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# Molecular Characterization of the Pneumococcal Teichoic Acid Phosphorylcholine Esterase

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## ABSTRACT

**A search to identify proteins with high affinity for choline-containing pneumococcal cell walls (choline-binding proteins) has permitted the localization, cloning, sequencing, and overexpression of a gene (*pce*), coding for a protein (Pce) that liberates phosphorylcholine from purified cell walls of *Streptococcus pneumoniae*. The *pce* gene of the pneumococcal strain R6 encodes a protein of 627 amino acids with a predicted $M_r$ of 72,104. Pce can remove a maximum of 20% phosphorylcholine residues from the cell wall teichoic acid. *In silico* analysis of Pce shows a modular organization of the enzyme where the choline-binding domain, involved in cell wall substrate recognition, and the catalytic domain are located at the carboxy- and amino-terminal moieties of the protein, respectively. Remarkably, a long tail of 85 amino acids follows the carboxy-terminal domain, a structural feature that had not been described for the published choline-binding proteins. The carboxy-terminal moiety of Pce is assembled by 10 repeated motifs, and the protein has also a cleavable signal peptide of 25 amino acids that renders after its cleavage a mature protein of 69,426 Da (602 amino acids). The *pce* gene has been expressed in *Escherichia coli,* and Pce was active when assayed on pneumococcal walls. We have also found that the signal peptide of Pce was functional in *E. coli*. Biochemical analysis suggested that Pce is the teichoic acid phosphorylcholine esterase of *S. pneumoniae* that had been biochemically characterized previously. Construction of two *pce* pneumococcal mutants (R6D and M31D) by insertion-duplication mutagenesis revealed that these mutants grew at a doubling-time similar to those of the parental strains of the wild-type R6 and the *lytA*-mutant M31, respectively. R6D and M31D were morphologically indistinguishable from the parental strains when whole-mounted cells were observed under the electron microscope and exhibited levels of competence for genetic transformation slightly lower than those reported for R6 and M31.**

## INTRODUCTION

*STREPTOCOCCUS PNEUMONIAE* (PNEUMOCOCCUS) is an important human pathogen as a common etiological agent in community-acquired pneumoniae and meningitis in adults and in acute otitis media in children. Pneumococcus is unique among the Gram-positive bacteria because of the nutritional requirement of this bacterium for choline[31] and because the teichoic acid (TA), and lipoteichoic acid (LTA) polymers of the cell wall membrane complex have the same chain structure. Interestingly, the aminoalcohol choline is a structural component of the TA and LTA and, for a given strain, an identical number of phosphorylcholine residues have been found per repeat. This is, in *S. pneumoniae* R6 strain, on the average, 78% of the repeats in LTA and TA are substituted with two phosphorylcholine residues and 22% with one.[6] The choline residues of the TA and LTA, among many other functions, serve as ligand for the attachment of a number of proteins to the cell surface, named choline-binding proteins (CBPs).[9] It has been pointed out that pneumococcus has evolved this unique mechanism to associate proteins to the cell surface.[4] In this sense, we had shown that two CBPs, the major pneumococcal autolysin (LytA amidase) and the Cpl1 lysozyme encoded by the pneumococcal Cp-1 phage, contain a homologous carboxy-terminal domain (choline-binding domain, ChBD) of six 20-amino acid tandem repeats (motifs) that allows the binding of these enzymes to the cell wall substrate through the choline residues of TA.[7] Up to now all the described CBPs contain this typical ChBD with a variable number of repeats either in its carboxy-terminal or its amino-terminal position.[11] Moreover, the criti-

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TABLE 1. BACTERIAL STRAINS, PLASMIDS, AND PRIMERS

| *Material* | *Description*[a] | *Reference or source* |
|---|---|---|
| Bacterial strains | | |
| *S. pneumoniae* | | |
| R6 | Wild type | Rockefeller University |
| M31 | Δ*lytA* R6 mutant | (22) |
| R6D | R6 (*pce*::*ermC*) | This study |
| M31D | M31 (*pce*::*ermC*) | This study |
| M222 | *hex-4, end-1, exo-2,* Lyt$^+$, S2$^-$, Str$^R$ | (5) |
| *E. coli* | | |
| DH5α | *supE44 hsdR17 recA1 endA1* Δ*lacU169 (*Δ*80 lacZ*Δ*M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | (21) |
| BL21 (DE3) | F$^-$ *ompT gal [cdm] [lon] hsdS$_B$* with DE3 | (27) |
| Plasmids | | |
| pUC18 | Ap$^R$ | BioLabs |
| pT7-7 | Ap$^R$ | (28) |
| pGL100 | *lytA*, Ap$^R$ | (8) |
| pLCC14 | *lytC*, Ap$^R$ | (11) |
| pRGR5 | *lytB*, Ap$^R$ | Unpublished |
| pUCE191 | Ln$^R$/Er$^R$, Ap$^R$ | (1) |
| pRGR11 | *pce*, Ap$^R$ | This study |
| pRGR12 | *pce* with deleted signal peptide encoding sequence, Ap$^R$ | This study |
| pRGR18 | 2.5-kb *Eco*RI–*Bam*HI fragment containing *pce*, Ap$^R$ | This study |
| pRGR191 | 1.1-kb *Hin*cII internal fragment of *pce*, Ln$^R$/Er$^R$, Ap$^R$ | This study |
| Primers | | |
| LytDEco | 5′-CCGAATTCGGCTGTAACTGCTAAAGGGAAG-3′ | This study |
| LytDBam | 5′-TTGGATCCATCAGCAATCTTAGTCGGACGC-3′ | This study |
| LytDN1 | 5′-CCGAATTCTGGAGATTGAGAAAGTAAGAATG-3′ | This study |
| LytDN2 | 5′-CCGAATTCAAGGAGATTAACATATGCAAGAAAGTTCAGGAAATAAAATCC-3′ | This study |
| LytDC | 5′-TTGGATCCCTACTACTGTTCTGATTCCGATTTG-3′ | This study |

[a]Str, streptomycin; Ap, ampicillin, Ln, lincomycin; Ery, erythromycin. The nucleotides underlined denote the restriction sites and start codons.

cal role of several CBPs in the physiology and pathogenicity of pneumococcus has been recently documented in the literature.[13]

The functions of some of the CBPs remain to be determined. In this sense, a teichoic acid phosphorylcholine esterase had been described in *S. pneumoniae*[14] and in *Streptococcusoralis*[20] as an enzyme that liberated phosphorylcholine residues from the choline-containing cell walls, but in spite of the fact that this enzyme might be a clear CBP candidate, it remained to be characterized at a molecular level. It should be pointed out that pneumococcus undergoes spontaneous and reversible phase variation marked by switching from opaque to transparent morphotypes, and vice versa, that implies changes in the amount of choline in the cell wall, *i.e.,* high amount in transparent and low amount in opaque variants[33] and it is tempting to speculate on a role of the esterase in this conversion. However, a fine characterization of the turnover of choline has not yet been determined although this aminoalcohol is essential for the biology of pneumococcus.

In this study, we have isolated, cloned, and overexpressed in *Escherichia coli* a new pneumococcal gene named *pce* that encodes an enzyme capable of liberating phosphorylcholine from choline-containing cell walls and that, according to its molecular structure, represents a new member of the CBP family. In a parallel and independent effort, Vollmer and Tomasz have also characterized the product of *pce* gene and studied some of its biological properties.[34]

## MATERIALS AND METHODS

### *Bacterial strains, plasmids, growth conditions, and transformation*

The bacterial strains, plasmids and oligonucleotide primers used in this study are listed in Table 1. *E. coli* was grown in Luria-Bertani medium.[21] *S. pneumoniae* was grown in C medium[16] supplemented with yeast extract (0.8 mg/ml; Difco Laboratories) (C+Y medium). The procedures used for the preparation of the competent pneumococci and the transforming DNA (Str$^r$) were essentially those described for analysis of genetic transformation in *S. pneumoniae*.[32] Transforming DNA was prepared from strain M222, and transformation frequency was calculated as the number of streptomycin-resistant transformants per milliliter times 100 divided by the total number of viable cells per milliliter. *E. coli* was transformed following a procedure previously described.[21]

### *Preparation of choline-binding proteins from autolytic walls*

To purify the choline-binding proteins bound to the cell envelope, we followed the procedure described by García *et al.*[11] In short, an exponential culture of the M31 strain was disrupted in a French Pressure Cell Press (American Inst. Comp.) and centrifuged (10,000 × *g*, 5 min), and the pellet washed twice

in 50 mM potassium phosphate buffer pH 6.5 containing 10 mM $MgCl_2$ and 0.1% Brij-58. After centrifugation, the pellet was resuspended in the same buffer containing 0.1% choline and allowed to autolyse by overnight incubation at 30°C ("autolytic walls"). Afterwards, the concentration of choline was raised to 2%, and, after incubation at 4°C for 15 min, the mixture was ultracentrifuged (120,000 × *g*, 1 hr). The supernatant was dialyzed against 50 mM potassium phosphate buffer pH 6.9 applied to a DEAE-cellulose column and washed with 1.5 M NaCl. CBPs were eluted from the column upon addition of 2% choline.[11] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with the buffer system described by Laemmli[17] in 12.5% polyacrylamide gels, and protein bands were detected by staining with Coomassie brilliant blue R250.

### *Amino-terminal amino acid sequence*

Amino-terminal amino acid sequence analyses were performed through Edman degradation by using an Applied Biosystem (Foster City, CA) model 470A gas-phase sequencer fitted with an on-line PTH analytical system, after transferring proteins to a membrane (Immobilon-P; Millipore), according to a published procedure.[26] Proteins were identified by brief staining with amido black, and paper strips were excised for sequencing.

### *DNA manipulations*

Routine DNA manipulations were performed essentially as described.[21] The oligonucleotide primers LytDEco and LytDBam (Table 1) were used to amplify and sequence the 2.5-kb fragment encoding the *pce* gene. These oligonucleotides were designed according to the sequence of contig number 4,139 that is included in The Institute for Genome Research (TIGR) *S. pneumoniae* genome database (http://www.ncbi.nlm.nih.gov/BLAST/tigrbl.html). The oligonucleotide primers LytDN1, LytDN2, and LytDC (Table 1) were used to clone the *pce* gene, with or without the region encoding the signal peptide, by using the pT7-7 vector. All primers for PCR amplification and nucleotide sequencing were synthesized on a Beckman model Oligo 1000M synthesizer. DNA fragments were purified by using the Geneclean II kit (Bio 101). DNA probes were labeled with the DIG Luminescent Detection Kit (Boehringer Mannheim). Southern blots and hybridizations were carried out according to the manufacturer's instructions. *S. pneumoniae* DNA digested with either *Sma*I, *Sac*II, or *Apa*I was analyzed by pulsed-field gel electrophoresis (PFGE) using a contour-clamped homogeneous electric field DRII apparatus (Bio-Rad Labs) as previously described.[2] DNA sequence was determined by the dideoxy chain-termination method[24] with an automated Abi Prism 377™ DNA sequencer (Applied Biosystems). The amino acid sequence was analyzed with the Protein Analysis Tool at the World Wide Web molecular biology server of the Geneva University Hospital and the University of Geneva. Protein sequence similarity searches were done with the BLASTP program via the National Center for Biotechnology Information server (http://www4.ncbi.nlm.nih.gov). Pairwise and multiple protein sequence alignments were done with the ALIGN and CLUSTAL W programs, respectively, at the Baylor College of Medicine-Human Genome Center server (http://kiwi.imgen.bcm.edu:8088). Analyses of blocks were performed at the Munich Information Center for Probe Sequences server (http://pedant.mips.biochem.mpg.de).

### *Purification of Pce*

*E. coli* BL21(DE3) (pRGR11) or *E. coli* BL21(DE3) (pRGR12) strains were incubated up to an $OD_{600}$ of 0.3. At this time, isopropyl-β-D-thio-galactopyranoside (40 nM) was added, and incubation proceeded for 6 hr at 22°C to minimize the presence of inclusion bodies. The culture was centrifuged (10,000 × *g*, 5 min) and the pelleted bacteria were resuspended in 20 mM sodium phosphate buffer pH 6.9 and disrupted in a French Pressure Cell Press. The insoluble fraction was separated by centrifugation (15,000 × *g*, 15 min) and the supernatant was loaded onto a DEAE-cellulose column to purify the Pce, in a single step, following a procedure previously described.[23]

### *Assay of Pce activity*

Pce activity was determined using pneumococcal cell walls radioactively labeled with [*methyl*-$^3$H]choline, as described.[14,19] One unit (U) of activity was defined as the amount of enzyme needed to release 700 cpm of labeled material per 10 min.[14] The Pce activity was also tested using as substrate [$^{14}$C]ethanolamine-labeled pneumococcal cell walls.[30] The hydrolytic products released from the [*methyl*-$^3$H]choline-labeled pneumococcal cell walls were analyzed by gel filtration in a Sephadex G-75 column.[14] In addition, to identify the labeled low-molecular-weight material released by Pce, a thin-layer chromatography (TLC) was carried out on silica gel using 0.2 M Tris-HCl buffer pH 8.0 in 50% ethanol as solvent.[14] Samples of 15 μl (about 20,000 cpm) of the soluble fraction released from the pneumococcal cell walls after digestion with Pce were spotted on the chromatograms together with samples of authentic phosphorylcholine and choline and developed for about 4 hr at room temperature. The chromatograms were dried in an oven at 100°C for 5 min and the single strips were cut into segments of 1 cm length. The radioactivity of each segment was determined by using a 1219 Rackbeta scintillation counter (LKB Wallack). Pce activity was also determined using *p*-nitrophenylphosphorylcholine (Sigma) as substrate as described for phospholipase C.[15] The time course of the reaction was followed at 410 nm ($\epsilon = 1.5 \times 10^4\ M^{-1}$) in a UV-260 Shimadzu spectrophotometer equipped with a thermostatized holder. The assay was performed in a final volume of 0.5 ml of 0.1 M Tris-HCl buffer pH 8.0 in the presence of 3 mM *p*-nitrophenylphosphorylcholine.

### *Electron microscopy*

Samples of bacteria were taken, washed three times with 0.1 M ammonium acetate pH 7.0 and mixed (1:1) with 2% potassium phosphotungstate, and a drop was placed on carbon-coated copper grids and kept there 1 min. The excess was then blotted off, and the grids were placed in a desiccator. Samples were examined in a Philips 300 electron microscope at 80 kV.

### *Nucleotide sequence accession number*

The nucleotide sequence data for the *pce*-containing fragment have been deposited in GenBank under accession number AJ272039.

## RESULTS

### *Identification of the* pce *gene*

In an effort to identify CBPs of pneumococcus using a procedure previously published to extract proteins tightly bound to the cell envelope,[10] we have recently identified, by SDS-PAGE, four protein bands with strong choline affinity.[11] One of these bands, of about 70 kDa, was excised from the acrylamide gel, and the amino-terminal sequence was determined. Comparison of this sequence with the translated version of the nucleotide sequence of the *S. pneumoniae* type 4 genome that has been released by TIGR (http://www.tigr.org) allowed the localization of a gene designed as *pce* for phosphorylcholine esterase. A 2.5-kb DNA fragment embracing the *pce* gene was PCR amplified using the primers LytDBam and LytDEco and pneumococcal R6 DNA as template. The resulting fragment was cloned into the pUC18 vector to produce the plasmid pRGR18. The sequence of the PCR-amplified fragment revealed the existence of 10-nucleotide changes compared to the equivalent type 4 pneumococcal sequence, which originates seven changes in amino acid composition (S99G, T162A, T164K, D407A, V424I, D488G, G494E). The *pce* gene encodes a putative protein of 72,104 Da (627 amino acids). According to the amino-terminal amino acid analysis, Pce contains a typical signal peptide of 25 amino acids that renders, after its cleavage, a mature protein of 69,426 Da (602 amino acids) (Fig. 1A). Further analysis of the nucleotide sequence revealed that Pce displays a modular organization typical of CBPs. This is, after the signal peptide, Pce contains an amino-terminal region that most probably represents the catalytic domain, followed by a carboxy-terminal moiety built up of 10 repeated motifs that is highly similar to the ChBDs of other characterized CBPs (data not shown) and a carboxy-terminal tail of 85 amino acids (Fig. 1B). Neither the amino-terminal region nor the terminal tail showed significant similarities with any other proteins of the data banks and, consequently, we could not assign through this analysis a precise catalytic role to this protein.

### In silico *analysis of the* pce*-containing region*

Comparison of the *pce* nucleotide sequence with the type 4 pneumococcal genome sequence provided by TIGR revealed that this gene mapped in contig 4,139. A detailed analysis of the *pce*-surrounding regions showed that this gene is located at the 3′ end of a putative transcriptional unit that contains three additional open reading frames (orfs) that we have named as *orf1, orf2,* and *orf3* (Fig. 1C,D). A putative promoter sequence TTGGAA-16 bp-TATAAT was found 28 bp upstream from the ATG start codon of *orf1*. In addition, a palindromic sequence that forms a hairpin loop of $\Delta G = -7.7$ kcal/mol that might act as a putative rho-independent terminator was located just downstream of the two tandem stop codons found in *pce*. Interestingly, downstream of *pce* we have found the *proABC* operon that encodes the enzymes responsible for proline biosynthesis.

The *orf1* gene encodes a putative protein of 34,976 Da (302 amino acids) that showed a 32.5% identity with CpsY, a LysR-like regulatory protein of *Streptococcus agalactiae* (accession number AF163833). The analysis of the Orf1 sequence revealed that the protein contains all of the typical sequence motifs of the regulators of the LysR family, strongly suggesting that the Orf1 protein could play a regulatory function. In this sense, we have detected a repeated sequence (AAAGATAAAG) two nucleotides downstream from the putative −10 promoter box that could act as a recognition sequence for the Orf1 regulator. Whether Orf1 plays a regulatory function controlling the expression of its own operon or other genes should be investigated.

The *orf2* gene encodes a protein of 17,150 Da (153 amino acids) that showed a 41.8% identity with the signal peptidase type II from *Lactobacillus lactis* (accession number U63724). The ATG start codon and the putative ribosome binding site of the *orf2* gene overlap the 3′ coding region of *orf1*. The high similarity of the Orf2 sequence with the enzymes of the type II signal peptidase family[29] supports the hypothesis that Orf2 could play a similar role in *S. pneumoniae*. A detailed analysis of the pneumococcal genome did not reveal the existence of other homologous signal peptidase II proteins in this microorganism.

The *orf3* gene encodes a protein of 32,850 Da (295 amino acids) that showed a 59.7% identity with the YlyB protein of *Bacillus subtilis* of unknown function (accession number U48870) that is apparently co-transcribed with the *lps* gene encoding the signal peptidase II of *B. subtilis*. Similar genes to *orf3* of unknown function have also been found contiguous to the signal peptidase II-coding genes in other microorganisms, *e.g., Thermotoga maritima* (accession number AE001724). In addition, Orf3 protein showed a 40% identity with the RluD pseudouridine synthase from *Pseudomonas aeruginosa* PAO1 (accession number AE004868) and 38.5% identity with the SfhB (RluD) pseudouridine synthase from *E. coli* (accession number ECU50134) suggesting that Orf3 could play a similar role in pneumococcus. The ATG start codon and the putative ribosome binding site of the *orf3* gene overlap the 3′-coding region of *orf2* strongly suggesting that these genes could be co-transcribed.

The start codon of the *pce* gene was located two nucleotides downstream from the stop codon of the *orf3* gene, and, thus, the putative ribosome binding site overlaps the 3′ coding region of *orf3*. This finding suggests that the *pce* gene most probably would be also transcribed together with the *orf1orf2orf3* genes, forming a single transcription unit. Supporting this hypothesis, we have not found any palindromic sequence within the *orf1orf2orf3*-coding region that could form a hairpin loop with a significant free energy value that might account for a putative *rho*-independent terminator. However, as pointed out above, a palindromic sequence was found just downstream of the *pce* gene that appears to constitute the terminator of the *orf1orf2orf3pce* operon.

### *Localization of the* pce *gene in the chromosome of* S. pneumoniae

The development of the physical map of *S. pneumoniae* provides a useful framework for gene localization. PFGE and Southern blot hybridization using pRGR18 as probe showed that the *pce* gene was localized in the 380-kb *Sma*I, the 330-kb *Apa*I, and the 160-kb *Sac*II fragments (Fig. 2). The accuracy of this physical localization of *pce* in the pneumococcal chromosome is best illustrated if we take into account the *in silico*

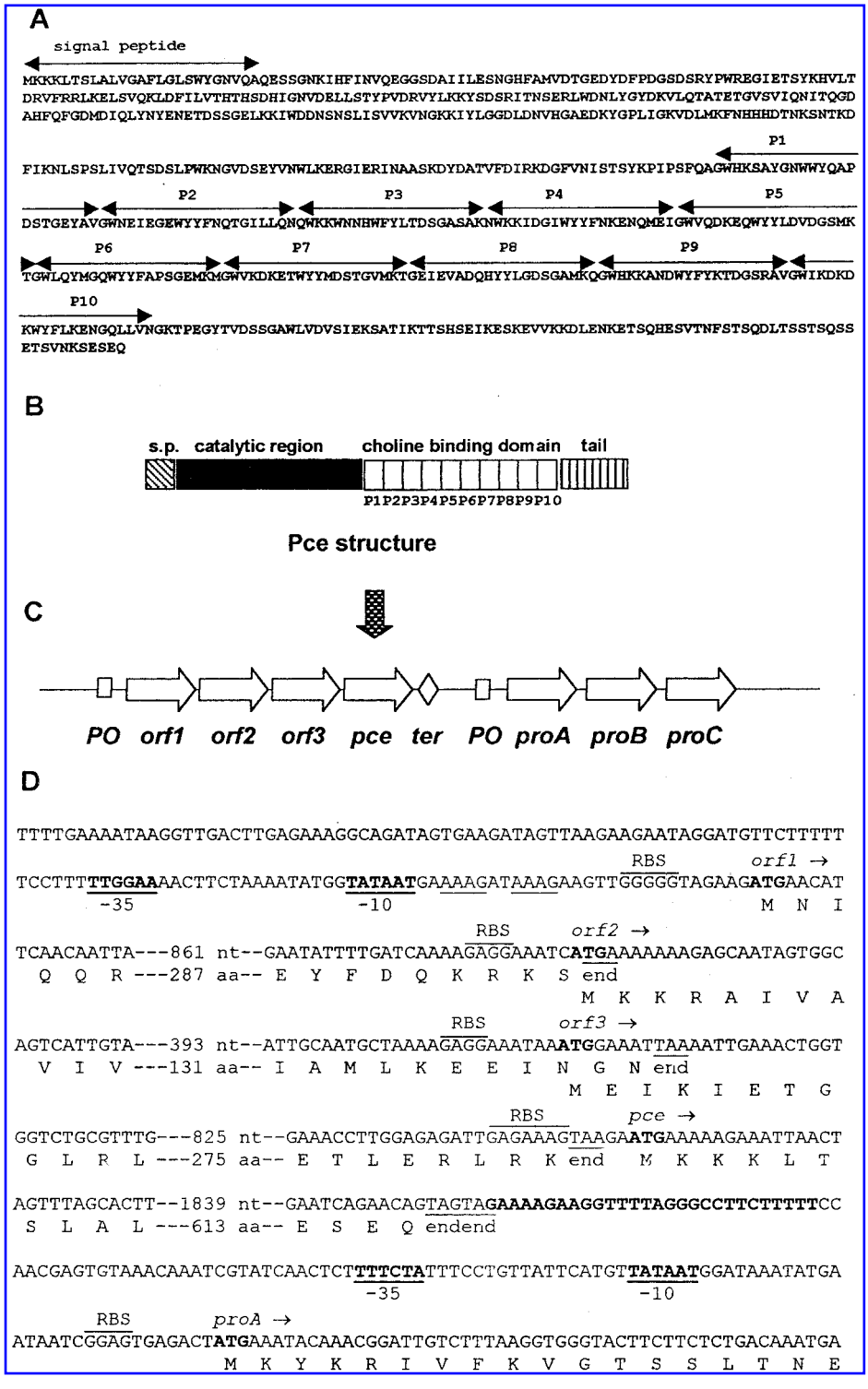


**FIG. 1.** Amino acid sequence and structural organization of Pce protein and genetic organization of contig 4,139 of *S. pneumoniae* chromosome containing the *pce* gene. (**A**) The signal peptide and the motifs that build up the ChBD (P1–P10) are overlined and marked with doubleheaded arrows. (**B**) Domain organization of Pce. s.p., signal peptide. (**C**) Genetic organization of the region of contig 4,139 containing the *pce* gene. Arrows represent putative genes, rectangles indicate putative promoters, and the diamond represents a putative transcription terminator. (**D**) Nucleotide sequence of the junction regions between the different orfs of the operon containing the *pce* gene and the upstream region of the *proABC* operon. The consensus promoter sequences (−35 and −10) are underlined and in boldface, and the start codons of *orf1, orf2, orf3, pce,* and *proA* genes are also in boldface and marked. Stop codons are underlined and marked as "end." Potential ribosome binding sites are indicated by RBS. The putative transcription terminator located downstream of *pce* gene is in boldface.

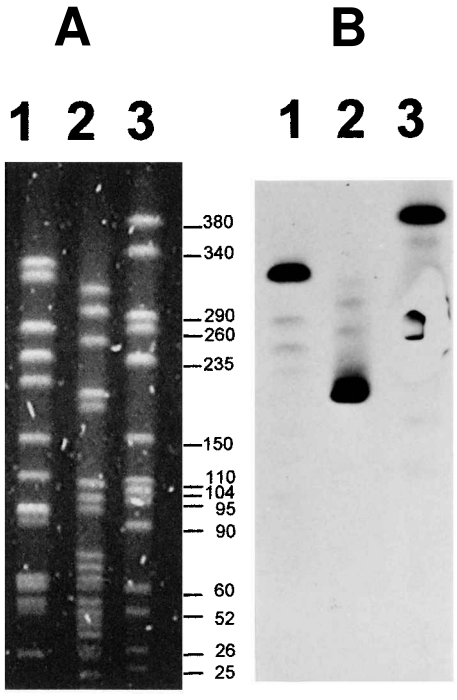


**FIG. 2.** Localization of *pce* gene in the physical map of *S. pneumoniae* R6. PFGE of the DNA prepared from strain R6 of *S. pneumoniae* digested with *Apa*I (lane 1), *Sac*II (lane 2), or *Sma*I (lane 3) (**A**), was blotted and hybridized with a *pce*-containing plasmid pRGR18 as a probe (**B**). The sizes (in kb) of the *Sma*I fragments are indicated.

analysis discussed above that revealed that *pce* is located upstream of the *proABC* operon, that has been recently postulated to map in the 380-kb *Sma*I fragment of the pneumococcal R6 chromosome.[3]

### *Cloning and overexpression of the* pce *gene and biochemical characterization of the Pce enzyme*

To determine the biological role of Pce, we have cloned and overexpressed the *pce* gene in *E. coli*. Plasmids pRGR11 and pRGR12 were constructed to express either the unprocessed or the mature forms of *pce* gene, respectively, under the control of $\phi$10 promoter (Fig. 3A). *E. coli* cells harboring pRGR11 or pRGR12 overexpressed a soluble 70-kDa protein, corresponding to the expected size of Pce (Fig. 3B). Determination of the amino-terminal amino acid sequence revealed that the protein expressed in *E. coli* BL21(DE3) (pRGR11) yielded a Pce with QESSG as amino-terminal sequence whereas *E. coli* BL21(DE3) (pRGR12) expressed a protein with MQESSG as amino-terminal sequence that corresponds to the mature form where the methionine codon was included during the construction of plasmid pRGR12. These results demonstrated that the signal peptide of Pce was functional in *E. coli*. The mature enzyme produced in *E. coli* was purified in a single-step by affinity chromatography on DEAE-cellulose (Fig. 3B). The protein was specifically eluted from the chromatographic support with choline, demonstrating that Pce has a high affinity for choline as the other members of the CBP family.[9]

To determine whether Pce could be a novel cell wall lytic enzyme, we tested its activity using [*methyl*-$^3$H]choline-labeled pneumococcal cell walls as substrate. Interestingly, crude extracts prepared from *E. coli* (pRGR11) and *E. coli* (pRGR12) cells liberated a significant fraction of the cell wall radioactivity, indicating that Pce produced in this heterologous system was active on pneumococcal cell walls. To characterize the type of cell wall hydrolytic activity of Pce, the soluble radioactive cell wall products released by this cell wall hydrolase were analyzed by chromatography on Sephadex G-75, revealing the presence of a low-molecular-weight material (data not shown). This material co-eluted with standard phosphorylcholine when

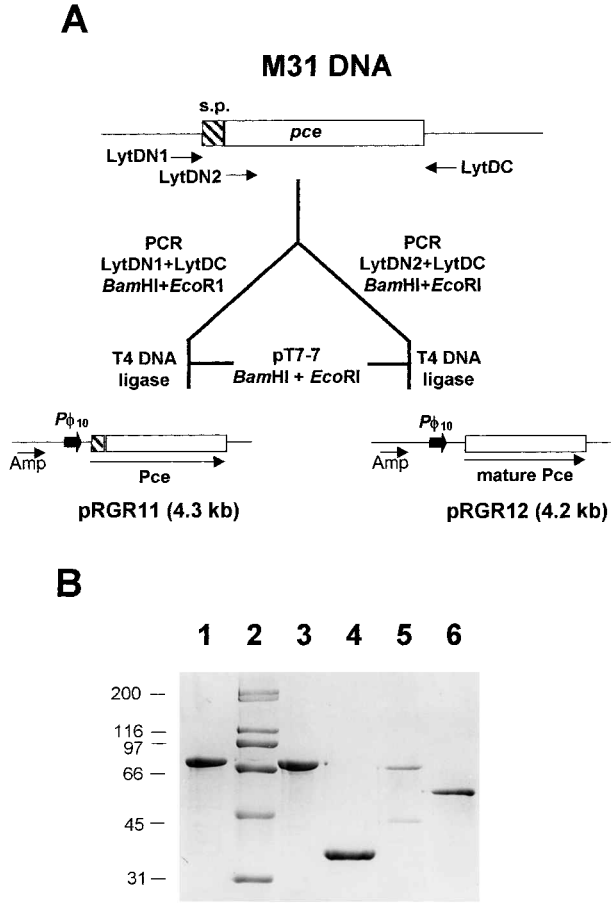


**FIG. 3.** Schematic representation of the cloning of the *pce* gene and analysis of the purified Pce enzyme. (**A**) Construction of plasmids pRGR11 and pRGR12. The relevant elements and restriction sites used to construct the recombinant plasmids coding for the unprocessed and the mature form of Pce, respectively, are indicated. (**B**) SDS-PAGE (12.5%) analysis of the purified Pce enzyme. All of the CBPs shown have been purified from crude extracts produced in *E. coli* by affinity chromatography on DEAE-cellulose. Lane 1, Purified Pce esterase prepared from *E. coli* BL21(DE3) (pRGR11); lane 2, molecular weight markers; lane 3, purified Pce esterase prepared from *E. coli* BL21(DE3) (pRGR12); lanes 4, 5, and 6, purified LytA amidase, LytB glucosaminidase, and LytC lysozyme, respectively. The molecular mass of the markers, in kDa, is indicated on the left.

analyzed by TLC, indicating that Pce is a pneumococcal phosphorylcholine esterase. The same characteristics had been previously reported for a minor hydrolytic activity detected in *S. pneumoniae*[14] and in *S. oralis*,[20] which led to the biochemical identification of the phosphorylcholine esterases in both species.

The enzyme was active between pH values of 7 and 8 and at temperatures from 30 to 37°C, but the maximum activity (2,400 U/mg protein) was obtained at pH 7.0 and 30°C using choline-containing pneumococcal cell walls as substrate. Ethanolamine-containing cell walls were not degraded by Pce. The addition of 5 mM choline chloride to the assay inhibited 50% of the initial activity whereas the addition of 20 mM ethanolamine did not.

In all the conditions assayed, including the presence of high salt concentration (300 mM NaCl), the enzyme was able to release only about 20% of the total radioactivity present in the cell wall substrate, suggesting that not all the phosphorylcholine residues were accessible to the enzyme or that the enzyme was inactivated during the assay. In this sense, we observed that whereas the addition of higher amounts of enzyme did not produce a further release of phosphorylcholine from the Pce-treated cell walls, the addition to the assay of fresh, untreated cell walls produced further release of phosphorylcholine, indicating that the enzyme was not inactivated during the assay. This unusual behavior was identical to that previously observed for the phosphorylcholine esterase of *S. pneumoniae*[14] and *S. oralis*[20] and supports the hypothesis that, at least *in vitro*, only a fraction of the phosphorylcholine residues of the cell wall are accessible to the Pce enzyme.

### *Analysis of the Pce activity using a model substrate*

To determine if the Pce was able to hydrolyze less complex substrate than the whole cell wall, we tested the activity of this enzyme on *p*-nitrophenylphosphorylcholine, a typical chromogenic substrate commonly used to assay phospholipase C.[15] Pure Pce enzyme prepared as mentioned above hydrolyzed this compound with a $K_{cat}$ of 0.048 $min^{-1}$ and a $K_m$ of 0.5 mM. The specific activity of Pce on this substrate is about ten-fold higher, and the $K_m$ almost 1,000-fold lower, than that described for phospholipase C of *Clostridium perfringens*.[15] Therefore, this substrate was used to determine several biochemical parameters of the enzyme. As expected, the activity was inhibited up to 40% by 5 mM choline chloride, but addition of 20 mM ethanolamine did not produce any effect. The enzyme did not appear to have any metal requirement because 10 mM EDTA only produced a very small reduction of the enzyme activity. Moreover, the addition of divalent cations (1 mM) as $Mg^{2+}$, $Zn^{2+}$, $Cu^{2+}$, and $Ca^{2+}$ did not cause a significant alteration of the enzyme activity. Only $Mn^{2+}$ and $Fe^{2+}$ showed a significant inhibition of 20% and 40% of activity, respectively. The enzyme activity remained also unaffected by 10 mM $\beta$-mercaptoethanol and 1 M NaCl. The enzyme conserved 50% of its original activity after incubation for 30 min at 45°C, although it was completely inactivated after heating for 30 min at 65°C.

### *Phenotype of the* pce *mutants*

To get insight into the biological role of the Pce enzyme, we constructed a *pce* mutant strain (R6D) by insertion-duplication mutagenesis using plasmid pRGR191 to transform *S. pneumoniae* strain R6 (Fig. 4A). Plasmid pRGR191 was obtained by subcloning the 1,081-bp *Hin*cII internal fragment of the *pce* gene into the pUCE191 vector. A lincomycin/erythromycin-resistant transformant of R6 was isolated and used for further studies. The accuracy of the construction was tested by Southern blot analysis, and Fig. 4B shows the results for R6 and R6D. A similar mutant (M31D) was prepared using M31 as recipient strain for transformation with plasmid pRGR191 (data not

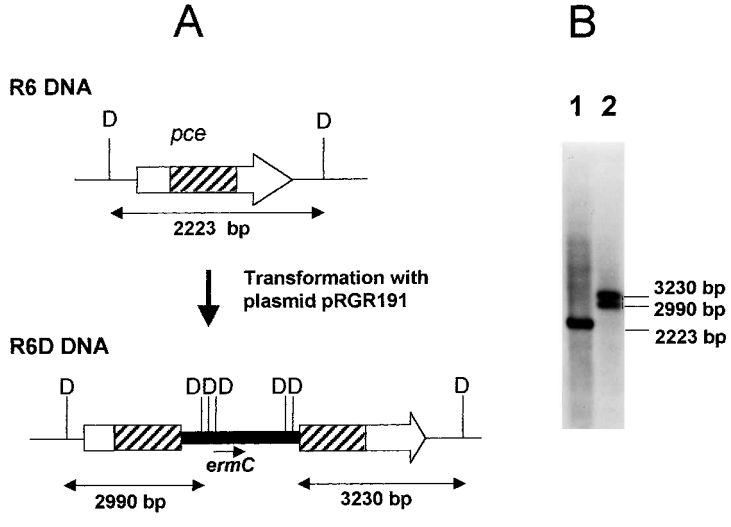


**FIG. 4.** Construction of a *pce* pneumococcal mutant. (**A**) Schematic representation of the DNA region surrounding the *pce* gene in the insertion-duplication strain R6D and its parent strain R6. The white arrow represents the *pce* gene. The hatched rectangle in *pce* indicates the 1,081-bp *Hin*cII DNA insert of pRGR191 used for insertion-duplication mutagenesis. The thick line corresponds to the vector plasmid pUCE191 used to construct pRGR191. Abbreviations: D, *Dra*I; *erm*C gene, encodes erythromycin/lincomycin resistance. (**B**) Southern blot hybridization analysis using the 1,081-bp *Hin*cII DNA fragment as probe. Lane 1, *Dra*I-digest of R6 DNA; lane 2, *Dra*I digest of R6D DNA. The size of the DNA fragments is shown on the right.

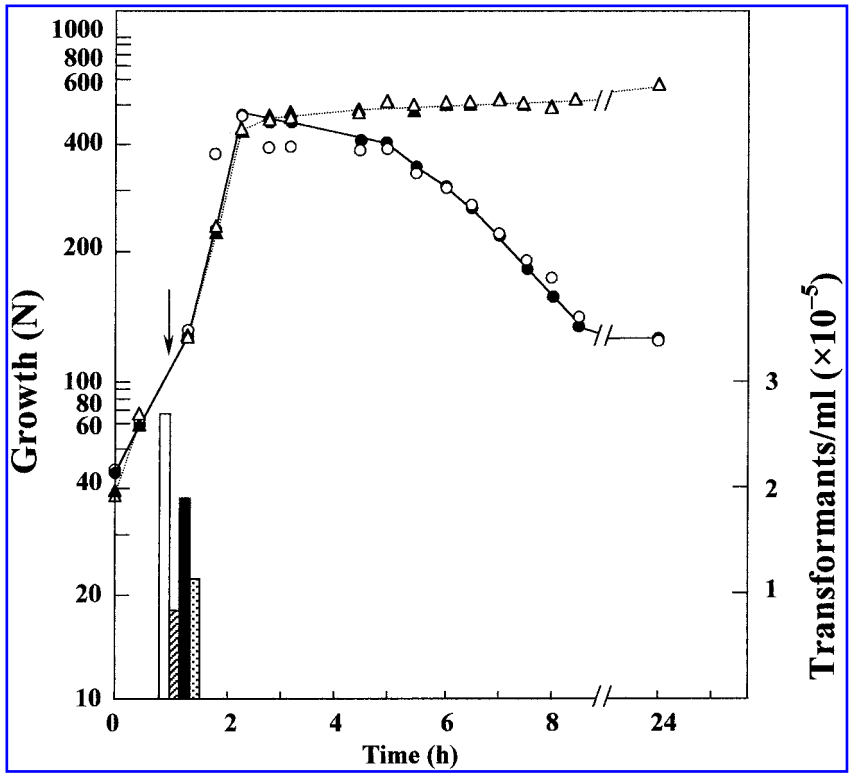


**FIG. 5.** Comparative analysis of growth, lysis, and genetic transformation of the wild-type strains and *pce* mutants. The strains were cultivated in C+Y medium at 37°C and growth and lysis for R6 (○), R6D (●), M31(△), and M31D (▲) were followed by nephelometry (N). Genetic transformation was tested at a cell concentration of about $2.1 \times 10^8$ bacteria/ml (arrow). Open bar, R6; striped bar, R6D; solid bar, M31; and, stippled bar, M31D.

shown). Both R6D and M31D mutants (the latter lacking the LytA amidase and Pce esterase) exhibited normal growth rates in C+Y medium (Fig. 5) as well as in other media tested (data not shown), and formed typical diplo cells (R6D) or small chains of about 10 cells (M31D) as reported for the parental strain. Observations at the electron microscope revealed that there were not noticeable changes when whole-mounted cells of the esterase-mutants were compared to the parental strains (data not documented). Moreover, R6D mutant normally autolysed at the end of the stationary phase of growth and R6D and M31D were competent for transformation. Transformation frequencies were: 1.1% and 1.5% for R6D and M31D, respectively, as compared with the 3.5% and 2.5% found for the parental strains R6 and M31, respectively (Fig. 5). These results indicate that there is only a limited influence of Pce in competence development.

## DISCUSSION

We have now identified and characterized the *pce* gene, coding for a novel member of the CBP family of pneumococcus, and we have been able to express the gene product as a mature protein in *E. coli.* The *pce* gene has been mapped in the vicinity of the *proABC* operon and most probably is transcribed together with the *orf1orf2orf3* genes, located upstream of *pce,* as a single transcription unit (Figs. 1 and 2).

Biochemical analyses demonstrated that this protein behaves as the pneumococcal phosphorylcholine esterase that had been biochemically characterized previously.[14] The structural analysis revealed that Pce shows a building plan similar to that observed for other CBPs, *i.e.,* the amino-terminal domain embraces the catalytic region of the enzyme whereas the carboxy-terminal domain is a typical ChBD responsible for substrate-binding specificity to choline. The structure of Pce represents a new example which reinforces our previous observation that the recruitment and genetic adaptation of the ChBD to provide a higher catalytic efficiency to the enzyme recognizing the cell wall as a substrate is extremely versatile in the pneumococcal system.[18] Moreover, the feasibility of the ChBD to become functional when it is located either at the amino or carboxyl ends of a protein was anticipated through the preparation of artificial fusion proteins containing the ChBD.[23] Recently, it has been confirmed by the characterization of two novel members of the CBP family, the LytB and LytC cell wall lytic enzymes, which contain the ChBD in the amino-terminal position in contrast with the carboxy-terminal arrangement of Pce and the other CBPs already described.[11,12]

The *pce* gene codes for a pre-protein, and the mature form is generated by the action of the signal peptidase at the cleavage site VQA ↓ QES that is in agreement with typical cleavage sites previously described.[35] Preparation of recombinant plasmids coding for the unprocessed and the mature form of Pce (Fig. 3), together with amino-terminal analysis of the purified

enzymes expressed in *E. coli,* revealed that the signal peptide of Pce was also functional in *E. coli.* Similar signal peptides have been also observed in other pneumococcal CBPs such as the murein hydrolases LytB and LytC.[11,12] The fact that we have localized the enzyme bound to the cell wall also indicates that Pce should play its physiological role in pneumococcus only after its secretion to the cell envelope. Actually, it is difficult to speculate on the biological role of the pneumococcal esterase. It has been suggested that the about 20% of residues removable by the enzyme might exist either in an anatomically unique position in the cell wall or might represent terminal residues in the teichoic acid chains.[14] More recently, the preparation of choline-independent pneumococcal strains that lead to noticeable physiological and morphological changes, together with observations available in the literature, suggest that teichoic acid chains may actually block the access of the enzyme molecules to the peptidoglycan substrate.[25,37] Also, the nutritional requirement for choline might reside in a recognition site for the phosphoaminoalcohol on TA, by which the TA-transferase is regulated, and the mutation may cause the activity of the transferase to become independent of this regulation. Insights into the mechanisms involved in synthesis of the TA and peptidoglycan of *S. pneumoniae* are required. The pneumococcal esterase activity might regulate the availability of choline residues required for activity of choline-dependent enzymes.

The biological role of choline during human infection is a subject of current interest. It appears that this aminoalcohol enhances pneumococcal attachment to the human cells by direct interaction with the platelet-activating factor, and, on the other hand, it has been suggested that bacteria with less choline, such as the opaque variant of pneumococcus would escape the innate clearance mechanism in the bloodstream.[36,38] According to these observations, we might expect that *pce* mutants should exhibit a higher amount of choline in their cell wall displaying a more transparent morphology and would have higher eukaryotic cell adherence. During the preparation of this manuscript, Gosink *et al.*[13] have studied the role of some CBPs in virulence of *S. pneumoniae*. An orf coding for a protein of 627 amino acids (CbpE; accession number AF278687) appears to be the esterase characterized here. These authors reported that *cbpE* mutants exhibited a lower cell adherence because a significantly reduced colonization of infant rat nasopharynx was found.

The observation that Pce can degrade phosphorylcholine-containing substrates that are not linked to a sugar moiety (*e.g., p*-nitrophenylphosphorylcholine) suggests that this enzyme might play other roles during infection, degrading different phosphorylcholine compounds of the host. In addition, the phase variation from opaque (cells with a low amount of choline) to transparent variants (pneumococcus with higher amount of choline) and vice versa, seems to establish an interrelationship between microbial physiology and determinants of disease.[33]

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