a number of base pairs could not be determined by the method used (Fig. 1, dotted arrows) possibly due to the existence of a palindrome, typical for a factor independent transcription termination signal (Brendel and Trifonov, 1984), as has been described for other porin genes (Gerbl-Rieger et al., 1991; Li et al., 1991). Codon usage is non-random. Of 59 possible sense codons (cysteine does not occur in the porin protein) 10 are not used and 8 are only used once. The following codons occur preferentially: GCC and GCU for alanine (31 and 11, respectively, of 45), CGU for arginine (8 of 12), AAC for asparagine (14 of 23), CAG for glutamine (13 of 15), GGU and GGC for glycine (35 and 14, respectively, of 50), AUC for isoleucine (5 of 7), CUG for leucine (29 of 33), AAG for lysine (9 of 11), UUC for phenylalanine (15 of 19), ACC for threonine (16 of 21), and GUG for valine (14 of 25). These data are similar to those of the codon usage of *E. coli*-porins (Mizumo et al., 1983). The polypeptide deduced from the porin gene contains the N-terminal protein sequence and all the tryptic peptides that were determined by amino acid sequence analysis. The last aa is a phenylalanine, which is supposed to be essential for the correct assembly of the protein. The last 10 aa are very similar to those of outer membrane proteins published by Struyvè et al. (1991).

A signal sequence of 22 aa is located before the N-terminal sequence start of the mature protein (Fig. 1, aa -22 to -1). It has the common features of prokaryotic signal peptides (Gierasch, 1989; von Heijne, 1985; Wickner et al., 1991): it has an average length and shows N-terminally two strongly basic aa followed by a hydrophobic core containing the secondary structure-breaking aa proline and glycine. The signal sequence-cleaving site is identified by A-M-A, which is in accordance with the common a-x-b (a and b are small, uncharged aa). Thus the mature protein consists of 352-aa residues and has a molecular mass of 37 140 Da. This is in good agreement with the $M_r = 37{,}160 \pm 30$ determined by MALDI-mass spectrometry (Wolf et al., 1996). The calculated isoelectric point of the mature protein is 4.15, similar to other porins Genomic Southern blot hybridization studies were carried out with the labelled *N-T31* probe and genomic DNA from *Ect. vacuolata* β1 digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, and *Xma*I. In all cases, even at low stringency, a single signal was

observed indicating that other sequences homologous to the gene do not occur in the genome.

3.3. Homology

In search for similarities to other published proteins we have performed a sequence comparison with the SWISSPROT, merged protein and DNA sequence data banks. Only two short regions of significant similarity to several proteins were found (Fig. 3, Boxes 1 and 2): various outer membrane proteins from *Neisseria menengitidis*, porins from *Neisseria gonorrhoeae* (Butcher et al., 1991), the porin from *Comamonas acidovorans* (Gerbl-Rieger et al., 1991), a hardly characterized ORF from *Alcaligenes eutrophus* (Valentin et al., 1995), and porins from *Bordetella pertusis* (Li et al., 1991) and *Burkholderia cepacia* (dbj, BCUOPCP1). The only other available sequence for a porin from a phototrophic purple bacterium shows no striking homology (Trieschmann et al., 1996a,b). Of the numerous neisserial outer membrane proteins, only 5 representative sequences are shown in Fig. 3.

No significant homology of *Ect. vacuolata* β1 and *E. coli* porins is found in either region. But box 1 turns out to be an important region not only for the porin from *Ect. vacuolata* and the porins from *Comamonas acidovorans* and *Neisseria*, but also for the *E. coli* porins OmpF and PhoE (Gerbl-Rieger et al., 1991). The alignment for OmpF and PhoE for box 1 is also shown in Fig. 3. The homologous region in the three dimensional structure of OmpF and PhoE extends from the first half of β-strand 2 and turn 2 to the first five aa of β-strand 3 (Cowan et al., 1992). It is located on the generally more highly conserved β-strand in the area where the monomers contact with each other. The second shorter box does not allow any indirect parallelism of the *Ect. vacuolata* porin to the three dimensional structure of the *E. coli* porins.

The extraordinary resemblance of the N-terminal 18-aa sequences of the porins from *Ect. vacuolata* β1 and *Ect. shaposhnikovii* N1 (Wolf et al., 1996) was the basis for cross-hybridization studies with DNA of further species of the Ectothiorhodospiraceae. We used a labelled DNA probe of the porin gene from *Ect. vacuolata*. The strains *Ect. shaposhnikovii* N1, *Ect. haloalkaliphila* 51/7, and *Hlr. halophila* 51/1 were selected to test possible homologies of porin genes. The PCR fragment *N-T31* was digested with *Pst*I to obtain the 5′ end of 126 bp as a probe (Figs. 1 and 2), which codes for N-terminal aa of the porin gene. Southern blot hybridization was performed under normal stringency with *Bam*HI-digested genomic DNA of *Ect. haloalkaliphila*, *Ect. shaposhnikovii*, *Hlr. halophila* and, as a control, *Ect. vacuolata*. For *Ect. haloalkaliphila*, *Ect. shaposhnikovii*, and *Ect. vacuolata* clear signals of nearly the same intensity were observed, while the probe did not show any cross-hybridization with the genomic DNA of *Hlr. halophila* (Fig. 4). The hybridization signals were at 3.2 kb, 6.5 kb and 4.3 kb for *Ect. haloalkaliphila*, *Ect. shaposhnikovii* and *Ect. vacuolata*, respectively.

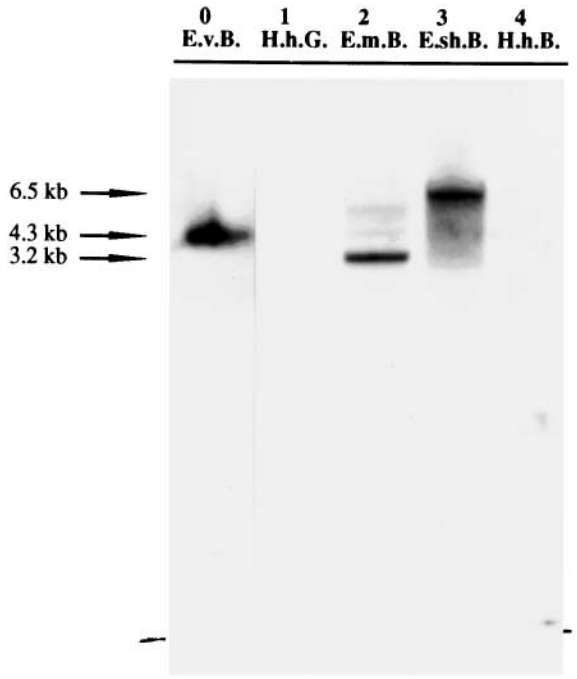

Fig. 4. Southern blot with genomic DNA and a 126-bp *Pst*I-fragment of *N-T31* as probe. Lanes: 0, E.v.B. = genomic DNA from *Ectothiorhodospira vacuolata Bam*HI-digested; 1, H.h.G. = genomic DNA from *Halorhodospira halophila* undigested; 2, E.m.B. = genomic DNA from *Ectothiorhodospira haloalkaliphila Bam*HI-digested; 3, E.sh.B. = genomic DNA from *Ectothiorhodospira shaposhnikovii Bam*HI-digested; 4, H.h.B. = genomic DNA from *Halorhodospira halophila Bam*HI-digested. **Methods**: After restriction digestion of 3–6 μg DNA with *Bam*HI and electrophoresis on a 1% agarose gel, DNA was transferred to Hybond-N + Nylonmembrane (Amersham, Braunschweig, Germany). The filter was hybridised at 56°C overnight with a probe generated by PCR on *Ect. vacuolata* β1 gemomic DNA, employing the 126-bp *Pst*I-fragment of *N-T31* (see Section 3.1 and Fig. 2), after radiolabelling (Nick Translation Kit, Boehringer, Mannheim, Germany, as described by the manufacturer). Washes were carried out at 56°C at 0.1 × SSC/0.1% SDS, and the film was exposed to the filter at −80°C for 48 h.

4. Discussion

In this paper we describe the cloning and analysis of the gene coding for the porin from *Ect. vacuolata β*1, as the first such gene from a purple sulfur bacterium, and cross-hybridization studies with DNA of related bacterial species. Cloning porin genes using plasmid systems has been difficult since the expression of an intact heterologous porin gene could be correlated with lethality for the host (Carbonetti and Sparling, 1987; Gotschlich et al., 1987). Although we cloned the whole gene in plasmids, we did not encounter serious problems.

We do not know whether this is an indication of the lack of expression of the porin gene in *E. coli*, or whether the *Ect. vacuolata* porin does not affect *E. coli* cell viability. Based on genomic Southern blot hybridization studies at low stringency with digested DNA of *Ect. vacuolata* β1, we concluded that genes homologous to the porin gene do not occur in the genome of this strain. Bacteria expressing various porins at the same time have been described (Butcher et al., 1991; Mizumo et al., 1983); however, other species exist (preferentially phototrophic bacteria), which can express only one porin (Robledano et al., 1996).

The taxonomic situation is reflected by the results of homology studies by cross-hybridization with a probe of the porin gene of *Ect. vacuolata* and genomic DNA of further Ectothiorhodospiraceae species. The species of the genus *Ectothiorhodospira* have been divided into two subgroups based on physiological and molecular data (Imhoff, 1989). *Ect. vacuolata*, *Ect. haloalkaliphila*, and *Ect. shaposhnikovii* were placed in subgroup I and *Hlr. halophila* (formerly *Ect. halophila*) in subgroup II. *Hlr. halophila* and other extremely halophilic relatives were recently removed from the genus *Ectothiorhodospira* and reassigned to the new genus *Halorhodospira* (Imhoff and Süling, 1996). In accordance with this scheme, the closely related species *Ect. haloalkaliphila* and *Ect. shaposhnikovii* showed hybridization signals similar to *Ect. vacuolata*, while genomic DNA from *Hlr. halophila* did not show any cross-hybridization. This indicates that *Hlr. halophila* did not share significant sequence similarity with the porin gene of *Ect. vacuolata*. Hence, the chosen probe could serve to fish the porin genes of *Ect. haloalkaliphila* and *Ect. shaposhnikovii* but would not help in the case of any gene of *Hlr. halophila*.

Two short regions of significant homology to other porins were found. It is not surprising that these small sections do not present a phylogenetical relationship. All the known bacteria showing homologous proteins, belong to the β-subclass of the *Proteobacteria*, whereas *Ectothiorhodospira* belongs to the γ-subclass (Stackebrandt et al., 1988). It is very likely that there is a structural or functional basis for these conserved portions of the sequences since all of the homologous proteins are porins, and the ORF9 from *Alcaligenes eutrophus* is supposed to be a porin too (Valentin et al., 1995). Possibly this homology is related to their strong anion specificity: protein I of *Neisseria gonorrhoeae* (Young et al., 1983), the porin of *Bordetella pertussis* (Armstrong et al., 1986) and Omp32 of *Comamonas acidovorans* (Gerbl-Rieger et al., 1991), as well as the porin of *Ect. vacuolata* β1 (Wolf et al., 1996), are anion specific. On the other hand, when compared over the whole sequence of the *Ect. vacuolata* porin the other porins showed little identity and are at best weakly related to it (Schiltz et al., 1991). In our sequence 11 (by Chou-Fasman) or 18 (by Garnier-Robson) β-sheet domains could be identified. As there is so little primary sequence homology to the members of the porin superfamily and as we do not have immunological or mutational data at our disposal yet, we cannot reliably make a more detailed secondary structure analysis, as has been done for other well studied porins (Jeanteur et al., 1991, 1994). Moreover, from the hybridization studies there is evidence that the porins of the genus *Ectothiorhodospira* form a new sequence family (Schiltz et al., 1991; Jeanteur et al., 1991, 1994).

Nonetheless the aa sequence as derived from the porin gene of *Ect. vacuolata* is that of a characteristic bacterial porin with respect to the distribution of charged and uncharged aa residues in the sequence, the high content of glycine (nearly 14%), and the absence of cysteine (Gerbl-Rieger et al., 1991). There was no obvious promoter consensus sequence in the -10 and -35 region. This is possibly explained by a 5′ untranslated leader sequence in the mRNA. With the assumed palindrome for factor-independent termination of transcription it is conceivable that the primary transcript extends out from the 5′ end rather than the 3′ end.

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