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FEMS Immunology and Medical Microbiology 22 (1998) 351–354

FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY

The identification of response regulators of *Branhamella catarrhalis* using PCR

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Received 4 May 1998; received in revised form 2 September 1998; accepted 18 September 1998

Abstract

Potential response regulator gene fragments from the genome of *Branhamella (Moraxella) catarrhalis* were isolated by PCR using degenerate oligonucleotide primers. DNA sequence analysis of several cloned PCR products with similar restriction endonuclease analysis (REA) patterns revealed that the cloned gene fragment had significant homology to members of the OMPR sub-family of response regulator genes, including 61% identity with the *pho*B gene of *Haemophilus influenzae*. The derived amino acid sequence showed greatest similarity to the PhoB response regulator protein of *Pseudomonas aeruginosa*. Characterisation of this *pho*B homologue and of other response regulators identified in this study should provide new knowledge of the physiology and pathogenic mechanisms of *B. catarrhalis*.

Keywords: *Branhamella catarrhalis*; Two-component signal transduction; Response regulator; PhoB

In the last 10–15 years, *Branhamella (Moraxella) catarrhalis* has emerged as one of the prominent human pathogens associated with infections of the ear and the lower respiratory tract where it is the major cause of exacerbations of chronic bronchitis in adults [2,4]. Little is known about the virulence mechanisms of this pathogen [4]. In this study we targeted the regulatory elements of virulence rather than the virulence genes themselves. Two-component signal transduction systems play a central role in the coordinate regulation of virulence in many bacteria and these systems are comprised of a sensor and a response regulator protein [3]. The aim of this study was to identify response regulator genes of *B. catarrhalis*.

B. catarrhalis clinical isolates K69, K70, K106 and K116 were recovered from sputum samples collected from patients at the Sir Charles Gairdner Hospital, Perth, Western Australia. Isolates were cultured on Mueller Hinton agar containing 0.5% yeast extract. DNA was extracted from these isolates and from

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Abbreviations: REA, restriction endonuclease analysis

The GenBank accession numbers for the N-terminal homologous region of the proposed phoB nucleotide sequence of *Branhamella (Moraxella) catarrhalis* are U80193 (clinical isolate K69) and U80194 (clinical isolate K70).

0928-8244/98/$19.00
PII: S0928-8244(98)00107-2

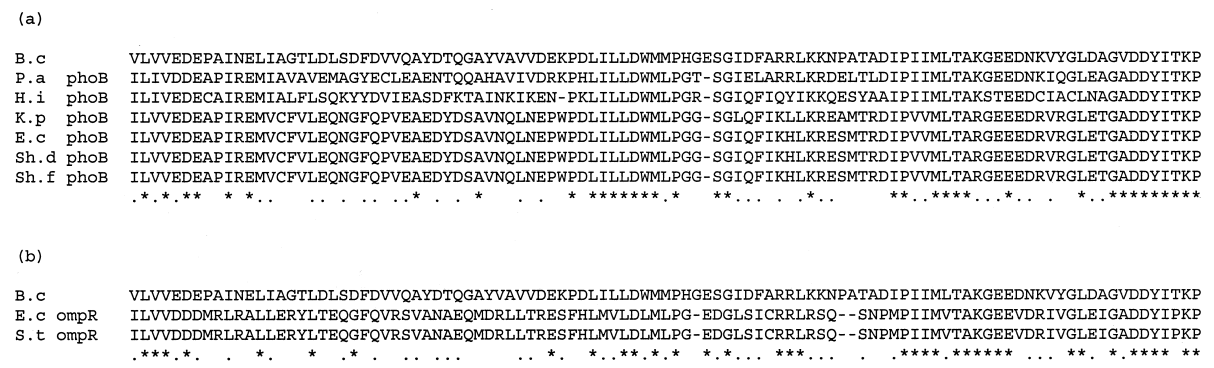

Fig. 1. a: Comparison of *B. catarrhalis* (B.c) derived amino acid sequence to the phoB sequences of *E. coli* (E.c), *H. influenzae* (H.i.), *K. pneumoniae* (K.p), *P. aeruginosa* (P.a), *Sh. dysenteriae* (Sh.d) and *Sh. flexneri* (Sh.f). b: Comparison of *B. catarrhalis* derived amino acid sequence to the ompR sequences of *E. coli* and *S. typhimurium*. (*) Identity; (.) biochemically similar amino acids.

Salmonella typhimurium LT2 [9] by the alkaline boiling method [8].

Degenerate oligonucleotide primers based on two regions of strong amino acid homology among members of the OMPR sub-family of response regulators [3] were designed by Wren et al. [12] using sequence data from *S. typhimurium* PhoP and *E. coli* PhoB response regulators. PCR using these primers was performed as described [12] with annealing temperatures of 45°C and 50°C. At both temperatures, an amplification product of 320 bp was obtained from *S. typhimurium* and from all four *B. catarrhalis* isolates. Non-specific background amplification products were reduced in the 50°C reaction.

The degenerate oligonucleotide primers contained restriction endonuclease sites for *Hin*dIII and *Pst*I at the 5′ ends. The K69 and K70 PCR products were digested with *Hin*dIII and *Pst*I, ligated with similarly digested plasmid vector pUC18 and transformed into *E. coli* DH5α [8]. Transformants were selected on Luria Bertani agar containing 100 μg ml^{-1} ampicillin and clones containing inserts were detected using histochemical screening. Plasmid DNA was extracted from transformants [8] and PCR performed using M13 universal 24mer primers, which recognise the flanking regions of the multiple cloning site, to provide template DNA for restriction endonuclease analysis (REA) and sequencing.

Double-stranded cycle sequencing was carried out according to the manufacturer's instructions, using the ABI PRISM™ Ready Reaction Dye Deoxy™ terminator cycle sequencing kit. One μg of template DNA was mixed with M13 forward or reverse sequencing primers (Promega) and using the PCR of 96°C for 30 s, 50°C for 30 s, and 60°C for 3.5 min for 28 cycles single-stranded terminator products were synthesised. The ABI 373A DNA automated sequencing system was used to sequence each template in both directions. Analysis was performed using the Australian National Genomic Information Service (ANGIS) database. Sequences of the homologous response regulators were extracted from both the GenBank and EMBL databases, and a multiple alignment using ClustalW (1.5) programme using cluster analysis Neighbour-joining method of Satou and Nei [10] set at default parameters with window size of 5. ClustalW (1.5) also allowed the generation of a phylogenetic tree based on a matrix of distances between sequences.

A total of 121 recombinant clones were investigated by the M13 PCR. Of these, 37 produced a PCR product greater than 320 bp; 15 K69 clones and 22 K70 clones. The 37 recombinants were analysed by REA with *Hin*fI and *Alu*I, and a total of 14 patterns were observed. Three K69 and two K70 clones with similar REA band patterns were selected for sequencing.

Sequence analysis revealed that a 320-bp region of a response regulator gene had been amplified and cloned on all five occasions. On one occasion, a 320-bp fragment had been correctly cloned in a *Hin*dIII-*Pst*I orientation as expected. However, on the remaining four occasions where a 320-bp fragment was cloned there appeared to be an extra 50 bp of

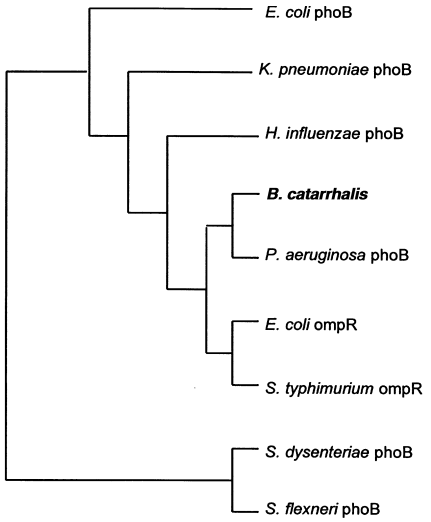

Fig. 2. Phylogenetic tree based on relatedness of amino acid sequences of response regulators of various Gram-negative bacteria. Tree created using the default parameters of the ClustalW (1.5) programme along with Phylip retree.

unrelated sequence. Sequence analysis of the unrelated region revealed that it was primer dimer in origin. Preliminary investigations were carried out on a third K70 clone which had a size estimated by M13 PCR of 650 bp. The first 320 bp in the forward direction showed homology to the five *B. catarrhalis* sequences described above.

These sequences were further analysed using UNIX-based programmes in the ANGIS database. The homology search for the 320-bp fragment to sequences on the GenBank database was performed using the FastA programme. Analysis of the nucleotide sequences of the six clones revealed closest homology to the OMPR subfamily of response regulators, in particular the *pho*B and *omp*R response regulator genes. The FastA algorithms indicated that at the nucleotide level, the 320-bp sequence of *B. catarrhalis* completely overlapped similar regions of response regulators and indicated there to be 61.4% identity with *Haemophilus influenzae pho*B and approximately 55% identity to the *pho*B response regulator sequences of various other Gram-negative bacteria. The generated phylogenetic tree indicated the *B. catarrhalis* 320-bp sequence was most similar to a phosphate response regulator in *Haemophilus influenzae* and the *omp*R response regulator gene of *Neisseria meningitidis*.

The derived amino acid sequence for the 320-bp fragment also showed homology to the OMPR subfamily of response regulators, as expected. The *B. catarrhalis* 103 amino acid sequence showed homology to the phoB (Fig. 1a) and ompR (Fig. 1b) sequences of *E. coli* and *S. typhimurium* and various other Gram-negative bacteria. A phylogenetic tree of the relatedness of the amino acid sequences was generated. Both the FastA and ClustalW (1.5) programmes indicated greatest homology to the *Pho*B response regulator of *Pseudomonas aeruginosa* at the amino acid level (Fig. 2). FastA indicated a 57.3% identity and a 87.4% similarity of the *B. catarrhalis* sequence to the amino acid sequence of *P. aeruginosa*. Analysis at both the nucleotide and amino acid level also revealed significant relatedness to the *omp*R sequences of *E. coli* and *S. typhimurium*.

Another set of clones with different sizes and REA patterns was sequenced with the following results. The first clone had most homology in its amino acid sequence (57% identity) to a family of ABC transporter proteins of *Klebsiella pneumoniae* [7]. The second clone of 750 bp was the largest of the PCR products sequenced and had unique *Hin*fI and *Alu*I REA patterns. Over 180 bp, there was 63.3% homology to the *pho*P gene of *Bacillus subtilis* [11]. Across 170 bp of the third clone there was 65.4% identity to the *ntr*A gene which codes for a *Pseudomonas putida* sigma factor [5], while the fourth clone had 58.6% identity over 140 bp to a *B. subtilis npr*B gene [6]. The final two clones had significant homology to the *dtx*R gene which regulates toxin production in *Corynebacterium diphtheriae* [1].

It is expected that the 320-bp sequence identified in this study is part of a *pho*B response regulator gene in *B. catarrhalis*. Mutagenesis studies similar to that described by Wren et al. [13] are now being performed to investigate the role of this and the other response regulator genes identified.

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