ENTEROBACTERIAL SPECIES-SPECIFIC DNA PROBES BASED ON THE GENE FOR OUTER MEMBRANE PROTEIN PhoE

Enterobacteriële species-specifieke DNA probes
gebaseerd op het gen coderend voor het
buitenmembraaneiwit PhoE

(met een samenvatting in het Nederlands)

P R O E F S C H R I F T

ter verkrijging van de graad van doctor aan de Rijksuniversiteit Utrecht, op gezag van de rector magnificus, Prof.Dr. J.A. van Ginkel, volgens besluit van het College van Dekanen in het openbaar te verdedigen op maandag 19 oktober 1992 des namiddags om 2.30 uur

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ALDEGONDA MARIA JOHANNA SPIERINGS

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Promotores: Prof.Dr. W.P.M. Hoekstra
(verbonden aan de Faculteit Biologie van de Rijksuniversiteit Utrecht
Prof.Dr. J.H.J. Huis in 't Veld
(verbonden aan de Faculteit Diergeneeskunde van de Rijksuniversitei
Utrecht)

Co-promotor: Dr. J.P.M. Tommassen
(verbonden aan de Faculteit Biologie van de Rijksuniversiteit Utrecht

The studies described in this thesis were performed in the Department of Molecular Cel
Biology, section Molecular Microbiology and the Institute for Molecular Biology an
Medical Biotechnology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Nether
lands.

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no probes, no assays.

aan mijn ouder

CONTENTS

INTRODUCTION

DNA hybridization as diagnostical tool.

Since the discovery by Robert Koch in 1877 (26) that bacteria can cause disease bacteriologists have been charged with the detection and identification of pathogeni agents in clinical samples, water and food. Two years earlier, Cohn proposed classification of bacteria based on their morphology (19) and in 1909 the biochemica characteristics of the bacteria were added as another major criterion in the classificatio (40). The conventional diagnostical assays, which are still based on these principals, hav however serious drawbacks. They can only be applied to pure cultures and therefore the require culturing steps. This makes these assays not only time-consuming and labou intensive but also inaccurate because they can not detect organisms that, although viabl are not culturable (16). Furthermore, the biochemical classification requires the phenotypi expression of the traits to be recognized.

The insight in the structure of DNA as postulated by Watson and Crick (55 revolutionized the approach to these diagnostic problems since it opened the possibilit to use the genotype rather than the phenotype of the organism for identification. In 1961 Marmur and Doty (32) first described the "in vitro" formation of the double helix fron two complementary DNA strands. The observation that the conditions that ruled thi phenomenon, designated nucleic acid hybridization, could be controlled (15, 58), led t the recognition of the potential use of this process.

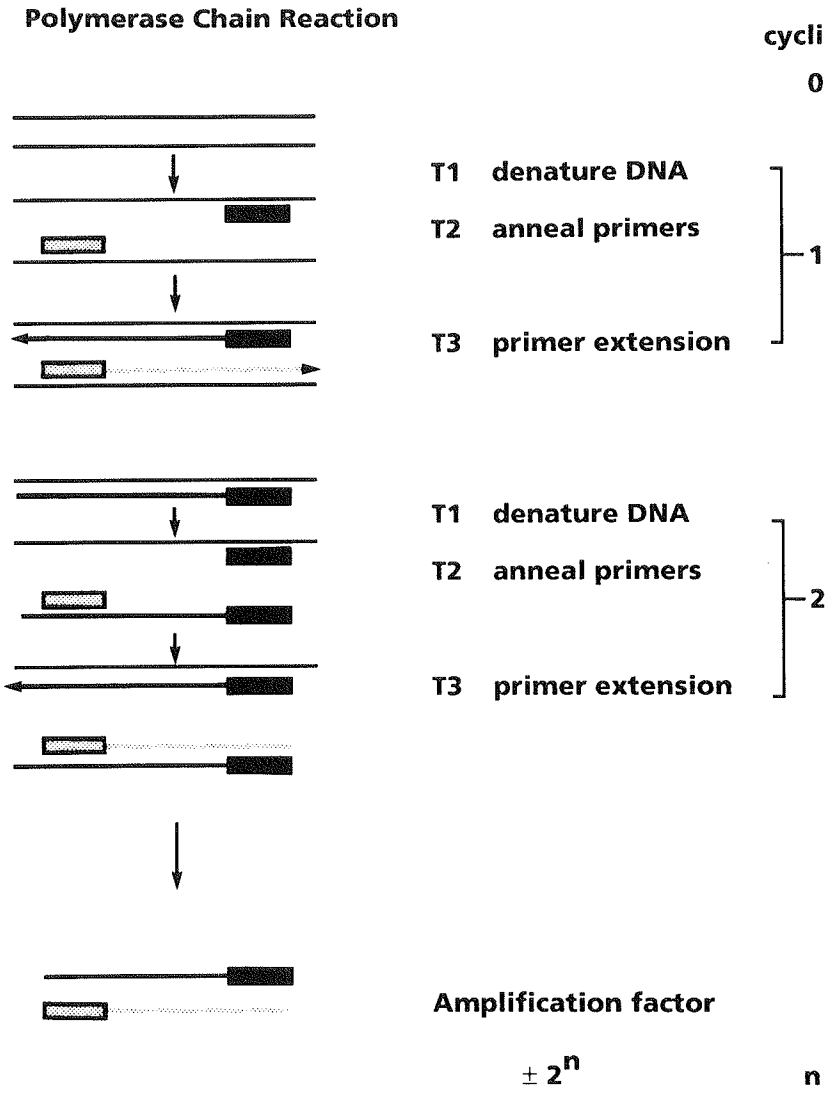

Fig. 1. Schematic presentation of the Polymerase Chain Reaction. At temperpature T1, the double stranded DNA is denatured. At temperature T2, the primers anneal to their complementary sequences. At temperature T3, the optimal temperature of the *Taq*-DNA polymerase, the primers are extended across the templates.

All genotypic tests are based on the following principals:

1. DNA can easily be denatured, by heat or by increasing the pH, which lea to the reversible separation of the two complementary strands.
2. A foreign nucleotide chain, introduced in a solution of denatured DNA, w only participate in the hybridization reaction if it contains a certain degr of complementarity with one of the strands.
3. The stability of the duplex formed depends on the extent of t complementarity between the two duplex-forming strands and can monitored by subjecting the duplex to heat and/or low-ionic streng buffers.

The first tests developed according to these principals were the DNA hybridizati assays (23). In these assays, the foreign nucleotide chain contains a label and is called t probe. After the actual hybridization reaction, the hybridized probe is separated from t unhybridized probe and is detected by its label. The clue in developing rapid and sensiti diagnostical assays lays in the amplification of the probe/target hybrids. One of t developed assays is the polymerase chain reaction (PCR). This technique uses the catalyt activity of a DNA polymerase to synthesize multiple copies of a DNA target region fro oligonucleotide primers which bind to opposite strands at the ends of the target sequenc (37, 45) (Fig. 1). Each cycle in the reaction involves the denaturing of the DNA at hig temperature, annealing of the primers and extending them with DNA polymerase acro the templates. Because each newly synthesized DNA segment, with the 5' termin consisting of the primer, acts as a substrate in the next cycle, the original target DNA exponentially amplified. In 1988, Saiki *et al.* (44) precluded the need to add fresh DN polymerase after each denaturing step by using the thermostable DNA polymerase fro the thermophilic bacterium *Thermus aquaticus*, and they were able to automate th reaction. Other developed amplifying tests are, for instance, the ligase chain reactio (LCR) (5), the transcription-based amplification system (TAS) (30) and the Qß replicas system (29).

A crucial role in all the assays is played by the foreign nucleotide chain (the prim or probe). In order to be specific for a group of organisms to be identified, it has to b both sensitive and selective, i.e. it has to recognize all strains and serotypes of this grou but it may not cross-react with other organisms. Depending on the application, differe types of oligonucleotides are required. They can be based on highly conserved genes, lik the rRNA genes, in order to obtain a specificity above the species-level (13). Protei encoding genes can be used to develop species-specific oligonucleotides (13). By usin virulence - or virulence-associated genes, it may be possible to develop oligonucleotid that can differentiate between pathogenic and non-pathogenic strains within a sing species (13).

Enterobacteriaceae.

The family of *Enterobacteriaceae* (Fig. 2) consists of a large, biochemically an genetically related group of bacteria that show substantial heterogeneity in their ecolog host-range and pathogenic potential for man, animals and plants (12). Enterobacteri strains that are pathogenic for man are water- and foodborne pathogens like *Escherichi coli, Shigella, Salmonella* and *Yersinia enterocolitica*, which cause gasterointestina

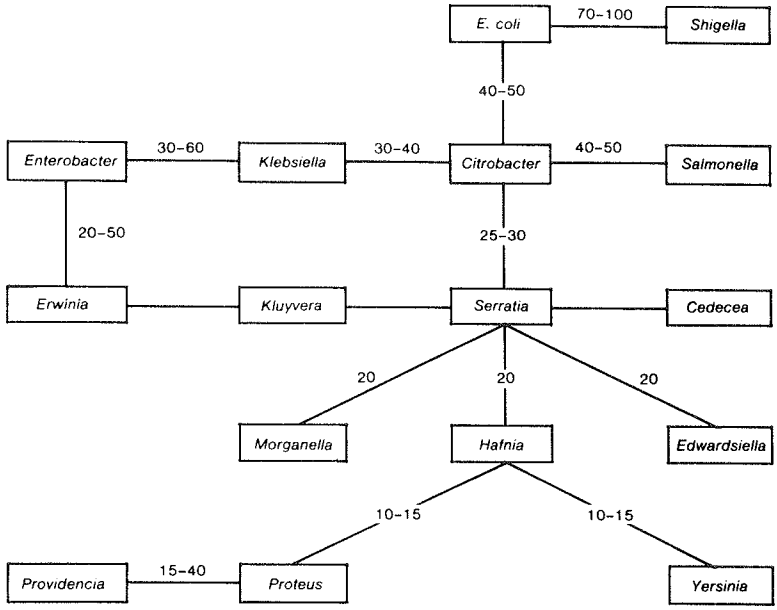

Fig. 2. DNA relatedness among *Enterobacteriaceae*. The numbers represent the approximate percentage of relatedness (Figure from reference 12).

infections. Furthermore opportunistic pathogens, like *Klebsiella, Enterobacter, Proteus Providencia* and *Serratia marcescens*, can cause a wide variety of extraintestinal infections in compromised hosts. Existing detection methods are generally slow and laborious. The test for *Salmonella* in food for instance uses bacteriological, biochemical and serological procedures and takes four to seven days to completion (43). A genetically based procedure like the PCR could potentially increase the rapidity and decrease the labour-intensiveness of the detection and could therefore be of great economical importance.

We were interested in developing a systematic approach to find species-specific DNA sequences for all members of the family *Enterobacteriaceae*. Such sequences could be used as tools in the specific detection of members of the family *Enterobacteriaceae*. In many cases one is specifically interested in detecting pathogenic species. Since pathogenicity is frequently determined by virulence-plasmids a species-specific oligonucleotide is no help in those cases. However the question why *Salmonellae* are virulent is still unanswered. Therefore all *Salmonella* strains should be treated as potential pathogens. In some cases, like for example when *E. coli* is used as an indicator organism in the determination of the possible faecal contamination of water and food, the detection is focused on the species. Also strains not known to be pathogenic for man can be of considerable economic importance because they cause diseases in animals or plants like for instance *Yersinia ruckeri* and *Erwinia herbicola*, respectively (21, 47). Furthermore the developed oligonucleotides could have potentials as taxonomic tools and be of help in the difficult classification of members of the family of *Enterobacteriaceae* [14].

Thus, since the detection and identification of members of the family *Enterobacteriaceae* is essential in a variety of studies, including fundamental and applied

research in the medical, food, environmental and agricultural sectors, the speci oligonucleotides are expected to have a broad application range. For the studies describ in this thesis, the structural gene for the outer membrane protein PhoE was used as starti point in the development of species-specific DNA probes for the members of the fam *Enterobacteriaceae*.

Outer membrane proteins.

The cell envelope of Gram-negative bacteria, including members of the fami *Enterobacteriaceae*, consists of an inner membrane, a peptidoglycan-containing periplas and an outer membrane (Fig. 3). The outer membrane functions as a molecular sieve. allows the passage of hydrophilic molecules of low-molecular weight by passive transpo but it creates a barrier for many, potentially harmful compounds, like detergen antibiotics and enzymes (39). It is composed of lipopolysaccharides (LPS), phospholipid lipoprotein and proteins. The outer membrane proteins can be divided into sever categories based upon their function, e.g. proteins that have a pore function, proteins th are important for maintaining the structure and stability of the outer membrane, such OmpA (46) and lipoprotein (11) and enzymes, such as the *pldA*-encoded phospholipa

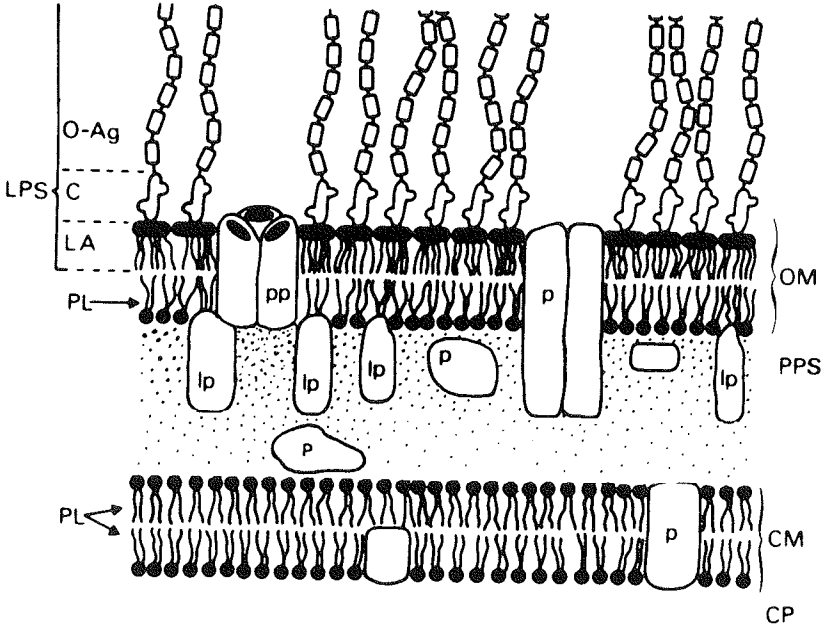

Fig. 3. Molecular organization of the cell envelope of *Enterobacteriaceae*. Th cytoplasm is surrounded by the cytoplasmic membrane (CM), the periplasm (PPS and the outer membrane (OM). The CM consists of proteins (P) and phospholipid (PL). The outer membrane consists of LPS, PL, lipoprotein (LP) and proteins The most abundant proteins are the trimeric pore proteins (PP) and OmpA. LP consists of three moieties, lipid A (LA), the core (C) and the O-antigen (O Ag). The dots in the PPS represent hydrated peptidoglycan.

(20) and the *ompT*-encoded protease (22, 24). The pore-forming proteins can be subdivided into proteins that form specific- and those that form general pores (38). The specific pores preferentially facilitate the permeation of specific classes of solutes. The best characterized protein of this group is the LamB protein. It contains a specific binding site for maltose and maltodextrins and, by binding these substrates, it facilitates their uptake (8, 48). The general pores form water-filled channels through which small hydrophilic solutes with molecular weights of up to about 700 Daltons can pass in a diffusion-like process.

Some of the outer membrane proteins that have been studied most extensively are the porins which form general pores in *E. coli* K-12. When grown under standard laboratory conditions, *E. coli* K-12 produces two porins, OmpC and OmpF (31). When cells are grown under phosphate-limitation, the synthesis of an extra porin, PhoE, is induced (42). The OmpC and OmpF pores have a preference for cations (7), whereas PhoE pores are more efficient for anions (27). The functional unit of all three porins is a trimer

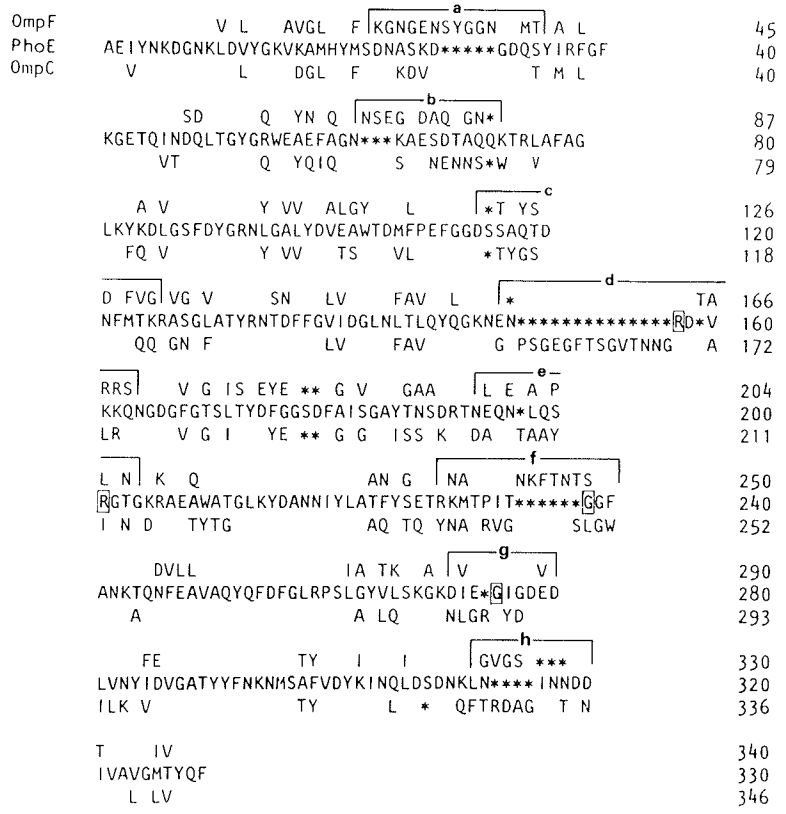

Fig. 4. Comparison of the amino acid sequences of the porins PhoE, (middle line), OmpF (upper line) and OmpC (bottom line). Amino acids in OmpF and OmpC are only indicated when they differ from PhoE. The proteins are aligned to give maximal homology. A* indicates a deletion of one amino acid residue. The regions with the most pronounced differences between the proteins are indicated and numbered a-h. The residues Arg-158, Arg-201, Gly-238 and Gly-275 of PhoE, which were shown to be cell surface-exposed, are boxed.

The *ompC* (33), *ompF* (25) and *phoE* (41) genes have been sequenced and comparison the predicted amino acid sequences revealed a homology of approximately 60% betwe the different proteins. Most variations are concentrated in eight regions (Fig. 4). Accordi to the postulated topology model, the PhoE protein traverses the outer membrane sixte times mostly as amphipathic ß-strands, thereby exposing eight regions at the cell surfa (Fig. 5) (49). This model was originally developed after the isolation and characterizati of missense mutations that interfered with the binding of monoclonal antibodies or t PhoE-specific bacteriophage TC45 (28, 53). It was concluded that the four altered ami acid residues, which are boxed in Fig. 5, must be located in cell surface-exposed regio These residues appeared to be regulary spaced, being approximately 40 amino ac residues apart in the primary sequence. Furthermore, the altered residues were found be located in hydrophilic maxima of the protein and in four of the regions that a hypervariable when the primary structures of PhoE, OmpC and OmpF were compar (Fig. 4). Combination of these types of observations led to the prediction that the fo additional hypervariable regions of the protein (numbered a, b, c and h in Fig. 4) whi also correspond to hydrophilic maxima, are cell surface-exposed as well. The origin model (53) was later refined (49) and the results of many different studies including t use of *phoE-ompC* and *ompC-phoE* hybrid genes (50, 52), small oligonucleotide insertio (4, 9) and the insertions of oligonucleotides encoding linear antigenic determinants (1- are consistent with this model. The PhoE topology correlates well with the thre dimensional structure of the *Rhodobacter capsulatus* porin as determined by Weiss *et a* (56, 57). The major difference between the model and the three dimensional structure the fact that the ß-strands of PhoE are presented to be embedded perpendicular in t outer membrane, whereas the ß-strands of *R. capsulatus* porin are embedded slangtingl The *phoE* genes of *Enterobacter cloacae* and *Klebsiella pneumoniae* have also bee sequenced (51). Comparison of the primary structures of the different PhoE protei

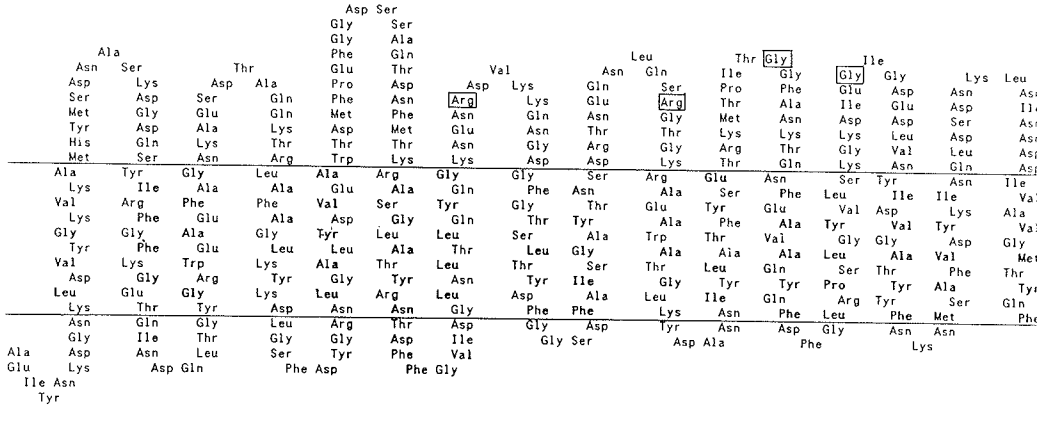

Fig. 5. Model for the topology of PhoE protein in the outer membrane. Th first identified cell surface-exposed amino acids Arg-158 (binding of phag TC45), Arg-201, Gly-238 and Gly-275 (binding of monoclonal antibodies) ar boxed. The membrane-spanning segments, located between the solid lines ar postulated to be in a ß-sheet conformation.

revealed a high degree of homology, i.e. 81%. Four hypervariable regions were discerned (Fig. 6), all of which corresponded to regions predicted to be cell surface-exposed (53).

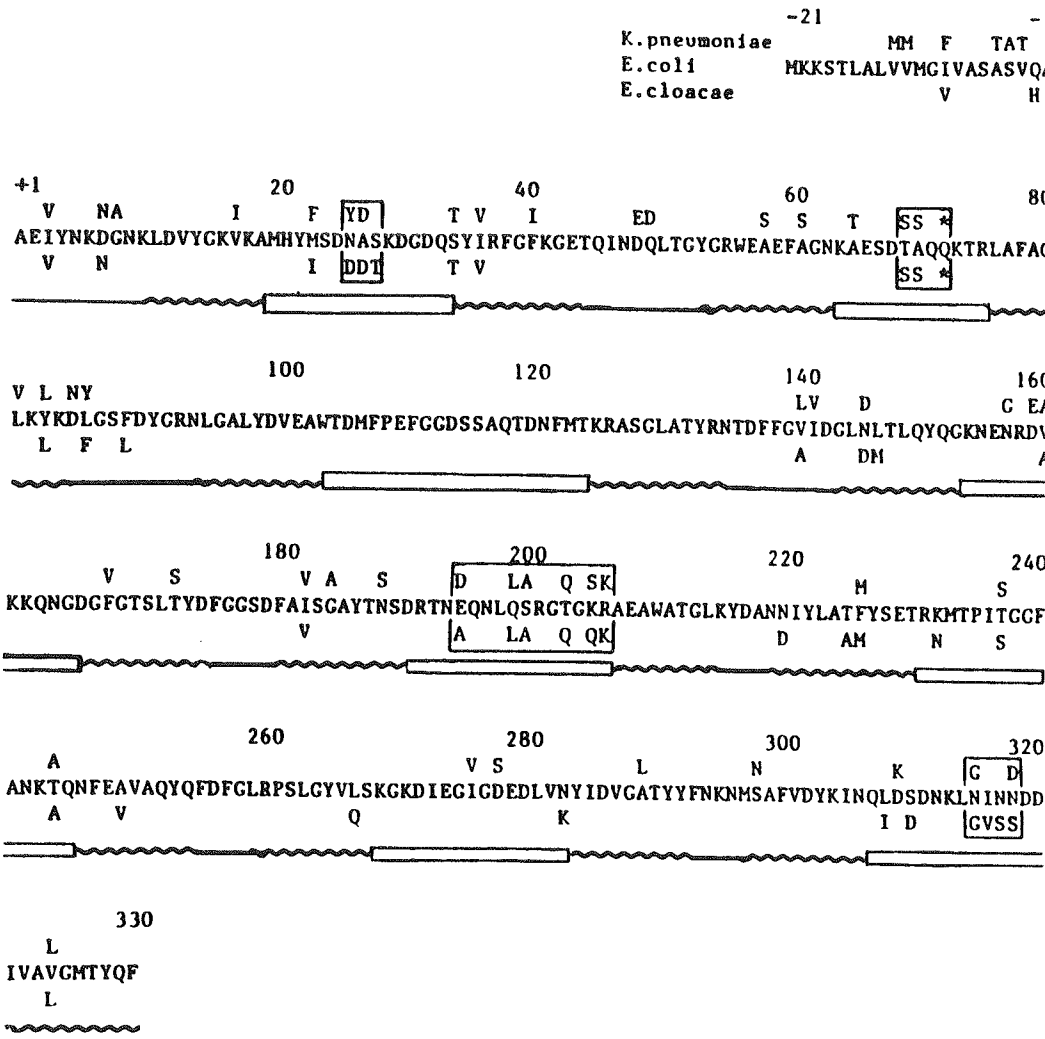

Fig. 6. Comparison of the primary structures of three enterobacterial PhoE proteins. The predicted amino acid sequences from *K. pneumoniae* and *E. cloacae* are compared with the *E. coli* sequence. Of the former two sequences only those residues that differ from the latter sequence are shown. Asterisks (*) represent deletions of amino acids. Residues -21 to -1 represent the signal sequences. The four regions where differences are most pronounced are boxed. Included are the following features of the proposed model for PhoE topology; (□), region predicted to be exposed at the cell surface; (~), transmembrane ß-sheet strand; (-), reverse turn at the periplasmic side.

Topology models, similar to the one presented for PhoE, have been proposed for LamB (18) and for the membrane-embedded domain of OmpA (35, 36, 54). OmpA is a protein of 325 amino acid residues, of which the N-terminal part is embedded in the outer

membrane, whereas the C-terminal part is located entirely in the periplasm. The proteins, which have no sequence homology with PhoE, are also supposed to traverse t outer membrane repeatedly, mostly as amphipathic ß-strands.The proposed cell surfac exposed regions that are also regulary spaced, correspond to hydrophylic maxima of t proteins and in case of OmpA, to regions that are hypervariable when OmpA sequenc of different enterobacterial species are compared (10).

In conclusion, outer membrane proteins appear to have a characteristic foldi pattern, consisting of conserved membrane-spanning regions and hypervariable c surface-exposed regions. Since the outer membrane forms the actual surface of t bacterial cell, it is involved in many interactions with the environment such as adhesi to animal and plant cells, interaction with molecules and cells of the immune system a binding of bacteriophages and bacteriocins. The fact that outer membrane proteins a involved in the specific binding of for instance bacteriophages and monoclonal antibodie indicates that (part of) the cell surface-exposed regions of these proteins are specific a is consistent with the discussed hypervariability of these domains. Therefore, DN sequences encoding (part of) hypervariable cell surface-exposed regions of out membrane proteins seem to be good candidates for the development of species-specif enterobacterial oligonucleotides. However, there is still one important point to considered, i.e. all members of the family *Enterobacteriaceae* should possess the gene question. So far, no *Enterobacteriaceae* have been described that lack the *ompA* or *pho* gene, whereas it is known that some *E. coli* strains lack *ompF* or *ompC* [34]. Therefor the *phoE* (or *ompA*) genes of *Enterobacteriaceae* seems to be a good potential source f species-specific DNA probes.

Scope of this thesis.

The aim of the investigations described in this thesis, was to study the possibilit of using nucleotide sequences encoding (part of) hypervariable cell surface-expose regions of outer membrane protein PhoE, as species-specific oligonucleotides. So far, w succesfully developed *E. coli/Shigella* - (Chapters 2, 3), *Salmonella* - (Chapter 4 *Citrobacter freundii* - (Chapter 5), *K. pneumoniae* - (Chapter 6) and *Klebsiella oxytoca* (Chapter 7) specific oligonucleotides. They were either used in DNA-hybridization (Chapters 2, 6) or in PCRs (Chapters 3-7). The use of oligonucleotides as taxonomic tool is described in Chapters 6 and 7. In Chapters 3 and 7 it is shown that, by using mor conserved sequences, the *phoE* gene can also be used to develop oligonucleotides with specificity above the species-level. In the general discussion (Chapter 8), the use of th *phoE* gene to develop specific oligonucleotides is compared to other systems and th potential application of the developed oligonucleotides is evaluated.

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DEVELOPMENT OF Enterobacterium-SPECIFIC OLIGONUCLEOTIDE PROBES BASED ON THE SURFACE-EXPOSED REGIONS OF OUTE MEMBRANE PROTEINS

Gonnie Spierings, Harmen Hofstra, Jos Huis in't Veld, Wiel Hoekstra and Jan Tommassen

SUMMARY

Outer membrane proteins of members of the family *Enterobacteriaceae* consi of conserved membrane-spanning segments and hypervariable, surface-expose regions. We demonstrate that the hypervariable DNA segments corresponding to t surface-exposed regions of these proteins can be used to develop specific DNA prob for the identification of members of the family *Enterobacteriaceae*.

The identification of microorganisms is essential in a variety of types of studies including fundamental research and applied research in the medical, environmental and agricultural sectors. Existing identification procedures are generally slow and laborious DNA hybridization techniques (5) could potentially increase the rapidity and decrease the labour-intensiveness of microbial identification. In addition, it could increase precision because it is independent of the phenotypical expression of identification markers.

The first step in developing specific DNA probes is to find a piece of DNA unique for a group of organisms to be identified. An ideal DNA probe for any particular group of organisms has to recognize all strains and serotypes belonging to the group, but it must not cross-react with other bacteria. In this study we investigated whether the genes encoding outer membrane proteins of members of the family *Enterobacteriaceae* can be used to develop specific probes.

Under standard growth conditions, *Escherichia coli* K-12 synthesizes two pore-forming outer membrane proteins, OmpC and OmpF. The synthesis of another porin PhoE, is induced by growth under phosphate-starvation (9). The genes encoding these three porins have been sequenced, and comparison of the deduced amino acid sequences revealed an overall homology of approximately 60% (7). A model for the topology of PhoE protein in the outer membrane has been proposed (12, 16). According to this model the polypeptide traverses the outer membrane 16 times in an anti-parallel ß-sheet structure. Eight areas are exposed at the cell surface. Comparison of the primary structures of OmpC, OmpF, and PhoE showed that the membrane-spanning segments are conserved whereas the surface-exposed regions are hypervariable. The *phoE* genes of *Klebsiella pneumoniae* and *Enterobacter cloacae* have also been sequenced (15). Comparison of the primary structures of the different PhoE proteins revealed a high degree of homology (81%). Four hypervariable regions were discerned, all of which corresponded to regions predicted to be cell surface-exposed (15).

OmpA protein is an outer membrane protein which is not related to the porins, and a model for the folding of this protein has been proposed (8). Comparison of the sequences of OmpA proteins of several members of the family *Enterobacteriaceae* showed that the cell surface-exposed regions are hypervariable (1).

Emanating from the idea that probes based on DNA sequences encoding surface-exposed regions of outer membrane proteins could be specific, we designed a 23-mer oligodeoxynucleotide (Fig. 1A) based on the fifth cell surface-exposed part of the PhoE protein of *E. coli* K-12 and tested its specificity in slot-blot hybridization experiments. The oligodeoxynucleotide was synthesized on a Biosearch 8600 DNA synthesizer, purified by high-pressure liquid chromatography, and labeled by the enzymatically catalyzed transfer of ^{32}P from [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham International) with T4 polynucleotide kinase according to the procedure described by Maniatis *et al.* (6).

The sensitivity and specificity of the probe was tested in slot-blot hybridization assays (Fig. 2). Strains were grown overnight at 37°C in L-broth (14). Approximately 10^8cells were filtered onto nitrocellulose filters (BA85; Schleicher & Schuell, Inc.) in a slot-blot apparatus (Minifold II; Schleicher & Schuell). The blots were prepared as described by Carter *et al.* (3). The DNA was fixed onto the filters by 4 min of UV irradiation (λ = 320 nm). The blots were prehybridized at 60°C for 45 min in 0.25% Protifar (Nutricia N.V., Zoetermeer, Holland), 6 x SSC (900 mM sodium chloride, 90 mM

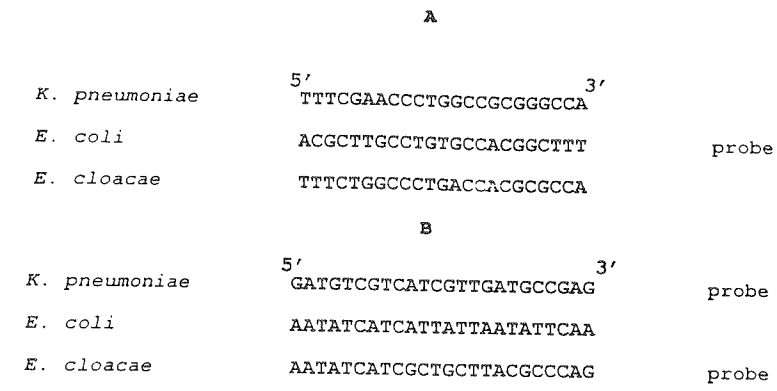


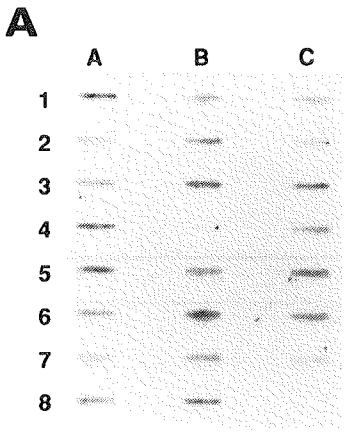
Fig. 1. Comparison of the DNA sequences of (A) the *E. coli* probe and (B) t *K. pneumoniae* and *E. cloacae* probes with the corresponding sequences of t other *phoE* genes.

sodium citrate, pH 7.0). After 20 pmol of radioactively labeled oligodeoxynucleotides w added, the filters were hybridized for 1.5 h at 60°C. The blots were washed twice for min in 6 x SSC at 60°C and autoradiographed.

The sensitivity assay, which tests the capacity of the probe to recognize all differe strains within a single species, was performed with *E. coli* strains with a variety of O a K serotypes (the serotypes are described in reference 10). Since *E. coli* and *Shigel* species belong to the same species according to *Bergey's Manual of Systema Bacteriology* (2), different serovars of *Shigella boydii, Shigella dysenteriae, Shigel flexneri* and *Shigella sonnei* were also tested (Fig. 2). The probe recognized all *E. coli* a *Shigella* strains, except for *E. coli* K-12 CE1194, which carries a deletion of the *phc* gene (13).

A successful DNA probe should not only show a high degree of sensitivity but should also be very specific, i.e. it should not cross-react with other bacteria. Figure 2 shows that the probe has a high degree of specificity within the fami *Enterobacteriaceae*, since it did not react with strains other than *E. coli* or *Shigella* strain Therefore, we did not expect to find cross-reactions with nonmembers of the fami *Enterobacteriaceae*. This was tested for a few species, i.e. *Aeromonas hydrophila, Bacill cereus, Pseudomonas aeruginosa, Sarcina flava* and *Staphylococcus aureus*. Indeed, r reactions were observed (data not shown). The results of these experiments show that t designed probe is specific for the species *E. coli-Shigella*.

To test the general applicability of the idea that specific DNA probes can b developed on the basis of the cell surface-exposed regions of outer membrane protein two other probes were tested. These probes were based on the DNA encoding the eig cell surface-exposed regions of the PhoE proteins of *K. pneumoniae* and *E. cloacae* (Fi 1B). Figure 3 shows the specificities of these probes. The tests were performed under t same conditions as those for Fig. 2 except that the hybridizations and the wash steps we performed at 63°C. The *Klebsiella* probe reacted exclusively with the *K. pneumonia* strain. The *Enterobacter* probe only reacted with *E. cloacae* and not with other member



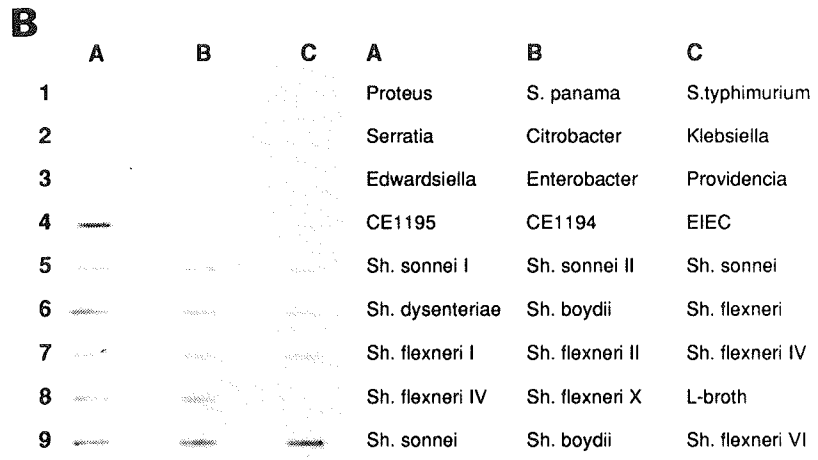


Fig. 2. Autoradiograms of slot-blot hybridizations using the *E. coli* probe. *E. coli* K-12 CE1194 carries a *phoE* deletion and CE1195 is its *phoE*$^+$ derivative (13), *E. coli* B and *E. coli* C were from our laboratory stocks. F, S and U denote *E. coli* strains isolated from faeces of healthy volunteers, from blood cultures of patients with bacteremia, and from urine of patients with urinary tract infections, respectively (10). Strain EIEC, an enteroinvasive *E. coli* and the *Shigella* (Sh) strains were obtained from the Institute for Public Health, Bilthoven, The Netherlands, *Salmonella typhimurium* SJ2353 (11) and the other enterobacterial strains (4) have been described previously.

of the family *Enterobacteriaceae*, including *Enterobacter aerogenes*.

In conclusion, it appears that DNA segments corresponding to cell surface-exposed regions of outer membrane proteins can be used to develop genus- and species-specific probes for the identification of members of the family *Enterobacteriaceae* and possibly also for other Gram-negative bacteria. These probes can be used for taxonomic research and for monitoring specific (genetically engineered) microorganisms in the environment. In addition, these probes can be used for the detection and identification of microorganisms in clinical material, food and feed.

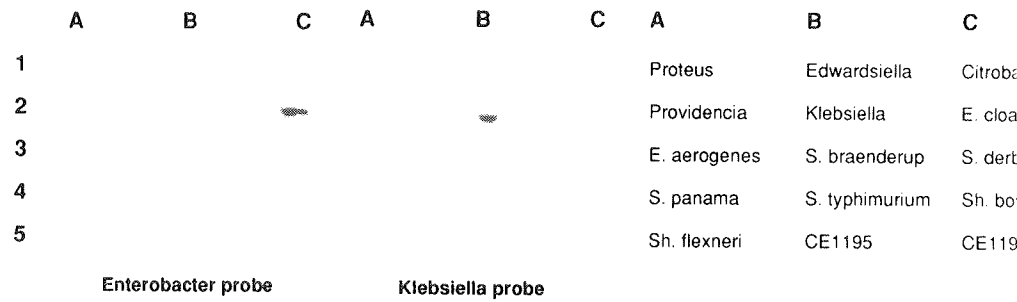


Fig. 3. Autoradiograms of slot-blot hybridizations using the *Enterobacter* a *Klebsiella* probes.

ACKNOWLEDGEMENTS

We thank D. van Brenk, J. Dankert, and T. Sato for providing different strains. This project was supported by the Netherlands Technology Foundation (S.T.W.).

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POLYMERASE CHAIN REACTIONS FOR THE SPECIFIC DECTECTION O *Escherichia coli/Shigella* AND FOR THE GENERAL DETECTION OF COLIFORI BACTERIA

Gonnie Spierings, Harmen Hofstra and Jan Tommassen

SUMMARY

The outer membrane protein PhoE of members of the famil *Enterobacteriaceae* consists of conserved membrane-spanning segments an hypervariable surface-exposed regions. Two oligonucleotides, based on DN sequences encoding two different cell surface-exposed regions of the *Escherichia co* K-12 PhoE protein, were tested for their specificity in polymerase chain reaction They were specific for the species *E. coli/Shigella*. In addition, a second primer couple based on two conserved regions of the *phoE* genes was tested. This primer couple was specific for coliform bacteria, and also *Salmonella* and *Shigella*. By usin the two primer-couples together in a multiplex PCR, a simple and rapid method wa developed for the simultaneous detection of *E. coli/Shigella*, coliform bacteria an *Salmonella* strains.

INTRODUCTION

Faecal contamination of food and drinking water forms a serious threat to public health. Therefore, the microbiological safety of these products is determined by monitoring the total coliform bacteria and the faecal coliform bacterium *Escherichia coli*. The existing conventional methods like the multiple tube fermentation test (1), are laborious and take 48-72 h to obtain results. The recently developed Colilert and Coliquick tests allow the simultaneous detection of specifically *E. coli* and of total coliforms within 24 h (5). They are based on the demonstration of ß-galactosidase activity, an enzyme specific to total coliforms, including *E. coli*, *Enterobacter*, *Klebsiella* and *Citrobacter*, and of ß-glucuronidase (GUR) activity, an enzyme specific to *E. coli* strains.

Although the GUR assay will give false-positive results due to *Shigella* (50%), *Salmonella* (25-30%) and some *Yersinia* strains (6, 9, 15), the main disadvantage of the assay is the large number of false-negative results (4). They are due to strains that have a GUR-negative phenotype or, because the test requires a culturing step to cells which are still viable but no longer culturable.

The fact that two major enteropathogenic bacteria of concern, i.e., *Salmonella* and *Shigella*, are not detected by the currently used methods seems somewhat unlogical. Bej *et al.* have reported on a polymerase chain reaction (PCR) that simultaneously detects total and faecal coliform bacteria by using two primer-couples (2). The total and faecal coliform-specific primer-couples were based on the *lacZ* and *uidA* gene of *E. coli*, respectively. Although also *Shigella* strains were detected by this PCR, *Salmonella* strains were not recognized.

In this article, two new PCR primer-couples are described. One of them can be used to detect specifically *E. coli* and *Shigella* strains, whereas the other recognizes coliform bacteria, and also *Salmonella* strains. The primer-couples are both based on the structural gene for the outer membrane protein PhoE. This protein forms anion-selective pores and is induced when bacteria are grown under phosphate-starvation (14). According to the postulated topology model, the polypeptide-chain traverses the outer membrane 16 times in an antiparallel ß-sheet structure and has eight areas exposed at the cell surface (21, 24). Comparison of the primary structures of the *E. coli* K-12, *Enterobacter cloacae* and *Klebsiella pneumoniae* PhoE proteins revealed four hypervariable regions, all predicted to be cell surface-exposed, whereas the membrane-spanning segments were found to be conserved (23). Previously, we have shown that DNA sequences encoding these surface-exposed regions can be used to develop species-specific oligonucleotides (18, 19). In the present study, *phoE* was used to develop a primer-couple for the specific detection of *E. coli/Shigella* by PCR. In addition, by taking the DNA sequences encoding the two most conserved regions of the protein, a primer-couple was developed for the detection of total coliform bacteria, and *Salmonella* strains. The two primer-couples can be combined in a multiplex PCR to detect simultaneously *E. coli/Shigella* strains, coliform bacteria and *Salmonella* strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Strains tested in the hybridization experiments included 15 *E. coli* strains isolat from clinical samples and three *E. coli* strains isolated from food. Fourteen of these stra have been described previously (25). The 16 non-*E. coli* strains tested were *pneumoniae, Yersinia enterocolitica, Yersinia pseudotuberculosis, Shigella flexne Salmonella typhimurium, Bacillus cereus, Bacillus subtilis, Corynebacterium pyogen Listeria monocytogenes, Micrococcus varians, Pseudomonas aeruginosa, Staphylococ aureus, Staphylococcus epidermis* and *Streptococcus faecium*, all described previously (2 and *Aeromonas hydrophila* and an unidentified yeast strain. All strains are from cultu collections at the National Institute for Public Health and Environmental Protectic Strains were grown overnight at 37°C in BHI-broth (Difco, Detroit, USA). Faecal sampl were obtained from 83 patients who suffered from diarrhoea of an unknown cause. T faeces were diluted in 0.8% sodium chloride and 0.1 ml samples were plated on END plates (7) and incubated at 37°C for 24 h.

Strains tested in PCRs were 21 *E. coli* and 14 *Shigella* strains (18), 77 *Klebsiel* K antigen reference strains, (13), 10 *Citrobacter* strains comprising six *C. freundii* strai including ATCC 8090 and ATCC 6750, three *C. diversus* strains including ATCC 271 and one *C. amalonaticus* strain ATCC 25405 (ATCC strains were obtained from t Phabagen Collection, Utrecht, The Netherlands), *Salmonella typhimurium* strain SJ23 (16), 19 *Salmonella* strains all of different serotypes and including 10, 4, 2, 1 and 2 strai belonging to subspecies I-V, respectively *Erwinia herbicola, Yersinia enterocolitica* (provided by W. Jansen at the National Institute for Public Health and Environment Protection), *Enterobacter cloacae, Edwardsiella tarda, Proteus mirabilis, Providenc stuartii, Serratia marcescens* (8), *Aeromonas hydrophila, Bacillus cereus, Pseudomon aeruginosa* and *Staphylococcus aereus*. Unless mentioned otherwise all strains are fro our laboratory stocks. Cells were grown overnight at 37°C in L-broth (22).

Synthesis and labeling of the oligonucleotides.

Oligonucleotides were automatically synthesized on an Applied Biosystem 381 DNA synthesizer. For hybridization assays, oligomers were labeled by the enzymatical catalyzed transfer of ^{32}P from [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Int.) with T polynucleotide kinase (Pharmacia, Uppsala, Sweden), according to the procedure describe by Maniatis *et al.* (11).

DNA hybridizations and PCRs.

Colony blots were performed as described earlier (12), except that t hybridization temperature was 58°C instead of 40°C. After the hybridization, the filte were washed twice for 15 min at 58°C in 6xSCC containing 1% sodium dodecyl sulfa and 0.1% sodium pyrophosphate. The membranes were exposed for 96 h to Konica A films with an intensifying screen at -70°C. PCRs were performed as described earlier (19 except that an annealing temperature of 52°C instead of 45°C was used. In the combine PCR, 4 pmol of each primer were used instead of 8 pmol.

API-20 systems.

API-20 systems (La Balme les Grottes, Montalieu-Vercieu, France) were used as described by the manufacturer.

A

	3′ 5′		
E. coli	TTTCGGCACCGTGTCCGTTCGCA	→	EC5c
	5′ 3′		
E. coli	AAAGCCGTGGCACAGGCAAGCGT	→	EC5
E. cloacae	tggcgCGTGGtcagGGCcAGaaa		
K. pneumoniae	tggcCCGcGGCcagGGttcGaaa		
	5′ 3′		
E. coli	TCAATTTGTTATCGCTATCCAGTTGG	→	EC8c$_2$
E. cloacae	cCAgTTTaTTATCGtcgTCaAtcTGG		
K. pneumoniae	cgAgTTTGTTATCGCTtTtCAGcTGG		

B

	5′ 3′		
K. pneumoniae	TGAAAAAGAGTACTCTGGCATT	→	C1
E. coli	TGAAAAAGAGcACTCTGGCATT		
E. cloacae	TGAAAAAGAGcACTCTGGCATT		
	5′ 3′		
K. pneumoniae	TTGGTCATAAAGTTATCGGTCTG	→	C2c
E. coli	TTGGTCATAAACTTgTCGGTCTG		
E. cloacae	TTGGTCATAAAGTTgTCGGTCTG		

Fig. 1A. Comparison of the DNA sequences of the *E. coli*-specific oligonucleotides with the corresponding sequences of the *phoE* genes of *E. cloacae* and *K. pneumoniae*. The names of the oligos are indicated behind the arrows.
Fig. 1B. Comparison of the DNA sequences of the coliform-specific oligonucleotides based on the *K. pneumoniae phoE* gene with the corresponding *E. coli* and *E. cloacae* sequences. C1 is based on DNA encoding part of the signal peptide, whereas C2c is based on DNA encoding amino acids 118-124 of the mature protein.
EC5 and C1 are based on the noncoding DNA strand. EC5c, EC8c$_2$ and C2c are based on the coding DNA strand.
Capital and small letters indicate identical and different nucleotides, respectively.

RESULTS

Specificity of *E. coli/Shigella* probe EC5c.

Recently, we tested an oligonucleotide, designated EC5c (Fig. 1a), on its suitability as a species-specific DNA probe (18). This probe was based on the DNA encoding part

of the fifth cell surface-exposed region of the *E. coli* K-12 PhoE protein. In DI hybridizations, EC5c was sensitive and selective, i.e. all 21 *E. coli* strains tested w recognized by the probe, whereas no cross-reactions were observed with 14 non-*E. c* strains, nine of which belonged to the family of *Enterobacteriaceae*. The probe a recognized the 14 *Shigella* strains tested, comprising different serovars of *Shigella boy Shigella dysenteriae, Shigella flexneri* and *Shigella sonnei*. This was not unexpected sir *E. coli* and *Shigella* strains are highly related and should be treated as one single spec (3). To test the utility and the specificity of the probe further, hybridization tests were n performed in another laboratory (National Institute for Public Health and Environmen Protection) and on another collection of strains. All 18 *E. coli* strains and the *Shige* strain tested (see Material and Methods) were recognized by the probe and no cro reactions were observed with 15 non-*E. coli/Shigella* strains tested (data not shown). Pro EC5c was further used for the identification of *E. coli* strains, following primary plati for the detection of presumptive faecal coliforms. A total of 83 different hum stoolsamples was plated on ENDO plates. Of each stoolsample, 48 characteristic colon were picked and tested in hybridizations. Of 77 and 1 stoolsamples, all 48 or only colonies, respectively, were recognized by the probe. In the remaining 5 stoolsamples, colonies were recognized by EC5c (results not shown). The colonies that gave negati results were identified in an API-20 system as *C. freundii, Hafnia alvei, K. pneumonic Klebsiella oxytoca* and *P. mirabilis*.

PCR primer-couple for the detection of *E. coli/Shigella*.

The experiments described in the previous paragraph confirm the specificity a the utility of the EC5c probe. However, the hybridization experiments require radioacti labeling of the probe, which will inhibit its application in routine- laboratories. Also t fact that exposure times of 96 h were necessary is a serious drawback of the assay. T PCR technique does not have these drawbacks. To develop an *E. coli/Shigella* specif PCR, two new oligonucleotides were synthesized. Oligonucleotide EC5 is complementa to EC5c and was therefore expected to have the same specificity, whereas $EC8c_2$, w based on DNA encoding part of the eighth cell surface-exposed region of the *E. coli* Phc (Fig. 1a). When EC5 and $EC8c_2$ correctly recognize their complementary sequences in test sample, a 348 basepairs (bp) fragment is expected to be amplified that can be detecte by electrophoresis of the PCR products on agarose gels. The expected amplified fragme was observed in all 21 *E. coli* and 14 *Shigella* strains tested, whereas no amplified produ could be detected with the 118 non-*E. coli/Shigella* strains, including 114 members of t family of *Enterobacteriaceae* (see Fig. 2 for examples). These results show that prime couple EC5/$EC8c_2$ is specific for the species *E. coli/Shigella*.

PCR primer-couple for the detection of total coliform bacteria.

Whereas the DNA sequences corresponding to the cell surface-exposed parts of t PhoE protein are apparently species specific, we considered the possibility that probe based on the conserved parts of the protein recognize total coliform bacteria. To test th possibility, oligonucleotides C1 en C2c, which are based on the *K. pneumoniae pho* sequence (Fig. 1b), were synthesized and tested in PCRs. The DNA sequence corresponding to these probes encode a part of the signal peptide and a highly conserve part of the mature protein, respectively. When C1 and C2c recognize their complementar

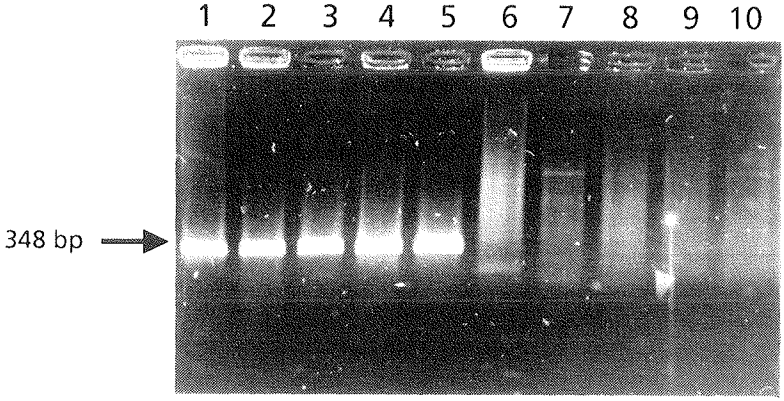

Fig. 2. Analysis of PCR products using EC5/EC8c$_2$ as primer-couple on a 1% agarose gel. Lane 1, *E. coli* K-12; lane 2, *Sh. boydii*; lane 3, *Sh. dysenteriae*; lane 4, *Sh. flexneri*; lane 5, *Sh. sonnei*; lane 6, *E. cloacae*; lane 7, *K. pneumoniae*; lane 8, *C. freundii*; lane 9, *S. typhimurium*,; lane 10, *E. tarda*. The position of the amplified segment is indicated at the left.

sequences in a test sample, a 436 bp or a 433 bp fragment will be amplified by PCR in case of *E. coli* or *E. cloacae* and *K. pneumoniae*, respectively. Primer-couple C1/C2c correctly recognized the *E. cloacae*, the 21 *E. coli*, the 14 *Shigella*, and the 10 *Citrobacter* strains tested, whereas no amplified product could be detected with *E. tarda, E. herbicola, P. vulgaris, P. stuartii, Y. enterocolitica, S. marcescens, A. hydrophila, B. cereus, P. aeruginosa* and *S. aureus* (see Fig. 3 for examples). In all, but one, of the 77 *Klebsiella* strains tested, a fragment of the expected size was amplified. The negative strain, K5, belongs to the species *K. pneumoniae* subspecies *ozaenae* (Fig. 3). Of the 20 *Salmonella* strains tested, only *S. brookfield* gave a negative result (Fig. 3).

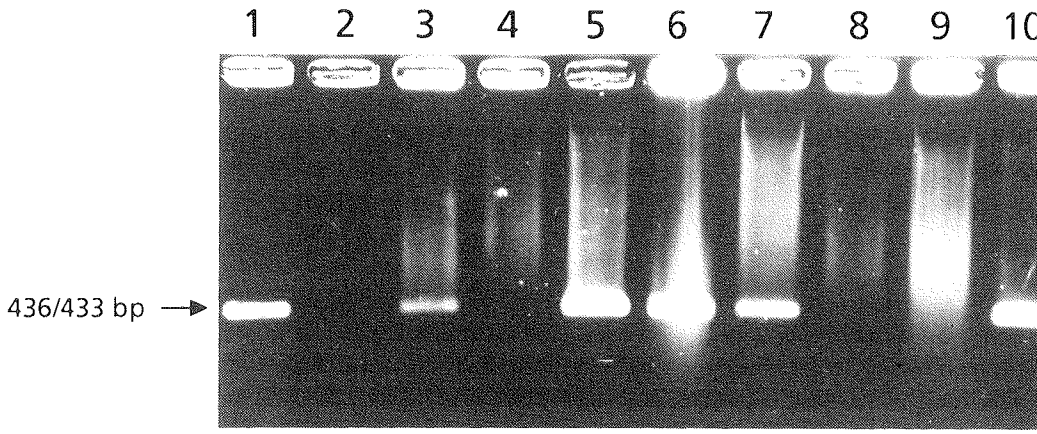

Fig. 3. Analysis of PCR products using C1/C2c as primer-couple on a 1% agarose gel. Lane 1, *K. pneumoniae*; lane 2, *K. pneumoniae* strain K5; lane 3, *S. typhimurium*; lane 4, *S. brookfield*; lane 5, *E. coli* K-12; lane 6, *E. cloacae*; lane 7, *C. freundii*; lane 8, *E. tarda*, lane 9, *S. marcescens*; lane 10, *Klebsiella* strain K74. The position of the amplified products are indicated at the left.

Multiplex PCR for the simultaneous detection of *E. coli* and total coliform bacter

For the ease and rapidity of diagnosis, it would be helpful if *E. coli* and to coliform organisms can simultaneously be detected in a single reaction. To test t possibility, the two primer-couples were combined in a single PCR. It should be not that, because all primers are based on the same gene, a third primer-couple, i.e., C1/EC is formed that is expected to result in the amplification of a 1005 bp fragment. All 21 *coli* and 14 *Shigella* strains tested showed amplification of the 436 and 348 bp fragme (see Fig. 4 for examples). In some cases, also the 1005 bp fragment could be detecte

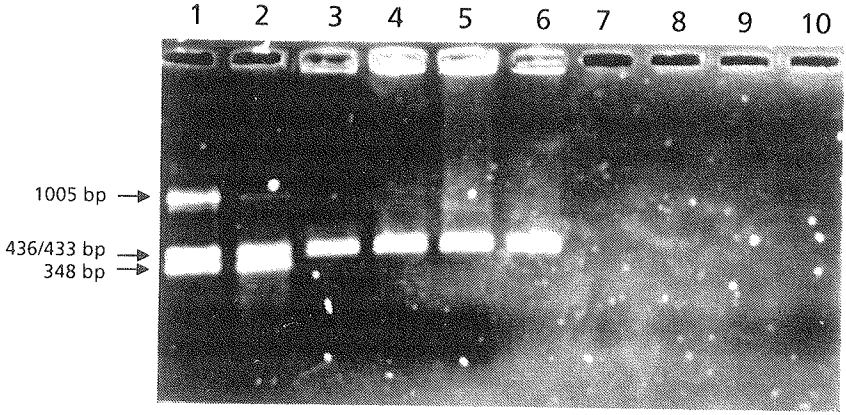

Fig. 4. Analysis of PCR products using EC5/EC8c$_2$ and C1/C2c together a primer-couples, on a 1% agarose gel. Lane 1, *E. coli* K-12; lane 2, *Sh flexneri*; lane 3, *S. typhimurium*; lane 4, *E. cloacae*; lane 5, *K. pneumoniae* lane 6, *C. freundii*; lane 7, *P. stuartii*; lane 8, *S. marcescens*; lane 9, *A hydrophila*; lane 10, *P. aeruginosa*. The position of the amplified products ar indicated at the left.

The amount of this product was always lower than that of the other two product probably because it serves as a substrate for the other two primer-couples, whereas th smaller products do not serve as substrates for the couple C1/EC8c$_2$. When other colifor bacteria and *Salmonella* strains (see Material and Methods) were tested only the 436/43 bp fragment was amplified, whereas no amplified product could be detected with *E. tard E. herbicola, P. vulgaris, P. stuartii, Y. enterocolitica, S. marcescens, A. hydrophila, B cereus, P. aeruginosa* and *S. aureus*. (see Fig. 4 for examples). Also in the combine PCR, only *Klebsiella* strain K5 and *S. brookfield* gave false-negative results.

DISCUSSION

In hybridization experiments performed at two different laboratories probe EC5c based on DNA encoding part of the fifth surface-exposed region of *E. coli* K-12 PhoE turned out to be specific for the species *E. coli/Shigella*. To develop an *E. coli/Shigella* specific PCR two other oligonucleotides were synthesized. In PCRs oligo EC5, having sequence complementary to that of EC5c, and oligo EC8c, based on DNA encoding par of the eighth cell surface-exposed region, correctly recognized all 21 *E. coli* and 14 *Shigella* strains tested, whereas no reaction was observed with the 118 tested non-*E*

coli/Shigella strain.

In addition to primer-couple EC5/EC8c_2, a second primer-couple, C1/C2c was developed. Whereas EC5/EC8c_2 are based on hypervariable parts of the *phoE* gene C1/C2c are based on the most conserved regions of this gene. C1/C2c turned out to be specific for coliform bacteria, *Salmonella* and *Shigella* strains. They correctly recognized 141 out of the 143 coliform strains tested, whereas no signal was obtained with ten other bacteria including six members of the family *Enterobacteriaceae*. One of the strains that gave a false-negative result was *K. pneumoniae* strain K5. Recently, we have reported on the development of a *K. pneumoniae*-specific PCR using primers based on the fifth and eighth surface-exposed regions of the *K. pneumoniae* PhoE (19). Also in that PCR, K5 was the only *K. pneumoniae* strain that was not recognized. The other strain that was not recognized in the combined PCR was *S. brookfield*. This strain was the only *Salmonella* not recognized by the *Salmonella*-specific oligonucleotides based on *S. typhimurium* PhoE (17). These results together with the fact that we were unable to detect PhoE proteins by sodium-dodecyl sulfate polyacrylamide gel electrophoresis after growth of these strains under phosphate-limitation (20) strongly suggests that these strains do not possess a *phoE* gene. Although it is not clear why certain *Salmonella* strains are virulent, it is known that strains most likely to be pathogenic for humans belong to subspecies I. Strains belonging to subspecies II and III are frequently isolated from the intestinal contents of cold-blooded animals and are rarely found in warm-blooded animals. Strains belonging to subspecies IV and V are mainly isolated from the environment and are rarely pathogenic for man (10). The only *Salmonella* strain that was not recognized by the PCR belongs to subspecies V and therefore this result has no dramatic consequences for the reliability of the method.

Finally, by using both primer-couples together in a multiplex PCR, a simpel and rapid assay is developed for the simultaneous detection of faecal and total coliform bacteria and the enteropathogenic bacteria of interest, i.e., *Salmonella* and *Shigella*.

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CHARACTERIZATION OF THE *Salmonella typhimurium phoE* GENE AN DEVELOPMENT OF *Salmonella*-SPECIFIC DNA PROBES

Gonnie Spierings, Rietje Elders, Bart van Lith, Harmen Hofstra and Jan Tommassen

SUMMARY

In *Escherichia coli* K-12, the *phoE* gene, encoding a phosphate-limitation inducible outer membrane protein (PhoE), is closely linked to the genes, *proA* an *proB*. When the corresponding fragment of the *Salmonella typhimurium* chromosom was transferred to *E. coli* K-12 using an RP4:: miniMu plasmid pULB113 n expression of *S. typhimurium* PhoE could be detected. However, DNA hybridizatio studies revealed that the corresponding plasmid contained *S. typhimurium phoE* Production of *S. typhimurium* PhoE in *E. coli* was detected after subcloning of th gene on a multicopy vector. Nucleotide (nt) sequence analysis showed extensiv homology of *S. typhimurium phoE* to the *E. coli* gene and revealed possibl explanations for the low expression of *S. typhimurium phoE* in *E. coli*. In addition the sequence information was used to develop *Salmonella*-specific DNA probes. Tw oligodeoxyribonucleotides (oligos) were synthesized based on DNA sequence encoding the fifth and eighth cell surface-exposed regions of PhoE. When used i polymerase chain reactions (PCRs), these probes turned out to be specific, i.e. n crossreactions occurred with the non-*Salmonella* strains, whereas 132 out of 13 tested *Salmonella* strains were recognized.

INTRODUCTION

The outer membrane of Gram-negative bacteria functions as a barrier for harmful compounds. Nutrients can pass this membrane via a number of pore-forming proteins, called porins (4). *Escherichia coli* K-12 contains three porins. OmpF and OmpC, whose synthesis is regulated by the osmolarity of the growth medium, and the phosphate-limitation-inducible PhoE protein. In contrast to the OmpF and OmpC pores, which are cation-selective, the PhoE pores are anion-selective (5). A model has been proposed for the topology of PhoE in the outer membrane (34, 41). According to this model, the polypeptide spans the membrane 16 times, mostly as amphipathic ß-strands, thereby exposing eight regions at the cell surface. In contrast to the membrane-spanning segments, which are rather well conserved, the cell surface-exposed regions appear to be hypervariable when the amino acid (aa) sequence of PhoE is compared to those of OmpF and OmpC or to those of the PhoE proteins of *Klebsiella pneumoniae* and *Enterobacter cloacae* (40).

As in *E. coli* K-12 (22), an anion-selective porin is induced when *Salmonella typhimurium* is grown under phosphate-limitation (2). However, a very recent immunological study suggested that *Salmonella* PhoE is not closely related to the general porins of *E. coli* and other *Salmonella* porins, including OmpF, OmpC and OmpD (27). Unlike *E. coli phoE*, which has been located at min 6 on the chromosomal map adjacent to the *proBA* operon (36), *S. typhimurium phoE* has not yet been localized. An R'plasmid carrying the *proBA* operon of the *S. typhimurium* chromosome did not evoke the expression of *Salmonella* PhoE in *E. coli* (2). A possible explanation for this observation is that *S. typhimurium phoE* is not expressed in *E. coli* K-12, for instance because the activator of the *pho*-regulon, PhoB (8, 46), does not recognize the heterologous promoter. However, it should be noted that *E. coli phoE* is normally expressed in *S. typhimurium* (1), suggesting that the regulatory system is conserved. Alternatively, *phoE* is not located next to the *proBA* operon as seems to be the case for *Serratia marcescens* (21) and *Yersinia enterocolitica* (30).

For several reasons, we were interested in cloning and characterizing *phoE* of *S. typhimurium* : (*i*) we wanted to solve the problem concerning the localization of *phoE* on the *S. typhimurium* chromosome, (*ii*) nt sequencing might give information on the relationship of PhoE to the other porins, and (*iii*) recently, we have shown that the DNA sequences encoding the hypervariable cell surface-exposed regions of PhoE protein are useful to generate species-specific oligos (28, 29). Thus, after establishing the nt sequence of *S. typhimurium phoE*, two oligos were designed and tested for their applicability in the rapid detection and identification of *Salmonella* strains in PCRs.

RESULTS AND DISCUSSION

Transfer of the *proA-proB* region of the *S. typhimurium* genome to *E. coli* K-12.

To investigate whether *S. typhimurium phoE* is located close to *proAB*, an *in vivo* cloning procedure with the RP4::miniMu plasmid pULB113 (42) was used to transfer the *proAB* region of the *S. typhimurium* genome to *E. coli* K-12. Plasmid pULB113, which renders cells resistant to the antibiotics Ampicillin (Ap), Kanamycin (Km) and

Tetracycline (Tc), was transferred to a spontaneous Rifampicin (Rif)-resistant derivati of *S. typhimurium* LT2 strain SJ2353 (26) by conjugation with donor str MXR[pULB113], as described by Van Gijsegem and Toussaint (42). One Km-and R resistant transconjugant was subsequently used as a donor strain in a mating with *ph recA hsdR hsdM E. coli* strain CE1226 (43) as the recipient. The latter host contains *proB-proA-phoE-gpt* deletion and is resistant to the antibiotic Streptomycin (Sm) due an *rpsL* mutation. One of the Sm-resistant *pro*$^+$ transconjugants, carrying an R'plasm designated pST_0, was further analysed.

Presence of *S. typhimurium phoE* on pST_0.

Consistent with the results described by Bauer *et al.* (2), sodium dodecyl sulpha polyacrylamide gel electrophoresis (SDS-PAGE) analysis of cell envelope protein patter of the pST_0 containing derivative of *E. coli* strain CE1226 did not reveal the expressi of *S. typhimurium* PhoE in *E. coli* (data not shown). For this observation there are tv possible explanations: either *S. typhimurium phoE* is not or poorly expressed in *E. c* K-12 or pST_0 does not contain *S. typhimurium phoE*. To study the latter possibility, DN DNA hybridization experiments were performed. Plasmid pULB113 and its derivati pST_0 were digested with several restriction enzymes and the fragments were separated a 1% agarose gel. The gel was denatured, dried and hybridized with a ^{32}P-labeled DN probe derived from the *phoE* gene of *E. coli* K-12 (Fig. 1). The probe did not hybridi with vector pULB113 (lane 4) but did recognize distinct fragments from pST_0 (lanes 1-3

These results show that pST_0 contains the *phoE* gene of *S. typhimurium*. The fa that the nt sequence revealed the presence of the *proBA* operon upstream from *phoE* (da not shown), further showed that the localizations of *phoE* on the chromosomal maps *S. typhimurium* and *E. coli* K-12 are identical.

Subcloning of the *S. typhimurium phoE* gene.

To study *S. typhimurium phoE* in further detail, it was subcloned. Hybridizatic studies (results not shown) revealed that *phoE* was located on an approximately 7 kb *Cl* fragment of pST_0. This fragment was cloned into the *Cla*I-site of the multicopy vect pBR322 (6). One of the constructs, designated pST_3 (Fig. 2), was introduced into the *coli phoE* mutant strains CE1224 (38) and CE1248 (14). To determine whether *typhimurium phoE* is expressed in *E. coli* when present on a multicopy plasmid, ce envelope patterns of the transformants isolated after growth of the cells on high- and lov phosphate medium were analysed by SDS-PAGE (Fig. 3). In cell envelopes of the pS containing derivative of strain CE1224, a band of the expected molecular weight w observed (Fig. 3, lane b). This band was not observed after growth of the cells in high phosphate medium (lane a) or in the plasmidless strain (lanes c,d). This protein w constitutively expressed in the pST_3 containing derivative of the *phoR* strain CE1248 (lan f). In Western blots, the band reacted with a monoclonal antibody raised against the *coli* PhoE (results not shown). These results show that *S. typhimurium phoE* is expresse in *E. coli* K-12 when present on a multicopy plasmid. Furthermore, this expression appea to be regulated under control of the *pho* regulon.

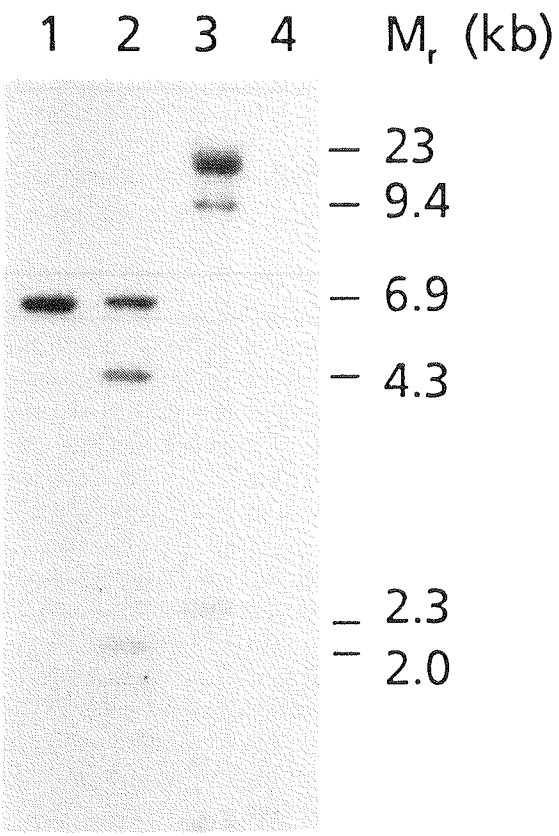


Fig. 1. Autoradiogram of a 1% agarose gel. Lanes 1, 2 and 3 contain pST$_6$ digested with *Eco*RV/*Hin*dIII, *Nde*I/*Eco*RV and *Nde*I/*Hin*dIII, respectively. Lane 4 contains pULB113 digested with *Eco*RV/*Hin*dIII. After electrophoresis, the gel was denatured, dried and hybridized with a labeled probe as described by Tsac *et al.* (39). As a probe, the 842 bp *Pst*I-*Bgl*II fragment of pJP$_{29}$ (7) containing most of the *phoE* gene of *E. coli* K-12 was used. The DNA fragment was labeled using [α - ^{32}P]dATP (3000 Ci/mmol, Amersham) and the random-prime labeling kit (Boehringer, Mannheim). The gel was prehybridized at 60°C for 1 h in 0.25% Protifar (Nutricia N.V., Zoetermeer, The Netherlands), 6 x SSC (0.15 M NaCl/0.015 M Na$_3$-citrate pH 7.0). After adding the probe, the gel was hybridized overnight at 60°C. The gel was washed twice for 15 min in 6 x SSC at 60°C and autoradiographed. The position of marker fragments, i.e. λ DNA digested with *Hin*dIII, are indicated at the right.

Sequence analysis.

The nt sequence of *S. typhimurium phoE* was determined on both strands (Fig. 4). In *E. coli*, the genes and operons of the *pho* regulon contain an apparently normal Pribnow box in their promoter regions, but no consensus -35 region. Instead, 10 basepairs (bp) upstream of the Pribnow box, a conserved 18 bp element, designated *pho* box, is found (18, 33). Such a *pho* box can be considered to consist of two direct, 7 bp repeats separated by 4 bp. In the *phoA* (13) and *phoB* (19) promoters, this *pho* box is present only once. In the promoter of the *pst* operon, a second *pho* box can be discerned, which is located 4 bp upstream of the first *pho* box, and which is essential for the full expression

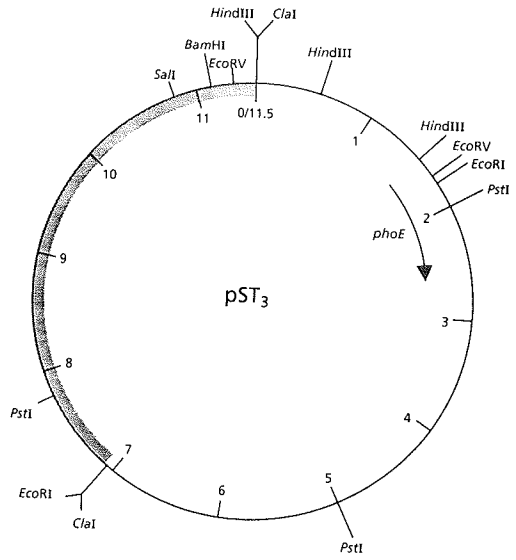


Fig. 2. Restriction map of pST_3. The pBR322 vector is indicated by the shade segment. Map units are in kb. The position of *phoE* is indicated.

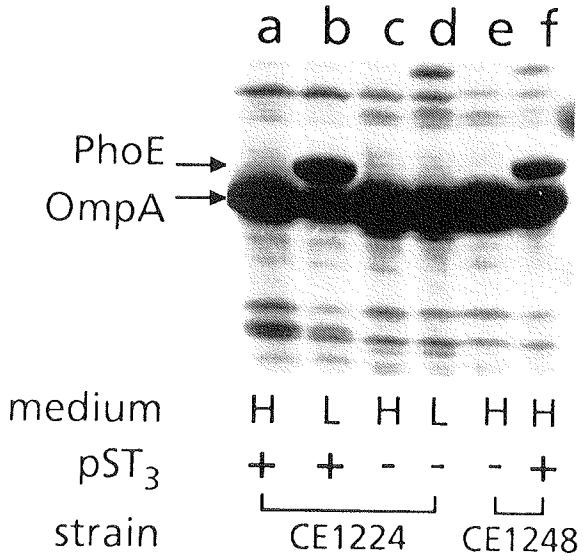


Fig. 3. SDS-PAGE protein patterns of cell envelope of the *E. coli* K-12 strain CE1224 and CE1248 and their pST_3 containing derivatives. *S. typhimurium* Pho and *E. coli* OmpA, are indicated by arrows. Only the relevant part of the ge is shown. Cells were grown overnight at 37°C under aeration in a medium (16 in which the phosphate concentration is limiting for growth (lanes marked L) Phosphate-replete conditions were obtained by supplementing the medium wit 660 µM K_2HPO_4 (lanes marked H). Cell envelopes were isolated by differentia centrifugation after ultrasonic disintegration of the cells (17) and thei protein patterns were analysed by SDS-PAGE, essentially as described b Lugtenberg *et al.* (17), except that the gels were supplemented with 6 M urea The gel was stained with Coomassie-Brilliant blue.

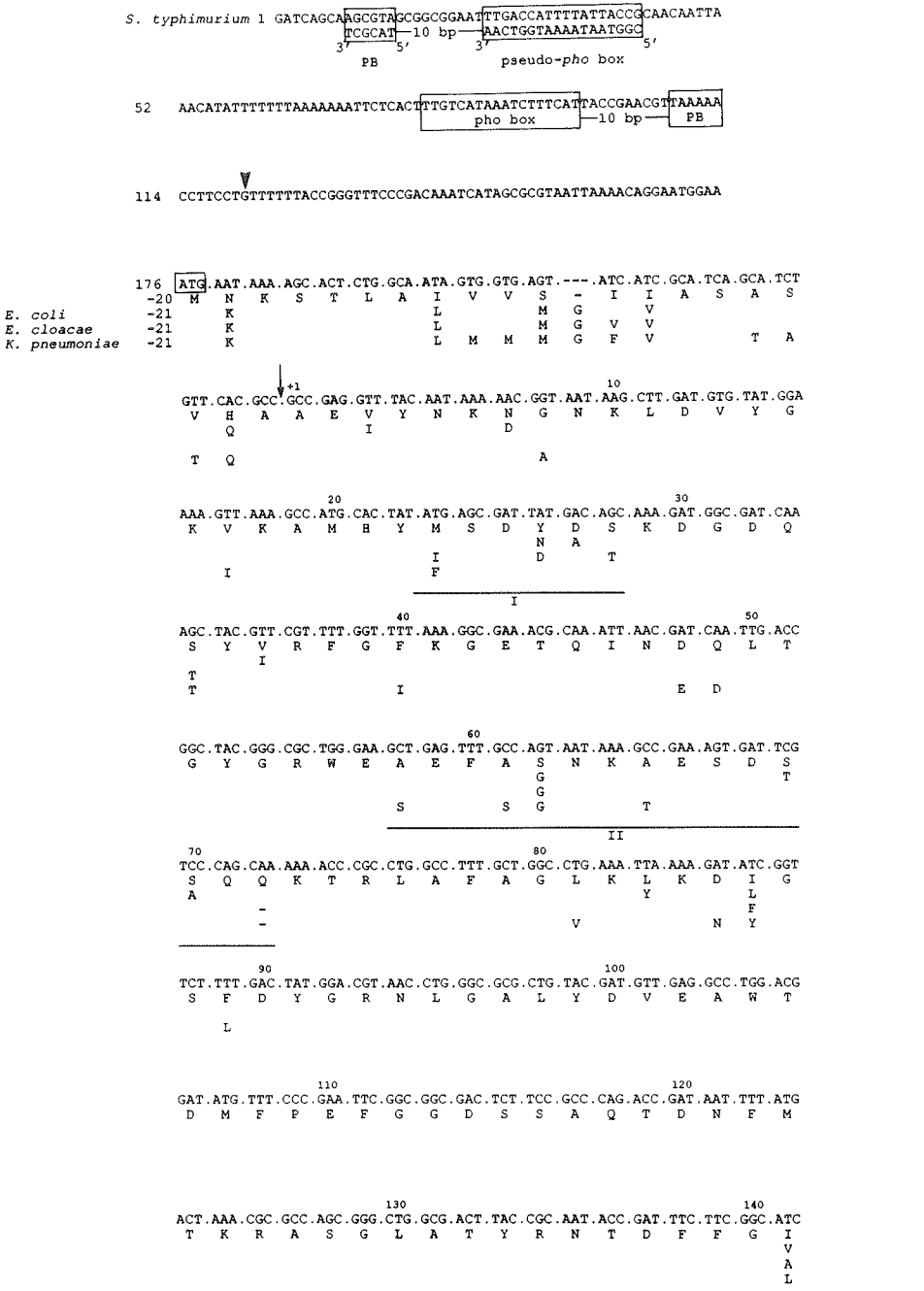
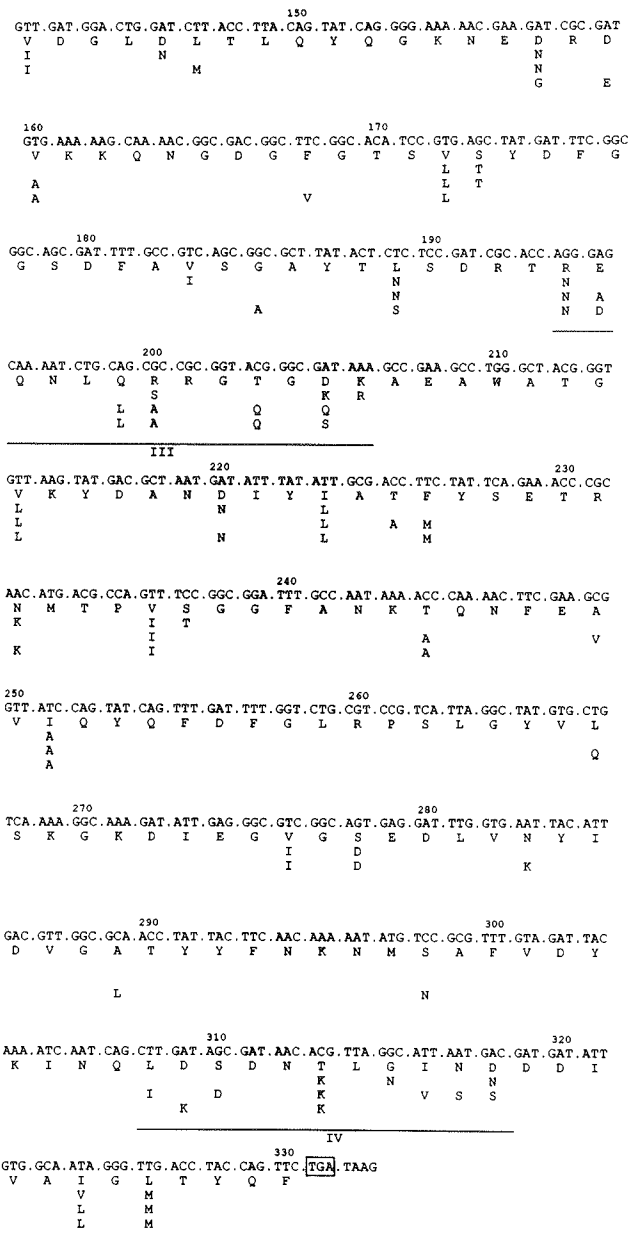


Fig. 4. Nt of *S. typhimurium phoE* and deduced aa sequence. The pseudo-*pho*, *ph* and Pribnow (PB) boxes are indicated. The nt corresponding with the *E. col* *phoE* transcription start point is indicated with a triangle. The translationa start and stop signals are boxed. The deduced aa sequence of *S. typhimuriu* PhoE is shown below the nt sequence in the one letter code for aa, and i compared with the corresponding sequences of *E. coli*, *E. cloacae* and *K pneumoniae*, respectively. In case of the latter three sequences only residue that differ from those of *S. typhimurium* PhoE are indicated. Residues -21/-2



to -1 represent the signal sequences. The arrow indicates the signal peptidase cleavage site. Dashes (-) represent deletions of aa. The poorly conserved regions are underlined and numbered I-IV. The nt sequence was determined by the dideoxynucleotide chain-termination method (25). Sequencing reactions were carried out with either single stranded or NaOH-denatured double stranded DNA templates and the T7 Sequencing ™ Kit (Pharmacia, Sweden). Sequences have been submitted to the EMBL Data Library under accession number X68023.

of the *pst* operon (18, 33). In the promoter of the *ugp* operon (23), 3 copies of the *pho* bo are present in a direct repeat. A *pho* box is also present in the promoter of *E. coli phoE* In addition, a region located 37 bp upstream of this *pho* box is required for efficien expression (35). This region, called pseudo-*phoE* promoter, contains sequence homologous to a *pho* box and a correctly-spaced Pribnow box, but in the reverse orientation. It is thought that the pseudo-*pho* box, like the *pho* box, binds th transcriptional activator PhoB (35). From all these *E. coli pho* boxes, a consensu sequence for the 7 bp repeats can be deduced (Fig. 5).

	position						
	1	2	3	4	5	6	7
C	12	1	0	0	11	0	5
A	2	1	1	2	6	17	1
T	3	16	3	16	1	1	12
G	1	0	14	0	0	0	0

consensus 7 bp repeat	C	T	G	T	A/C	A	C/T
pseudo-*pho* box	C	g	G	T	A	A	T
S. typhimurium	t	g	G	T	C	A	a

Fig. 5. Comparison of the *S. typhimurium* pseudo-*pho* box with the *pho* bo consensus sequence. The upper panel shows the occurrence of the different n at each position in 18 different 7 bp repeats of *E. coli pho* boxes. From thi panel, a consensus sequence for the 7 bp repeat is deduced. Below thi consensus sequence, the two 7 bp repeats of the pseudo-*pho* box of *S typhimurium phoE* are shown. Capital and small letters indicate identical an different nt, respectively.

In the *S. typhimurium phoE* promoter, a Pribnow box with identical sequence a in *E. coli phoE*, and a correctly-spaced *pho* box can be discerned (Fig. 4). Furthermore a pseudo-*phoE* promoter, consisting of a *pho* box and a correctly-spaced Pribnow box an oriented opposite to the direction of transcription, is present. Like in *E. coli phoE* th pseudo-*phoE* promoter is separated from the *pho* box by a 37 bp, AT-rich stretch However compared to the consensus *E. coli pho* box (Fig. 5), there is a notable difference Position 2, in both 7 bp repeats, is occupied by a G residue, which is never found at thi position in the 18 *E. coli* sequences. Therefore, the presence of these G residues in th pseudo-*pho* box might explain the poor expression of *S. typhimurium* PhoE in *E. coli.*

Fig. 4 also shows the deduced aa sequence of *S. typhimurium* PhoE and comparison of this sequence with the corresponding sequences of *E. coli, E. cloacae* an

K. pneumoniae. In contrast to the suggestion based on immunological studies (27) th PhoE proteins appear to be remarkably well conserved, i.e. the homology in terms o identical aa between the primary sequence of *S. typhimurium* PhoE and those of *E. coli E. cloacae* and *K. pneumoniae* is 87%, 85% and 84%, respectively. Only at two position one in the signal sequence and one in the mature domain, a deletion/insertion of one a is observed.

The *S. typhimurium* PhoE signal peptide contains only 20 aa, instead of the 2 residues observed in the signal peptides of the other PhoE proteins. The hydrophobic core of signal peptides usually contain a helix-breaking Gly or Pro residue (12). The deletio in the signal peptide of *S. typhimurium* PhoE removes this Gly (Fig. 4). Interestingly, site directed mutagenesis has been applied to replace the Gly in the signal peptide of *E. co* PhoE by Ala (20). The mutant precursor was still normally translocated across the inne membrane, but expression of the mutant protein was drastically reduced. Thus, althoug it is not yet clear how the mutation affects the expression of *E. coli* PhoE, the absence o a Gly in the signal peptide of *S. typhimurium* PhoE may provide an alternative explanatio for the poor expression of the protein in *E. coli.*

The PhoE proteins of *S. typhimurium* and *E. coli* contain an extra Gln residue a position 72 of the mature sequences. Although the overall homology between the fou PhoE proteins is high, four regions can be discerned with much lower homology (Fig. 4)

According to the postulated topology model, PhoE traverses the outer membran 16 times and has eight regions located at the cell surface (34, 41). The four hypervariabl regions are all predicted to be cell surface-exposed. Interesting is the remarkably wel conserved region between aa 90 and 140, which contains the third cell surface-exposed region. This region probably corresponds with the "eyelet" in the 3-dimensional structur of the *Rhodobacter capsulatus* porin as determined by Weiss et al. (44, 45) and contain the Lys residue in position 125 that is very important for the anion-selectivity of PhoE pores (3). Also the other aa reported to contribute to the selectivity of the *E. coli* PhoE pores, i.e. the Lys residues at position 18, 29 and 64 (3) and those important for the biogenesis of the protein, i.e. Glu (31), Gly (9) and Phe (32) at position 2, 144 and 330 respectively, are also conserved in *S. typhimurium* PhoE.

Design of *Salmonella*-specific oligos.

The genus *Salmonella* consists of only one species (15), designated *S. choleraesuis* However, many investigators prefer the name *S. enterica* because *choleraesuis* is also the name of a serotype (10). The species can be divided into 5 different subspecies namely subspecies I named *enterica*, subspecies II named *salamae*, subspecies III, subdivided into *arizonae* and *diarizonae*, subspecies IV named *houtenae* and subspecies V named *bongor* (10). It is not yet clear why certain *Salmonella* strains are virulent and therefore all strains should be treated as potential pathogens. However, the *Salmonella* strains most likely to be pathogenic for humans belong to subspecies I. Strains belonging to subspecies II and III are frequently isolated from the intestinal contents of cold-blooded animals and are rarely found in warm-blooded animals. Strains belonging to subspecies IV and V are mainly isolated from the environment and are rarely pathogenic for man (15). The classical *Salmonella* detection methods are slow and laborious. The test for *Salmonella* in food, for instance, uses bacteriological, biochemical and serological procedures and will take 4-7 days to complete (24). A genetically based procedure like the PCR could

potentially increase the rapidity and decrease the labour-intensiveness of the detection.

To determine whether the *S. typhimurium phoE* nt sequence can be used to develo species-specific DNA probes, two oligos were synthesized based on the hypervariabl regions where the homology between the different PhoE proteins was the lowest (Fig. 6).

	5′	3′
S. typhimurium	AGCGCCGCGGTACGGGCGATAAA →	ST5
E. coli	AaaGCCGtGGcACaGGCaAgcgt	
E. cloacae	tGgcgCGtGGTcaGGGCcAgAAA	
K. pneumoniae	tGgcCCGCGGccaGGGttcgAAA	

	5′	3′
S. typhimurium	ATCATCGTCATTAATGCCTAACGT →	ST8c
E. coli	AatATCaTCATTAtTaatattCaa	
E. cloacae	AatATCaTCgcTgcTtaCgccCag	
K. pneumoniae	gatgTCGTCATcgtTGatgccgag	

Fig. 6. Comparison of the DNA sequences of the *S. typhimurium* oligos with the corresponding sequences of the *phoE* genes of *E. coli* K-12, *E. cloacae* and *K pneumoniae*. The oligos named ST5 and ST8c, are based on the DNA sequences encoding the fifth and eighth surface-exposed regions of the PhoE protein, respectively. Capital and small letters indicate nt identical and different to *S. typhimurium phoE* nt, respectively. ST5 is based on the non-coding DNA strand, whereas ST8c is based on the coding DNA strand. Oligos were synthesized in a Applied Biosystem 381 A DNA-synthesizer.

Oligos ST5 and ST8c were based on DNA sequences encoding the fifth- and eighth surface-exposed regions of PhoE, respectively. They correspond to segments of the non coding-and the coding DNA strands, respectively, so that they can be used in PCRs. When the primers recognize their complementary sequences, a 365 bp DNA fragment should be amplified that can be detected by electrophoresis on agarose gels. To test whether the developed oligos are specific, both their sensitivity and their selectivity have to be tested In the sensitivity assay, the capacity of the primers to recognize different *Salmonella* strains is tested, whereas in the selectivity assay the ability to discriminate between *Salmonella* and non-*Salmonella* strains is tested.

The sensitivity of the primers was tested on 130 *Salmonella* strains all of different serotypes, including 96 strains belonging to subspecies I, 18 strains belonging to subspecies II, 13 strains belonging to subspecies III, one strain belonging to subspecies IV and two strains belonging to subspecies V. In addition, strain SJ2353 (26), on which sequence the primers are based, and two *S. enteritidis* strains phage type 4, one isolated from a human and one from a chicken source, were tested (except for strain SJ2353 all strains were provided by W. Jansen, National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands). An example of a PCR analysis is shown in Fig. 7. With one exception, from all 133 *Salmonella* tested strains the expected 365 bp DNA fragment was amplified. The *Salmonella* strain that was not

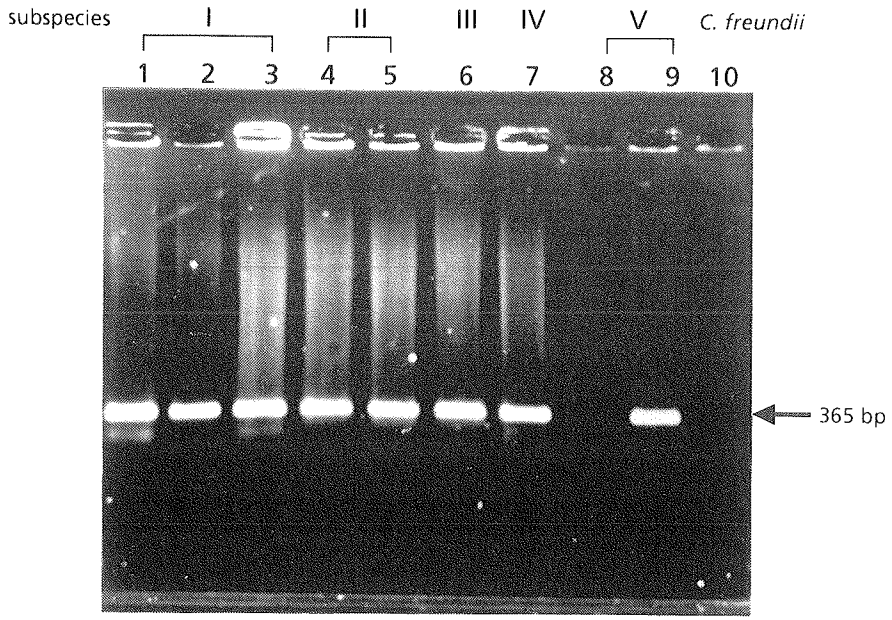


Fig. 7 Analysis of PCR products on a 1% agarose gel. Lane 1, *S. typhimuriu* LT2 strain SJ2353; lanes 2 and 3, *S. enteritidis* strains phage type 4 isolate from a human and a chicken source, respectively; lane 4, *S. Zeist*; lane 5, *S bulawayo*; lane 6, unnamed *Salmonella*; lane 7, *S. wassenaar*; lane 8, *S brookfield*; lane 9, *S. malawi*; lane 10, *Citrobacter freundii* previously name *Salmonella ballerup*. The subspecies to which the *Salmonella* strains belong ar indicated. Strains were grown overnight at 37°C in 1 ml L-broth (38). Cell were harvested by centrifugation, resuspended in 100 µl demineralized wate and boiled for 5 min. Cell debris was removed by 2 min centrifugation a 14,000 x g. PCR amplifications were performed on 5 µl samples of th supernatant fraction by using a DNA thermal cycler (PHC-1, New Brunswick plc. Int.). One unit of *Taq*-polymerase (Promega, Madison) was used as recommende by the manufacturer except that RNAse (Boehringer, Germany) was added to final concentration of 20 µg/ml and the total volume was adjusted to 25 µl A total of 25 PCR cycles was run under the following conditions: denaturatio at 94°C for 1 min, primer annealing at 45°C for 2 min and DNA extension a 72°C for 2 min but 7 min in the last cycle. Of the PCR products, 5 µl sample were analysed on the gels.

recognized by the primers, belongs to subspecies V serovar *Brookfield*.

The selectivity of the primers was tested on 14 different non-*Salmonella* strain belonging to the *Enterobacteriaceae* being *E. coli* K-12 strain CE1195 (37), *Citrobacte freundii*, *Edwardsiella tarda*, *Enterobacter aerogenes*, *E. cloacae*, *K. pneumoniae Providencia stuartii*, *Proteus mirabilis*, *Serratia marcescens*, *Shigella boydii* and *Shigell flexneri* described previously (11), *Citrobacter freundii* type *Ballerup*, *Erwinia herbicol* and *Yersinia enterocolitica*, (provided by W. Jansen) and *Citrobacter diversus* (provide by J. van der Plas, division for Nutrition and Food Research TNO, Zeist, Th Netherlands). With none of these strains, a DNA segment was amplified in the PCRs (dat not shown). Also the four tested non-*Enterobacteriaceae*, i.e. *Aeromonas hydrophila Bacillus cereus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, all from ou

laboratory stocks, were not recognized by the primers (data not shown).

Apparently, the two selected oligos form an excellent primer-couple with high specificity for the detection and identification of *Salmonella* by PCR.

CONCLUSIONS.

1. The localizations of *phoE* on the chromosomal maps of *S. typhimurium* and *E. coli* K-12 are identical.
2. *S. typhimurium* PhoE was only detected in *E. coli* K-12 when expressed from a multicopy plasmid. The poor expression of the *S. typhimurium phoE* gene in *E. coli* K-12 may be due to aberrancies in the pseudo-*pho* box and/or the absence of a Gly residue in the signal sequence of the protein.
3. *S. typhimurium* PhoE is structurally closely related to the *E. coli* protein and to the other general porins of *E. coli*.
4. Two oligos, based on the fifth and eighth surface-exposed regions of *S. typhimurium* PhoE, are specific for *Salmonella* in PCR.

ACKNOWLEDGEMENTS

We thank W. Coers and A. Herrewegh for technical assistance, M. Havekes for synthesizing the oligos and W. Jansen and J. van der Plas for providing strains. This work was supported by Holland Biotechnology (H.B.T.) and by The Netherlands Technology Foundation (S.T.W.).

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CHARACTERIZATION OF THE *Citrobacter freundii phoE* GENE AND DEVELOPMENT OF *C. freundii*-SPECIFIC OLIGONUCLEOTIDES

Gonnie Spierings, Corrine Ockhuijsen, Harmen Hofstra and Jan Tommassen

SUMMARY

The *phoE* gene of *Citrobacter freundii*, encoding a pore-forming outer membrane protein, was cloned and its nucleotide sequence was determined. The homologies in terms of identical amino acids between the *C. freundii* PhoE protein and those of *E. coli*, *E. cloacae* and *K. pneumoniae* are 90%, 86% and 84% respectively. Two synthetic oligonucleotides, corresponding to hypervariable, cell surface-exposed regions of the protein, were tested for their specificity in polymerase chain reactions. They were specific for the species *C. freundii*, i.e. no reaction was detected with 35 non-*C. freundii* strains tested, including 17 *Salmonella*, two *Citrobacter amalonaticus* and three *Citrobacter diversus* strains, whereas all five *C. freundii* strains tested were correctly recognized.

INTRODUCTION

In the current classification, the genus *Citrobacter* is divided into three species, i.e *Citrobacter freundii, Citrobacter amalonaticus* and *Citrobacter diversus* (12). Howeve it is also known that considerable heterogeneity exists within the species and suggestion have been made to reexamine the classification (3). Although *C. freundii* is normall considered as a common non-pathogenic inhabitant of the human intestine, reports on C *freundii* as a possible cause of diarrhoea and of extraintestinal infections have bee published (5, 6). Already in 1940 the organism, originally designated *Salmonell ballerup*, was connected with a severe case of gastritis (8). This, and the fact that C *freundii* strains that ferment lactose slowly, are often misidentified as *Salmonella* strain (12), makes the correct identification of *C. freundii* not only of importance in the researc laboratory but also in clinical diagnostics and in the food industry.

Recently, we have shown that species-specific DNA probes can be developed based on the *phoE* genes of different *Enterobacteriaceae* (15-18). The *phoE* gene encode a pore-forming outer membrane protein, the synthesis of which is induced unde phosphate-limitation. According to a topology model, the polypeptide traverses the oute membrane 16 times, mostly as amphipathic ß-strands, thereby exposing eight areas at th cell surface (19, 24). Comparison of the primary structures of PhoE proteins of differen *Enterobacteriaceae* revealed four hypervariable regions, all corresponding to predicted cel surface-exposed domains, whereas the membrane-spanning regions were found to b highly conserved (23). By using DNA sequences encoding these hypervariable, surface exposed regions, we succesfully developed *E. coli/Shigella* (16, 17), *Klebsiell pneumoniae* (17, 18) and *Salmonella* (15) -specific probes. In this study, we have clone and determined the nucleotide sequence of the *C. freundii phoE* gene and we hav evaluated the specificity of a polymerase chain reaction (PCR) using primers based on thi gene.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions.

The *E. coli* strains used were MXR containing plasmid pULB113 (25), strai CE1226, containing as relevant markers Δ (*proB-proA-phoE-gpt*), *phoS, recA, hsdR, hsdM* (26) and strain CE1195 (21). *Citrobacter* strains used were *C. amalonaticus* strains ATC 25405 and 25406, *C. diversus* strains ATCC 27156, 29936 and 27028, a *C. freundii* isolat described earlier (7) and now designated CE1345 and ATCC strains 8454, 8090, 29935 6750 and 10625. The latter strain has previously been described as *S. ballerup*. The ATC strains were obtained via the Phabagen Collection, Netherlands Culture Collections o Microorganisms, Utrecht, The Netherlands. Other enterobacterial strains used wer *Edwardsiella tarda, Enterobacter cloacae, Klebsiella pneumoniae, Proteus vulgaris Providencia stuartii* and *Serratia marcescens*, all described previously (7), *Salmonell typhimurium* strain SJ2353 (14) and *Erwinia herbicola, Yersinia enterocolitica* and 1 *Salmonella* strains with different serotypes, including 9, 2, 2, 1 and 2 strains belonging t subspecies I-V, respectively (all provided by the National Institute for Public Health an Environmental Protection, Bilthoven, The Netherlands). The non-enterobacterial strain

used, i.e. *Aeromonas hydrophila, Bacillus cereus, Pseudomonas aeruginosa* a *Staphylococcus aureus*, were from our laboratory stocks. The RP4::mini Mu plasm pULB113 (25) and pBR322 (2) were used as vectors for cloning the *C. freundii ph* gene.

Strains were grown overnight at 37°C under aeration in L-broth (22). Whe necessary, the following antibiotic concentrations were added: ampicillin (Ap) 50 µg/m kanamycin (Km) 50 µg/ml, rifampicin (Rif) 100 µg/ml, streptomycin (Sm) 100 µg/ml a tetracycline (Tc) 10 µg/ml.

Genetic techniques.

Matings between donor strains carrying RP_4-derived plasmids and recipient strai were performed as described (25). Preparation, restriction enzyme analysis and ligation plasmid DNA were performed as described by Maniatis *et al.* (10). The nucleotid sequence was determined by the dideoxynucleotide chain-termination method (13) and t reactions were carried out on either single-stranded or NaOH denatured double-strand DNA templates using the T7 Sequencing™ kit (Pharmacia, Sweden).

Isolation and characterization of cell envelopes.

Cell envelopes were isolated by differential centrifugation after ultrasonic disi tegration of the cells (9). The protein patterns were analysed by sodium dodecyl sulpha (SDS)-polyacrylamide gel electrophoresis (9) using gels containing 6M urea. Western bl analysis was performed as described (1).

Synthesis of the oligonucleotides and PCR conditions.

Oligonucleotides were automatically synthesized on an Applied Biosystem 381 DNA synthesizer. PCRs were performed as described earlier (18), except that t following conditions were used: denaturation at 94°C for 1 min, primer annealing at 55° for 2 min and DNA extension at 72°C for 2 min, but for 7 min in the last cycle.

Enterotubes.

Enterotubes II Roche (Hoffmann-La Roche, Basle, Switzerland) were used a described by the manufacturer.

RESULTS AND DISCUSSION

Cloning of the *C. freundii phoE* gene.

In *E. coli* K-12, the *phoE* gene is located in the vicinity of the *proA* and *pro* genes (20). By using an *in vivo*-cloning procedure with RP4::mini Mu plasmid pULB11 (25), the corresponding region of the *C. freundii* genome was transferred to *E. coli* K-1 Plasmid pULB113, which renders cells resistant to the antibiotics Ap, Kn, and Tc, wa transferred to a spontaneous Rif resistant derivative of *C. freundii* strain CE1345 b conjugation with donor strain MXR. One Kn and Rif-resistant transconjugant wa subsequently used as a donor strain in a mating with *phoS recA* strain CE1226 as th recipient. The latter host contains a *proA-proB-phoE-gpt* deletion and is resistant to th antibiotic Sm due to an *rpsL* mutation. One of the Sm resistant *pro*$^+$ transconjugant

carrying an R' plasmid designated pCF_0, was further analysed. Cell envelope proteins o strains CE1226 and its pCF_0 containing derivative, were analysed by SDS-PAGE. The cel envelope protein pattern of the transconjugant revealed the presence of an extra protei of the expected apparent molecular weight that reacted with a monoclonal antibody raise against the *E. coli* K-12 PhoE on Western blots (data not shown). Apparently pCF contains the *C. freundii phoE* gene and this gene is normally expressed in *E. coli* K-12

To study the *C. freundii phoE* gene in further detail, an approximately 5 kb *Cla* fragment containing the gene was subcloned from pCF_0 into the *Cla*I-site of the multicop vector pBR322. One of the contructs, designated pCF_9 is depicted in Fig. 1.

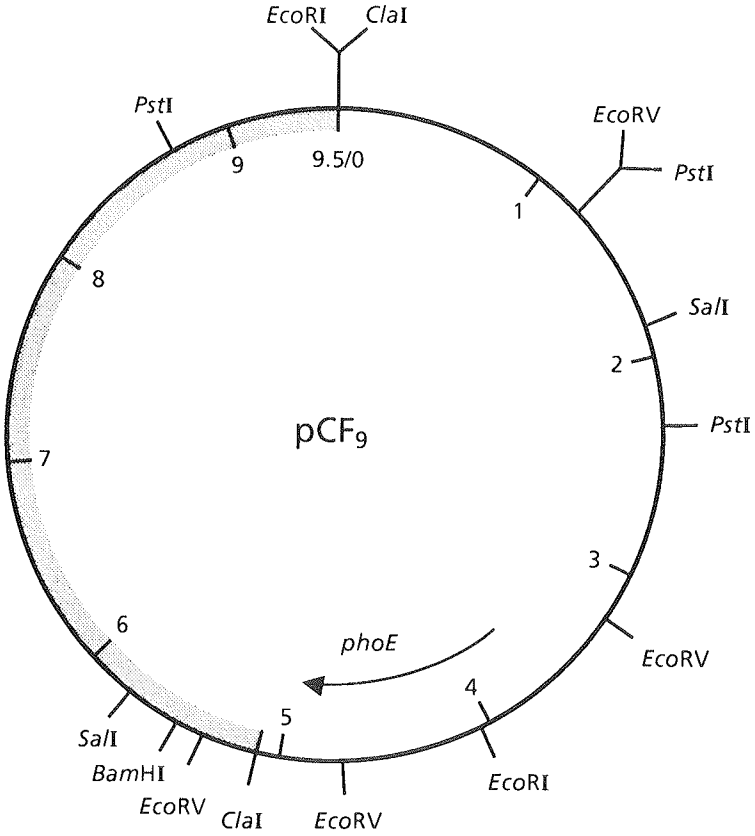

Fig. 1. Restriction map of plasmid pCF_9. The pBR322 vector is indicated by the shaded segment. The location and direction of transcription of the *C. freundii phoE* gene are indicated by the arrow. Map units are in kb.

Sequence analysis.

The nucleotide sequence of the *phoE* gene of *C. freundii*, which was determine entirely on both strands, and the deduced amino acid sequence of the protein are show in Fig. 2. The protein is synthesized as a precursor composed of a signal peptide and mature domain of 21 and 330 amino acid residues, respectively. The PhoE proteins o

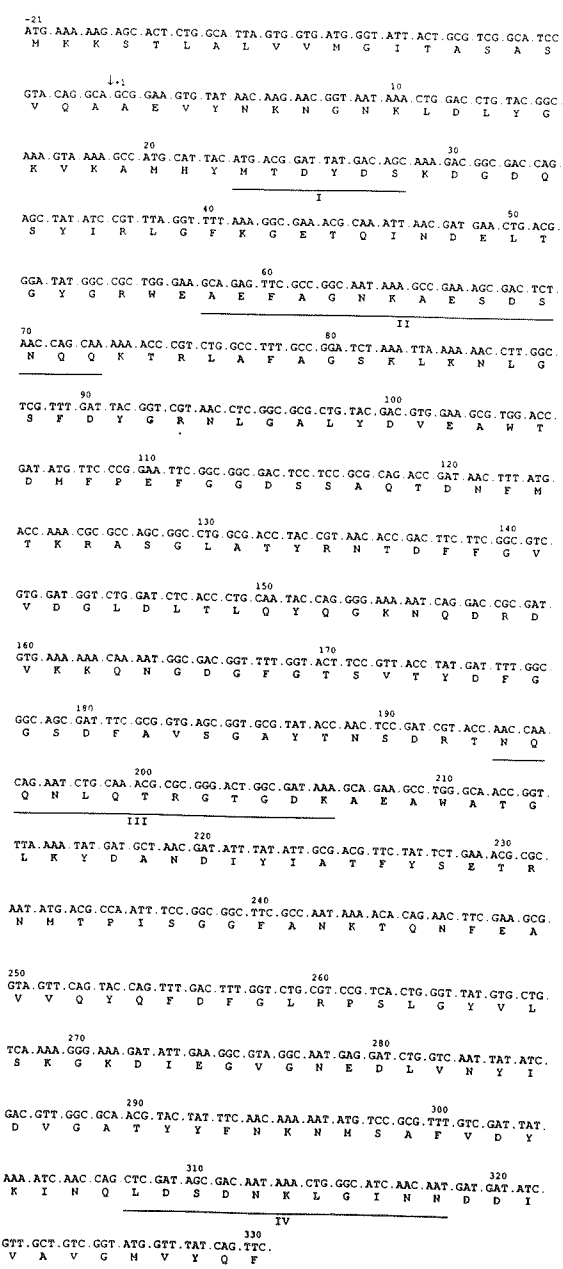

Fig. 2. Nucleotide sequence of the *C. freundii phoE* gene and deduced amino acid sequence. The deduced amino acid sequence of *C. freundii* PhoE is shown below the nucleotide sequence in the one letter code for amino acids. Residues -21 to -1 represent the signal sequence. The arrow indicates the signal peptidase cleavage site. The regions with low homology between the PhoE proteins of *E. coli, E. cloacae* and *K. pneumoniae* (23) are underlined and numbered I-IV. Sequences have been submitted to the EMBL Data Library under accession number X68021.

different members of the family *Enterobacteriaceae* appear to be remarkably well conserved, i.e. the homologies in terms of identical amino acids between the PhoE of *C. freundii* and those of *E. coli* [11], *E. cloacae* (23) and *K. pneumoniae* (23) are 90%, 86% and 84%, respectively. Previous studies on the PhoE proteins of different *Enterobacteriaceae* showed that most sequence variations are concentrated in four hypervariable regions which are all predicted to be cell surface-exposed (23). Also in case of the *C. freundii* PhoE, most differences with the other PhoE proteins occur in these four hypervariable regions (not shown).

Design of *C. freundii*-specific oligonucleotides.

To develop a *C. freundii*-specific PCR, two oligonucleotides were synthesized based on DNA encoding parts of the hypervariable fifth and eighth cell surface-exposed regions, respectively (Fig. 3). Oligonucleotide $CF5_2$ corresponds to amino acids 194-200, whereas $CF8c_2$ corresponds to amino acids 314-321. When the primers correctly recognize their complementary sequences on a template, a 387 basepairs DNA fragment is expected to be amplified in PCRs, which can be detected when the PCR products are analysed by electrophoresis on agarose gels.

	5′ 3′		
C. freundii	CCAACCAACAGAATCTGCAAACGC	→	$CF5_2$
S. typhimurium	CCAgggAgCAaAATCTGCAgcgcC		
E. coli	CCAACgAgCAGAAcCTGCAAAgcC		
E. cloacae	CCAACgcACAGAAcCTGCtggCGC		
K. pneumoniae	CCAACgAtCAGAAcCTGCtggCcC		

	5′ 3′		
C. freundii	CGATATCATCATTGTTGATGCCCAG	→	$CF8c_2$
S. typhimurium	CaATATCATCgTcaTTaATGCCtAa		
E. coli	CaATATCATCATTaTTaATattCAa		
E. cloacae	CaATATCATCgcTGcTtAcGCCCAG		
K. pneumoniae	CGATgTCgTCATcGTTGATGCCgAG		

Fig. 3. Comparison of the DNA sequences of the *C. freundii* oligos with the corresponding sequences of the *phoE* genes of *E. coli* K-12, *E. cloacae* and *K. pneumoniae*. Capital and small letters indicate nucleotides identical to and different from the *C. freundii phoE* nucleotides, respectively. $CF5_2$ is based on the noncoding DNA strand, whereas $CF8c_2$ is based on the coding strand.

The selectivity of the probes was tested on 35 non-*C. freundii* strains of which 31 belonged to the family of *Enterobacteriaceae* (see Material and Methods). With these strains which included 17 *Salmonella*, two *C. amalonaticus* and three *C. diversus* strains no amplified product could be detected (for examples, see Fig. 4). In contrast, five out of six *C. freundii* strains tested, i.e., CE1345 and ATCC 8090, 29935, 6750 and 10625 were

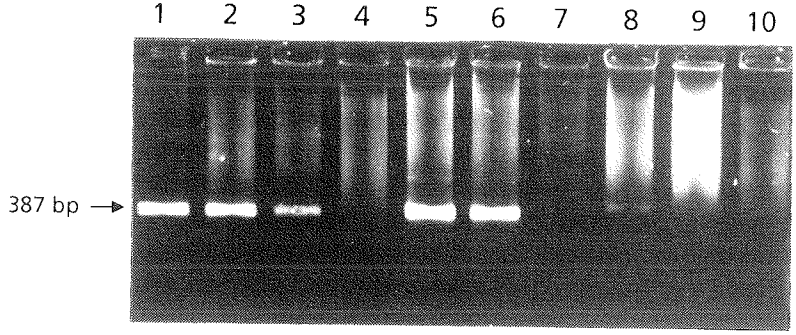

Fig. 4. Analysis of PCR products using primer-couple $CF5_2/CF8c_2$ on a 1% agaros gel. Lane 1. *C. freundii* strain CE1345; lane 2, *C. freundii* ATCC 8090; lar 3, *C. freundii* ATCC 29935; lane 4, *C. freundii* ATCC 8454?; lane 5, *C. freundi* ATCC 6750; lane 6, *C. freundii* ATCC 10625; lane 7, *C. amalonaticus* ATCC 254(; lane 8, *C. diversus* ATCC 27156; lane 9, *S. typhimurium* SJ2353; lane 10, *E. coli* strain CE1195.

correctly recognized by the primers and showed amplification of the 387 basepai fragment (Fig. 4). However, the primers failed to recognize strain ATCC 8454. When th latter strain was characterized biochemically by using the Enterotube system, it wa identified as *Salmonella* in contrast to the former strains, which were identified as *freundii*. Furthermore, when the six strains were tested in a *Salmonella*-specific PCR using primers based on DNA sequences encoding parts of cell surface-exposed regions o the *S. typhimurium* OmpA protein (17), strain ATCC 8454 was the only strain that wa recognized by the *Salmonella*-specific primers (Fig. 5). These results strongly suggest tha

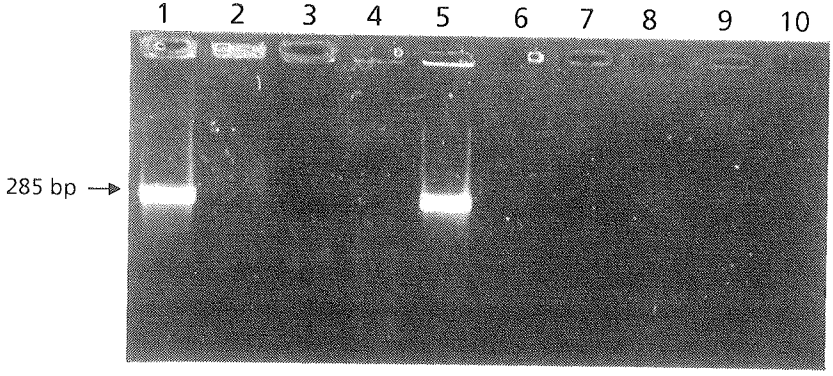

Fig. 5. Analysis of PCR products using the *Salmonella*-specific primer-couple ST1/ST3c on a 1% agarose gel. Lane 1, *S. typhimurium* SJ 2353; lane 2, *C. freundii* strain CE1345; lane 3, *C. freundii* ATCC 8090; lane 4, *C. freundii* ATCC 29935; lane 5, *C. freundii* ATCC 8454?; lane 6, *C. freundii* ATCC 6750, lane 7, *C. freundii* ATCC 10625; lane 8, *C. amalonaticus* ATCC 25405; lane 9, *C. diversus* ATCC 27156; lane 10, *E. coli* strain CE1195.

the tested strain ATCC 8454 is in fact a *Salmonella* strain and not a *C. freundii* strain. The fact that *C. freundii* strain ATCC 10625, which has previously been described as a *bethesda/ballerup*, is normally recognized by primer-couple $CF5_2/CF8c_2$ is in agreement

with the conclusion of Crosa *et al.* that there is no reason to distinguish the *bethesda* group from *C. freundii* solely on the basis of slow lactose fermentation (4).

In conclusion, by using DNA sequences encoding two different cell surface-exposed regions of the *C. freundii* PhoE, a primer-couple is developed that is specific for the species *C. freundii*. The developed PCR will be of great help in the correct identification of *C. freundii* strains, especially in discriminating between the slow lactose fermenting strains and the *Salmonellae*.

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IDENTIFICATION OF *Klebsiella pneumoniae* BY DNA HYBRIDIZATION AN FATTY ACID ANALYSIS

Gonnie Spierings, Albert van Silfhout, Harmen Hofstra and Jan Tommassen

SUMMARY

On the basis of the idea that DNA sequences encoding cell surface-expos regions of outer membrane proteins are genus or species specific, two oligonucleoti probes, which were based on the PhoE protein of *K. pneumoniae* were evaluated. slot-blot hybridizations and in polymerase chain reactions, no cross-hybridizatio were observed with non-*Klebsiella* strains. When the probes were tested on different K antigen reference *Klebsiella* strains, 16 strains were not recogniz although they did produce PhoE protein under phosphate-starvation. To determi whether these 16 strains belong to (a) different species, the reference strains we also tested for their ability to produce indole and to grow at 10°C and their whol cell fatty acid patterns were analysed by gas chromatography. A strong correlati was observed between (i) reaction with the probes (ii) inability to produce indole, (i inability to grow at 10°C and (iv) presence of the hydroxylated fatty acid $C_{14:0-2O}$ From these results we conclude that the two oligonucleotides are specific for t species *K. pneumoniae*. Furthermore, analysis of fatty acid patterns appears to be useful tool to distinguish *K. pneumoniae* from other *Klebsiella* species.

INTRODUCTION

The genus *Klebsiella* consists of four species, i.e. *K. pneumoniae* (with subspecies *pneumoniae, ozaenae* and *rhinoscleromatis*), *K. oxytoca, K. planticola* and *K. terrigena* (12). Although the present definition of the genus is confined to nonmotile strains proposals have been made to transfer *Enterobacter aerogenes* to the genus as *K. mobilis* (2,5). The current classification of *Klebsiella* strains into the different species is based on their biochemical properties. The results of these assays are in many cases difficult to interpret. For instance, there is no key discriminatory test to differentiate the indole negative *K. planticola* strains from *K. pneumoniae* (1). A genetically based procedure like the polymerase chain reaction (PCR) could potentially increase the precision and the rapidity and decrease the labour-intensiveness of the identification.

The first step in the development of such an identification system is the selection of species-specific oligonucleotide probes. Recently, we have shown that DNA sequences encoding surface-exposed regions of outer membrane proteins can be used as specific probes (16). The *phoE* gene of members of the family *Enterobacteriaceae* encodes a pore-forming outer membrane protein which is induced when the bacteria are grown under phosphate-limitation (14). According to the proposed topology model, the polypeptide traverses the outer membrane 16 times in an antiparallel ß-sheet structure, thereby exposing eight areas at the cell surface (17, 22). Comparison of the primary structures of different PhoE proteins revealed four hypervariable regions, all of which predicted to be cell surface-exposed, whereas the membrane-spanning regions were found to be highly conserved (21).

In this study, we have evaluated the potential of two oligonucleotide probes, based on the *phoE* gene of *K. pneumoniae*, to discriminate between different *Klebsiella* species. The sensitivity of the probes was tested on the K antigen reference strains described by Ørskov and Ørskov (13). The capsular polysaccharide, or K antigen, is most commonly used for serological typing of *Klebsiella* strains. The (lack of) reaction of the probes was correlated to species classification by testing the ability of the strains to produce indole and testing their growth at 10°C. *K. pneumoniae* strains are negative in the indole test and are the only *Klebsiella* strains inable to grow at 10°C (12). As an independent method, the fatty acid content of the strains was analysed by gas-liquid chromatography, which is also considered as a powerfull approach to differentiate between bacterial species (6).

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Escherichia coli K-12 strain CE1194, which carries a chromosomal *phoE* deletion and its *phoE*$^+$ derivative CE1195 (19) and strain K10 (CGSC4234) (18) have been described elsewhere. Other enterobacterial strains used were *Citrobacter freundii, Edwardsiella tarda, Enterobacter aerogenes, Enterobacter cloacae, Klebsiella pneumoniae, Providencia stuartii, Proteus mirabilis, Salmonella braenderup, Salmonella derby, Salmonella panama, Serratia marcescens, Shigella flexneri, Shigella boydii*, all described by Hofstra and Dankert (4) and *Salmonella typhimurium* strain SJ2353 described by Sato and Yura (15). Of the 77 different K antigen reference strains described by

Ørskov and Ørskov (13), 75 (missing K6 and K49) were kindly provided by W. Jans at the National Institute for Public Health and Environmental Protection in Bilthoven (T Netherlands). The non-enterobacterial strains used, i.e. *Aeromonas hydrophila, Bacill cereus, Pseudomonas aeruginosa, Sarcina flava* and *Staphylococcus aureus* were from o laboratory stocks.

Unless mentioned otherwise, strains were grown overnight at 37°C under aerati in L-broth (20). When strains were grown at 10°C, incubation was continued for 48 h

Isolation and characterization of cell envelopes.

Strains were grown overnight at 37°C under aeration in a medium (7) in whi the phosphate concentration is limiting for growth. Phosphate-replete conditions we obtained by supplementing the medium with 660 µM K_2HPO_4. Cell envelopes we isolated by differential centrifugation after ultrasonic disintegration of the cells (8). Tl protein patterns were analysed by SDS-polyacrylamide gel electrophoresis (8) using ge containing 6M urea.

Indole reaction.

Indole tests were performed as described by Frobisher (3).

Synthesis and labeling of the oligonucleotides.

Oligonucleotides were automatically synthesized on a Biosearch 8600 DN synthesizer. When used in hybridization assays, the oligomers were labeled by th enzymatically catalized transfer of ^{32}P from [γ-^{32}P]ATP (3000 Ci/mmol; Amersha International) with T4 polynucleotide kinase (Pharmacia, Uppsala), according to th procedure described by Maniatis *et al.* (9).

DNA hybridizations and PCR-assays.

Slot-blot hybridization assays were performed as described previously (16), excep that the incubation temperature during (pre)hybridization and washings was 63°C instea of 60°C. DNA samples to be used in PCRs were isolated from 1 ml overnight ce cultures. Cells were harvested by centrifugation, resuspended in 100 µl demineralize water, and boiled for 5 min. Celldebris was removed by a 2 min centrifugation step a 14.000x*g*. PCR amplifications were performed on 5 µl samples of the supernatant fractio by using a DNA-thermal cycler (PHC-1, New Brunswick plc. Int.). One unit *Taq* polymerase (Promega, Madison, Wis.) was used as described by the manufacturer excep that RNase (Boehringer Mannheim, Germany) was added to a final concentration of 2 µg/ml and the total volume was adjusted to 25 µl. A total of 25 PCR cycli was run unde the following conditions: denaturation at 94°C for 1 min, primer annealing at 45°C for min and DNA extension at 72°C for 2 min but for 5 min in the last cycle. Of the PCR products, 10 µl samples were analysed by electrophoresis on 1% agarose gels.

Gas-liquid chromatographic-analysis of fatty acids.

For analysis of fatty acid patterns bacteria were harvested after growth for 24 h at 37°C on Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) solidified with 1.5% agar. Whole-cell fatty acids were analysed as fatty acid methyl esters Fatty acid methyl ester extracts were made by the techniques described by Miller an

Berger (10). They were analysed by high performance capillary gas-liquid chromatography using the MIDI Microbial Identification System (Microbial ID, Inc. Newark, Del.). The system configuration contained a Hewlett-Packard HP 5890 A gas chromatograph with autosampler and was equipped with a 25-m Ultra 2 column and a flame ionization detector. The system was completed with a HP9000 series 300 computer system for analysis control, data acquisition and data evaluation, using the MIDI libary generating software.

RESULTS

The *phoE* gene of *K. pneumoniae* has been cloned and sequenced previously (21). Comparison of the DNA sequences of the *phoE* genes of *K. pneumoniae, E. cloacae* and *E. coli* revealed four hypervariable regions, corresponding to cell surface-exposed segments of the PhoE proteins (21). The hypervariable regions where the homology between the proteins was the lowest were chosen for two oligonucleotide probes. Probes KP5 and KP8c were based on DNA sequences encoding the fifth and the eighth surface exposed region of PhoE, respectively (Fig. 1). They correspond to segments of the

E. coli	$^{5'}$AAAGCCGTGGCACAGGCAAGCGT$^{3'}$	
K. pneumoniae	$^{5'}$TGGCCCGCGGCCAGGGTTCGAAA$^{3'}$	KP5
E. cloacae	$^{5'}$TGGCGCGTGGTCAGGGCCAGAAA$^{3'}$	

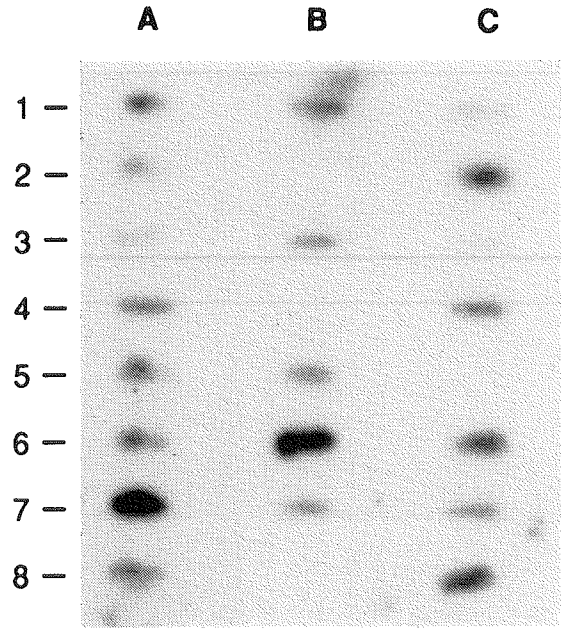
E. coli	$^{5'}$AATATCATCATTATTAATATTCAA$^{3'}$	
K. pneumoniae	$^{5'}$GATGTCGTCATCGTTGATGCCGAG$^{3'}$	KP8c
E. cloacae	$^{5'}$AATATCATCGCTGCTTACGCCCAG$^{3'}$	

Fig. 1. Comparison of the DNA sequences of the *Klebsiella* probes with the corresponding sequences of the *phoE* genes of *E. coli* and *E. cloacae*. KP5 is based on the DNA complementary to the sequence encoding the fifth and KP8c is based on the DNA sequence encoding the eighth surface-exposed region of the *phoE* gene of *K. pneumoniae*, respectively.

noncoding and the coding DNA strands, respectively, so that they can be used in PCRs. An ideal DNA probe has to be both sensitive and selective, i.e. it has to recognize all strains and serotypes of a certain taxon, but it should not cross-react with other bacteria. These conditions were tested in hybridization and PCR assays.

Sensitivity assays.

The capacity of the probes to recognize different *Klebsiella* strains was tested on 75 of the 77 K antigen reference strains (13). The strains are named K1 to K82; K73 and K75 to K78 have been eliminated because of misdiagnosis and overlapping (13). Strains K6 and K49 were lost from our collection. The probes were tested on these strains in slot blot hybridization assays. An example of an autoradiogram is shown in Fig. 2. Both



	A	B	C
1 —	K31	K32	K33
2 —	K34	K35	K36
3 —	K37	K38	K39
4 —	K40	K41	K42
5 —	K45	K43	K44
6 —	K46	K47	K48
7 —	K50	K51	K52
8 —	K53	*E. coli*	*Kpn*

Fig. 2. Autoradiogram of a slot-blot hybridization using probe KP5. The strains are different *Klebsiella* K antigen reference strains. The *E. col* strain used was CE1195. *K.pn* is an unserotyped *K. pneumoniae* strain, the *pho* gene of which has been sequenced previously (21). The probes were based o this sequence.

probes reacted with 59 out of the 75 strains and each of them failed to recognize 1 strains, which were the same strains in both cases. The probes were designed in such way that they could also be used as primers in PCRs. When the primers recognize thei complementary sequences, a 368 basepairs DNA fragment will be amplified which ca be detected when the PCR products are analysed by electrophoresis on agarose gels. A example of such an analysis is shown in Fig. 3. The expected amplified fragment wa observed in the same 59 strains that reacted positively in the slot-blot hybridization assays whereas the remaining strains again were negative (Table 1).

Selectivity assays.

To test the selectivity of the probes, the 14 different non-*Klebsiella* strain belonging to the *Enterobacteriaceae* and 5 non-*Enterobacteriaceae* strains, mentioned i Material and Methods were tested in slot-blot hybridizations. The results of probe KP8 have been published earlier (16), and showed that only the *K. pneumoniae* strain fro which the *phoE* nucleotide sequence was established was recognized. Similar results were found when KP5 was used as probe or when KP5 and KP8c were used together in PCR (data not shown).

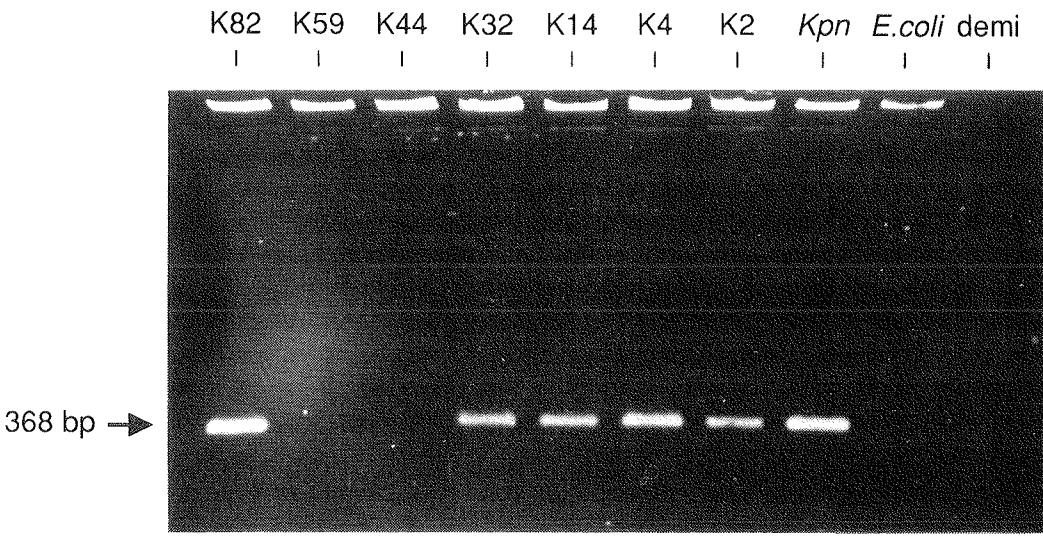


Fig. 3. Analysis of PCR products on a 1% agarose gel. The K strains are different *Klebsiella* K antigen reference strains. The *E. coli* strain used was CE1195. *K.pn* is an unserotyped *K. pneumoniae* strain, the *phoE* gene of which has been sequenced previously (21). The probes were based on this sequence. Demi means demineralized water was used as substrate.

Expression of the *phoE* genes.

The two probes turned out to be selective, since they did not recognize any other strains except *Klebsiella*, but when tested on their sensitivity, they failed to recognize 16 out of 75 *Klebsiella* strains, i.e. K5, K26, K29, K35, K41, K44, K59, K65 to K70, K72, K74 and K79. To test whether these strains contain a *phoE* gene, a number of them were grown under high- and low-phosphate conditions and their cell envelope protein patterns were analysed. Fig. 4 shows that all tested *Klebsiella* strains induced a membrane protein of the expected apparent molecular weight when grown under low-phosphate conditions. Of the strains tested K26, K29, K44, K59 and K65 did not react with the probes. The DNA sequence of their *phoE* genes must therefore differ from those of strains recognized by the probes.

Species classification reactions.

To determine whether the different reactions with the DNA probes can be correlated to differences at the species level, more information about the 75 *Klebsiella* strains was necessary. Therefore, the strains were tested for their ability to produce indole and to grow at 10°C (Table 1). Strains that are positive in the indole test belong either to the species *K. oxytoca* or *K. planticola* and of the different *Klebsiella* species only *K. pneumoniae* is not able to grow at 10°C (12). All strains found to produce indole, being K26, K29, K41, K44, K66, K70, K72 and K74, were not recognized by the probes. Furthermore, we found a strong correlation between positive results in the probe assays and no or poor growth at 10°C (Table 1). The strains that gave negative results in the probe assays, on the other hand, could all grow at 10°C, except for strains K5 and K70 which showed no and poor growth, respectively.

TABLE 1: Characteristics of the *Klebsiella* strains

strain	PCR results	growth at 10°C[a]	indole production	presence of fatty acid $C_{14:0\text{-}2OH}$
K. pneumoniae[b]	+	-	-	+
K1	+	-	-	+
K2	+	-	-	+
K3	+	-	-	+
K4	+	+/-	-	+
K5	-	-	-	+
K7	+	-	-	+
K8	+	+/-	-	+
K9	+	-	-	+
K10	+	+/-	-	+
K11	+	-	-	+
K12	+	+/-	-	+
K13	+	+/-	-	+
K14	+	+/-	-	+
K15	+	-	-	+
K16	+	-	-	+
K17	+	-	-	+
K18	+	-	-	+
K19	+	+/-	-	+
K20	+	-	-	+
K21	+	-	-	+
K22	+	+/-	-	+
K23	+	-	-	+
K24	+	+/-	-	+
K25	+	-	-	+
K26	-	++	+	-
K27	+	+/-	-	+
K28	+	-	-	+
K29	-	++	+	-
K30	+	+/-	-	+
K31	+	+/-	-	+
K32	+	+/-	-	+
K33	+	-	-	+
K34	+	+/-	-	+
K35	-	++	-	-

TABLE 1: Characteristics of the *Klebsiella* strains

strain	PCR results	growth at 10°C[a]	indole production	presence of fatty acid $C_{14:0\text{-}2OH}$
K36	+	+/-	-	+
K37	+	-	-	+
K38	+	-	-	+
K39	+	-	-	+
K40	+	-	-	+
K41	-	++	+	-
K42	+	+/-	-	+
K43	+	+/-	-	+
K44	-	++	+	-
K45	+	+/-	-	+
K46	+	+/-	-	+
K47	+	+/-	-	+
K48	+	+/-	-	+
K50	+	-	-	+
K51	+	-	-	+
K52	+	-	-	+
K53	+	+/-	-	+
K54	+	+/-	-	+
K55	+	+/-	-	+
K56	+	+/-	-	+
K57	+	-	-	+
K58	+	+/-	-	+
K59	-	++	-	-
K60	+	-	-	+
K61	+	-	-	+
K62	+	-	-	+
K63	+	-	-	+
K64	+	-	-	+
K65	-	++	-	-
K66	-	++	+	-
K67	-	++	-	-
K68	-	++	-	-
K69	-	++	-	-
K70	-	+/-	+	-

TABLE 1: Characteristics of the *Klebsiella* strains

strain	PCR results	growth at 10°C[a]	indole production	presence of fatty acid $C_{14:0\text{-}2OH}$
K71	+	-	-	+
K72	-	++	+	-
K74	-	++	+	-
K79	-	++	-	-
K80	+	+/-	-	+
K81	+	-	-	+
K82	+	+/-	-	+

[a] symbols: Optical density values at λ = 660 nm after 48 h
- OD < 0.1
+/- 0.1 ≤ OD < 0.5
++ OD ≥ 1

[b] *K. pneumoniae* is an unserotyped strain, the *phoE* gene of which has been sequenced (21). The probes were based on this sequence.

Gas-liquid chromatographic analysis of fatty acids.

The analysis of fatty acid content has been propagated for the identification o bacterial species (6) but has, to our knowledge, never been used to differentiate betwee different *Klebsiella* species. Therefore, as a totally independent determination method, th

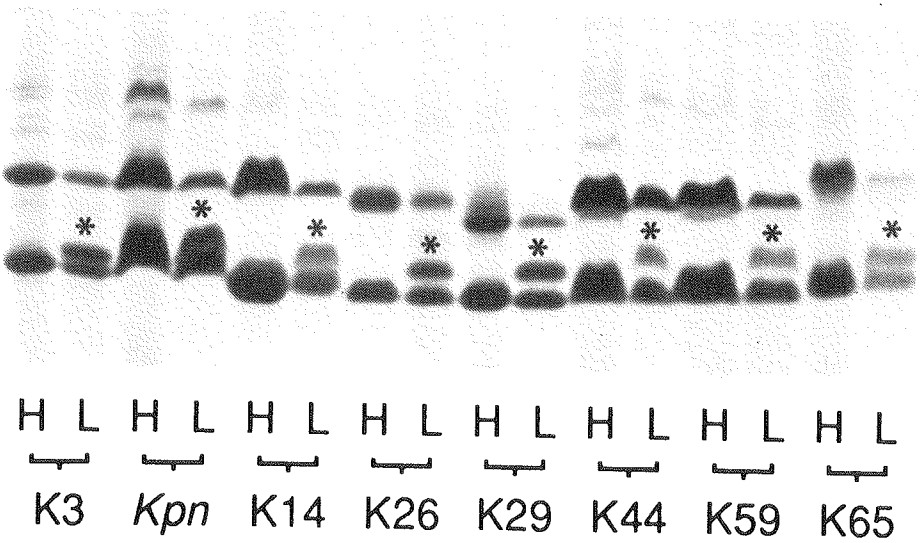


Fig. 4. SDS-polyacrylamide gel electrophoresis patterns of cell envelope proteins of *Klebsiella* strains either grown under high- (H) or low-phosphate (L) conditions. The PhoE proteins are indicated with asterisks. Only the relevant part of the gel is shown.

fatty acid methyl esters of the isolated phospholipids of the 75 *Klebsiella* strains were analysed. The most common fatty acids found in all the *Klebsiella* strains were $C_{16:0}$ (34%), $C_{17:0\ cyclo}$ (14%) and $C_{14:0}$ (9%). The major hydroxylated fatty acid found was $C_{14:0-2OH}$ (2%). Interestingly, only those *Klebsiella* strains that were recognized by the probes contained this hydroxylated fatty acid whereas the strains which were not recognized by the probes did not (Table 1). The only exception is strain K5, which, although negative in the probe assays, contains fatty acid $C_{14:0-2OH}$. Strain K60, which is positive in the probe assays, does contain fatty acid $C_{14:0-2OH}$, albeit at a lower level (1%).

DISCUSSION

The two *Klebsiella* probes evaluated in this study were found to be selective, i.e. no cross-reactivity occurred with tested *Enterobacteriaceae* other than *Klebsiella* strains and with *non-Enterobacteriaceae* strains. When the probes were tested on 75 of the 77 K antigen reference strains, 16 strains were not recognized by the probes. Among these strains are K26, K29, K44, K59 and K65, which normally induced a PhoE protein when grown under phosphate-limitation (Fig. 4). Therefore, the *phoE* gene is probably commonly present in *Klebsiella* strains. An explanation for the fact that 16 of the *Klebsiella* strains were not recognized by the probes might be that the probes are specific for the species *K. pneumoniae*. It has been known for some time that a number of the K antigen reference strains are *K. oxytoca* strains (12) and more recent research has indicated that also *K. planticola* and *K. terrigena* strains might be present in this collection (11). To obtain more information on the species classification of the *Klebsiella* strains their ability to produce indole and to grow at 10°C and their whole-cell fatty acid patterns were analysed. A good correlation was observed between (i) reaction with the probes, (ii) inability to produce indole, (iii) inability to grow at 10°C and (iv) presence of the hydroxylated fatty acid $C_{14:0-2OH}$. Only two exceptions were found. Strain K5, despite of its inability to grow at 10°C and the presence of $C_{14:0-2OH}$ in its fatty acid profile, was not recognized by the probes. This strain, like K4, which normally reacted with the probes, has been classified as a *K. ozaenae* strain (12a). Strain K70, although positive in the indole test, negative in the probe assays and missing fatty acid $C_{14:0-2OH}$ showed only poor growth at 10°C.

From the results, we conclude that recognition by the probes is probably restricted to the species *K. pneumoniae* and that therefore, with the exception of strain K5, all strains which are not recognized by the probes belong to other *Klebsiella* species. The finding that K26, K29, K35, K41, K44, K59, K65, K66, K70, K72, K74 and K79 do not belong to the species *K. pneumoniae* is in agreement with the results of Mori *et al.* (11). However strains K67, K68 and K69 which, according to our tests, did not behave like *K. pneumoniae* strains were classified as such by Mori *et al.* On the other hand strains K8, K14, K32, K39, K48, K49, K56, K57, K58 and K71 classified by Mori *et al.* as *K. planticola* strains, behaved in our tests as normal *K. pneumoniae* strains. Most differences occur in differentiating *K. pneumoniae* strains from the indole-negative *K. planticola* strains. Since there is no key discriminatory test in the biochemical classification, Mori *et al.* used, as recommended by Bagley *et al.* (1), a combination of an L-sorbose fermentation test and an hydroxy-L-proline utilization test to distinguish between the two

species. Also, two other tests, namely the faecal coliform reaction at 44,5°C and grow at 10°C, were used. Mori *et al.* found only 65% of the faecal coliform negative and h growth at 10°C to be positive in the tests recommended by Bagley *et al.*, whereas Bagl *et al.* found a correlation of more then 90%. According to I. Ørskov some *Klebsiel* strains are biochemically hard to classify (12a).

The above-mentioned results demonstrate the difficulty in the biochemic classification assays to distinguish *K. pneumoniae* from the indole-negative *K. planticol* In our opinion, the use of the DNA probes as described in this article and the analysis fatty acid content will contribute to a more precise classification of *Klebsiella* strain Moreover, the use of these probes will lead to more rapid detection and classificatic assays. Presently, we are developing similar DNA probes for the other *Klebsiella* speci to enable the specific detection and identification of these species as well.

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POLYMERASE CHAIN REACTIONS FOR THE SPECIFIC DETECTION O *Klebsiella oxytoca* AND FOR THE GENERAL DETECTION OF *Klebsiellae*

Gonnie Spierings, Corrine Ockhuijsen, Harmen Hofstra and Jan Tommassen

SUMMARY

The *phoE* gene of *Klebsiella oxytoca*, encoding a pore-forming outer membran protein, was cloned and its nucleotide sequence was determined. The homology i terms of identical amino acids between *K. oxytoca* PhoE and that of *K. pneumonia* is 95%. The region with lowest homology corresponds to a part of a cell surface exposed region of the protein. Two oligonucleotides, based on parts of thi hypervariable region, were synthesized and used together with an oligonucleotid corresponding to a more conserved region, in polymerase chain reactions. On primer-couple was specific for the species *K. oxytoca*, whereas the other recognize all indole-positive *Klebsiella* strains suggesting that indole-positive *Klebsiella planticol* strains are more closely related to *K. oxytoca* than to indole-negative *K. planticol* strains. In addition, a third primer-couple, consisting of two oligonucleotides base on two conserved regions of the *phoE* gene, was tested. This primer-couple wa specific for the genus *Klebsiella*.

INTRODUCTION

The current classification of *Klebsiella* strains into the different species, i.e *Klebsiella pneumoniae, Klebsiella oxytoca, Klebsiella planticola* and *Klebsiella terrigena* is based on their biochemical properties (8). The tests to differentiate between the indole negative *K. planticola* strains and *K. pneumoniae*, or between the indole-positive *K. planticola* strains and *K. oxytoca* are in many cases difficult to interpret (2). Genetically based procedures like polymerase chain reactions (PCRs) could potentially increase the precision and the rapidity of the identifications provided that species-specific nucleotide sequences can be found. Recently, we have presented a *K. pneumoniae*-specific PCR using oligonucleotides based on two different surface-exposed regions of the *K. pneumoniae* PhoE protein (17).

The *phoE* genes of members of the family *Enterobacteriaceae* encode a pore forming outer membrane protein, the synthesis of which is induced when the bacteria are grown under phosphate-starvation (11). According to the proposed topology model, the polypeptide traverses the outer membrane 16 times as amphipathic ß-strands, thereby exposing eight areas at the cell surface (19, 24). Comparison of the primary structures of PhoE proteins of bacteria belonging to different genera of the family of *Enterobacteriaceae* revealed four hypervariable regions, all predicted to be cell surface exposed, whereas the membrane-spanning regions were found to be highly conserved (23). The nucleotide sequences encoding these hypervariable regions could be used to develop species-specific DNA probes (14-17).

In this study, we have cloned and determined the nucleotide sequence of the *K. oxytoca phoE* gene and compared it with the corresponding sequence of *K. pneumoniae*. This information was used to develop species- and genus-specific oligonucleotide probes for *K. oxytoca* and *Klebsiella*, respectively

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions.

The *E. coli* strains used were MXR containing plasmid pULB113 (25), strain CE1226, containing as relevant markers, Δ (*proB-proA-phoE-gpt*), *phoS, recA, hsdR, hsdM* (26), and strain CE1195 (21). The *Klebsiella* strains used were a *K. pneumoniae* isolate described earlier (4) and now designated CE1346 and the 77 different K antigen reference strains described by Ørskov and Ørskov (9). The strains are named K1 to K82; K73 and K75 to K79 have been eliminated because of misdiagnosis and overlapping. Other enterobacterial strains used were *Salmonella typhimurium* SJ2353 (13), *Citrobacter freundii, Edwardsiella tarda, Enterobacter cloacae, Proteus mirabilis, Providencia stuartii, Salmonella braenderup, Salmonella derby, Salmonella panama, Serratia marcescens, Shigella boydii* and *Shigella flexneri*, all described earlier (4) and *Erwinia herbicola* and *Yersinia enterocolitica* which were kindly provided by W. Jansen at the National Institute for Public Health and Environmental Protection in Bilthoven, The Netherlands. The non enterobacterial strains used, i.e. *Aeromonas hydrophila, Bacillus cereus, Pseudomonas aeruginosa* and *Staphylococcus aureus*, were from our laboratory stocks.

The RP4::miniMu plasmid pULB113 (25) and pACYC184 (3) were used as vector

for cloning *K. oxytoca phoE*. Strains were grown overnight at 37°C under aeration in broth (22). Where necessary, antibiotics were added in the following concentrations: ampicillin (Ap) 50 µg/ml, kanamycin (Km) 50 µg/ml, rifampicin (Rif) 100 µg/m streptomycin (Sm) 100 µg/ml and tetracycline (Tc) 10 µg/ml.

Genetic techniques.

Matings between donor strains carrying RP4-derived plasmids and recipient strai were performed as described (25). Preparation, restriction enzyme analysis and ligation plasmid DNA were performed as described by Maniatis *et al.* (6). The nucleotide sequen was determined by the dideoxynucleotide chain-termination method (12). The reactio were carried out using NaOH-denaturated double stranded DNA templates and the T Sequencing ™Kit (Pharmacia, Sweden).

Isolation and characterization of cell envelopes.

Cell envelopes were isolated by differential centrifugation after ultrason desintegration of the cells (5). The protein patterns were analysed by sodium dodec sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (5) using gels containing 6 urea. Western blot analysis was performed as described (1).

Synthesis of the oligonucleotides and PCR conditions.

Oligonucleotides were automatically synthesized on an Applied Biosystem 381 A DNA synthesizer. PCRs were performed on a DNA thermal cycler (PHC-1, Ne Brunswick plc., Int.) as described previously (17).

Pectate degradation test.

Pectate lyase activity was tested on L-broth plates with an overlay containing 0.7 polygalacturonic acid (PGA). After growth for 48 h. at 37°C, the plates were stained wit a solution of 10% copper acetate which forms a blue percipitate with PGA.

RESULTS

Cloning of the *K. oxytoca phoE* gene.

In *E. coli* K-12, the *phoE* gene is located in the vicinity of the *proA* and *pro* genes (20). By using an *in vivo*-cloning procedure with RP4::miniMu plasmid pULB11 (25), the corresponding region of the *K. oxytoca* genome was transferred to *E. coli* K-12 Plasmid pULB113, which renders cells resistant to the antibiotics Ap, Kn and Tc wa transferred to a spontaneous Rif-resistant derivative of the K26 antigen reference strain o *K. oxytoca* by conjugation with donor strain MXR. One Kn- and Rif-resistan transconjugant was subsequently used as a donor strain in a mating with *phoS recA* strai CE1226 as the recipient. The latter host contains a *proA-proB-phoE-gpt* deletion and i resistant to the antibiotic Sm due to an *rpsL* mutation. One of the Sm-resistant *pro* transconjugants, carrying an R' plasmid designated pKO_{26}, was further analysed. Cel envelope protein patterns of strains CE1226 and of its pKO_{26}-containing derivative wer analysed on SDS-PAGE. The cell envelope protein pattern of the transconjugant revealed the presence of an extra protein that had the expected apparent molecular weight, i.e

38,000 and that reacted on Western blots with a monoclonal antibody raised against the *E. coli* K-12 PhoE protein (data not shown). These results indicated that the *K. oxytoca phoE* gene was cloned on pKO_{26} and that the gene was normally expressed in *E. coli* K-12.

To study the *K. oxytoca phoE* in further detail an approximately 3 kb *Sal*I fragment containing the gene was subcloned from pKO_{26} into the *Sal*I-site of the multicopy vector pACYC184. One of the constructs, designated $pKO_{26\text{-}3}$ is depicted in Fig. 1.

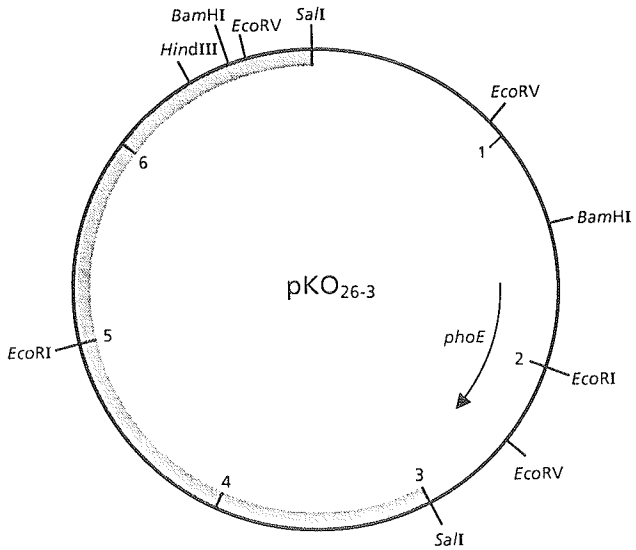


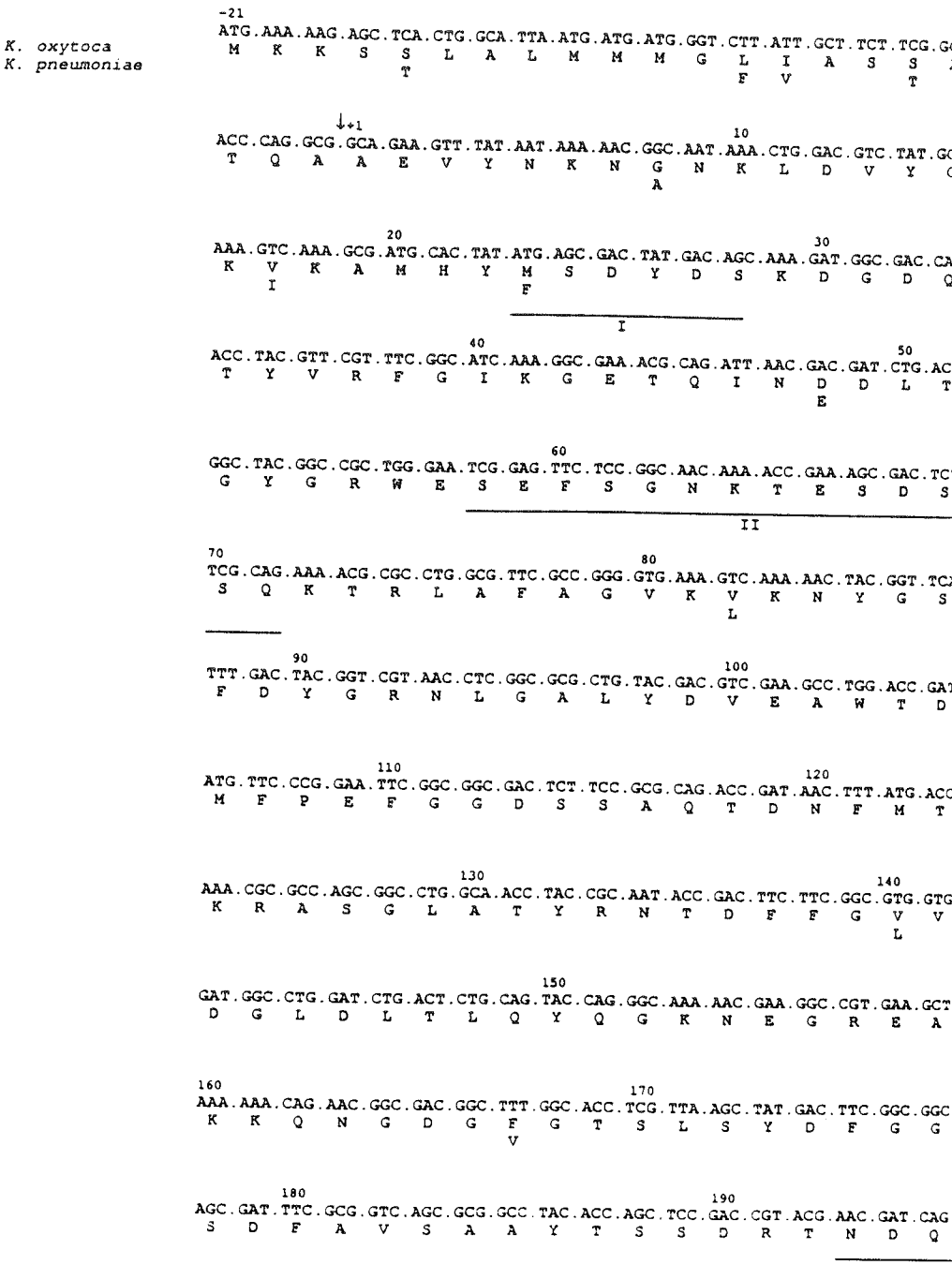
Fig. 1. Restriction map of plasmid $pKO_{26\text{-}3}$. The pACYC184 vector is indicated by the shaded segment. The location and direction of transcription of the *K. oxytoca phoE* gene are indicated by the arrow. Map units are in kb.

Sequence analysis.

The nucleotide sequence of the *K. oxytoca phoE* gene was determined on both strands (Fig. 2). In Fig. 2 also the deduced amino acid sequence of the *K. oxytoca* PhoE protein and, for comparison, the corresponding sequence of *K. pneumoniae* (23) are shown. The protein is synthesized as a precursor, composed of a signal peptide and a mature domain of 21 and 328 amino acid residues, respectively. The PhoE proteins appear to be well conserved, i.e. the homology in terms of identical amino acids between the PhoE of *K. oxytoca* and those of *K. pneumoniae* (23), *E. coli* (10), *S. typhimurium* (14) *E. cloacae* (23) and *C. freundii* (16) is 95%, 85%, 84%, 84% and 85%, respectively.

Previous studies on the PhoE proteins of bacteria belonging to the family of *Enterobacteriaceae* showed that most sequence variations are concentrated in four hypervariable regions which are all predicted to be cell surface-exposed. Also in case of the *K. oxytoca* PhoE, most differences with those of bacteria belonging to other genera occur in these four hypervariable regions, designated I-IV in Fig. 2 (comparisons not shown). As expected, the homology between the *K. oxytoca* PhoE and that of *K. pneumoniae* is even more extensive, i.e. 95%. Most differences concern conservative replacements. Only one region with lower homology can be discerned, namely

hypervariable region III, which includes the fifth surface-exposed domain (Fig. 1
Interestingly, the *K. oxytoca* PhoE contains a deletion of one amino acid residue in th
region (Fig. 2). This explains why *K. oxytoca* strains were not recognized in the
pneumoniae-specific PCR since one of the primers used was based on this region (17



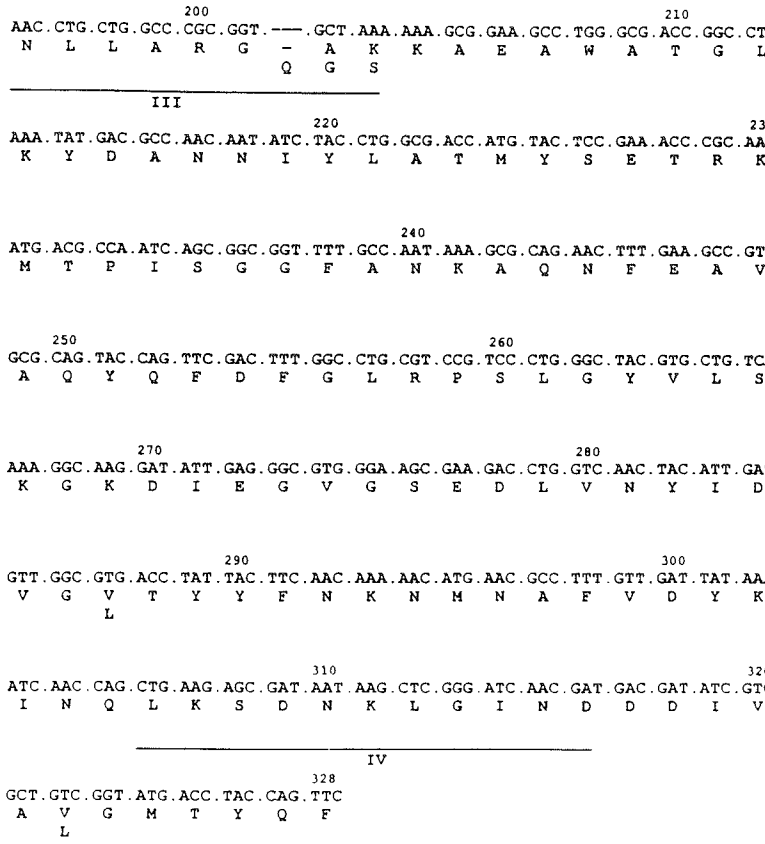


Fig. 2. Nucleotide sequence of the *K. oxytoca phoE* gene and deduced amino acid sequence. The deduced amino acid sequence of *K. oxytoca* PhoE is shown below the nucleotide sequence in the one letter-code for amino acids and is compared with the corresponding sequence of *K. pneumoniae* of which only those residues that differ from *K. oxytoca PhoE* are indicated. The dash (-) represents the deletion of an amino acid. Residues -21 to -1 represent the signal sequence. The arrow indicates the signal peptidase cleavage site. The regions with low homology between the PhoE proteins of *E. coli, E. cloacae* and *K. pneumoniae* (23) are underlined and numbered I-IV. Sequences have been submitted to the EMBL Data Library under accession number X68022.

Development of a *K. oxytoca*-specific PCR.

To develop a *K. oxytoca*-specific PCR, two oligonucleotides were synthesized based on DNA encoding parts of the fifth and eighth surface-exposed regions, respectively (Fig. 3A and B). Oligonucleotide KO5$_1$ corresponds to amino acids 197-204, whereas KO8c corresponds to amino acids 312-319. When the primers recognize their complementary sequences on a template, a 368 basepairs DNA fragment is expected to be amplified in PCRs, which can be detected when the PCR-products are analysed by electrophoresis on agarose gels.

The selectivity of the primer-couple was tested on 19 non-*Klebsiella* strains, 15 of which belonged to the family of *Enterobacteriaceae* (see Material and Methods). With

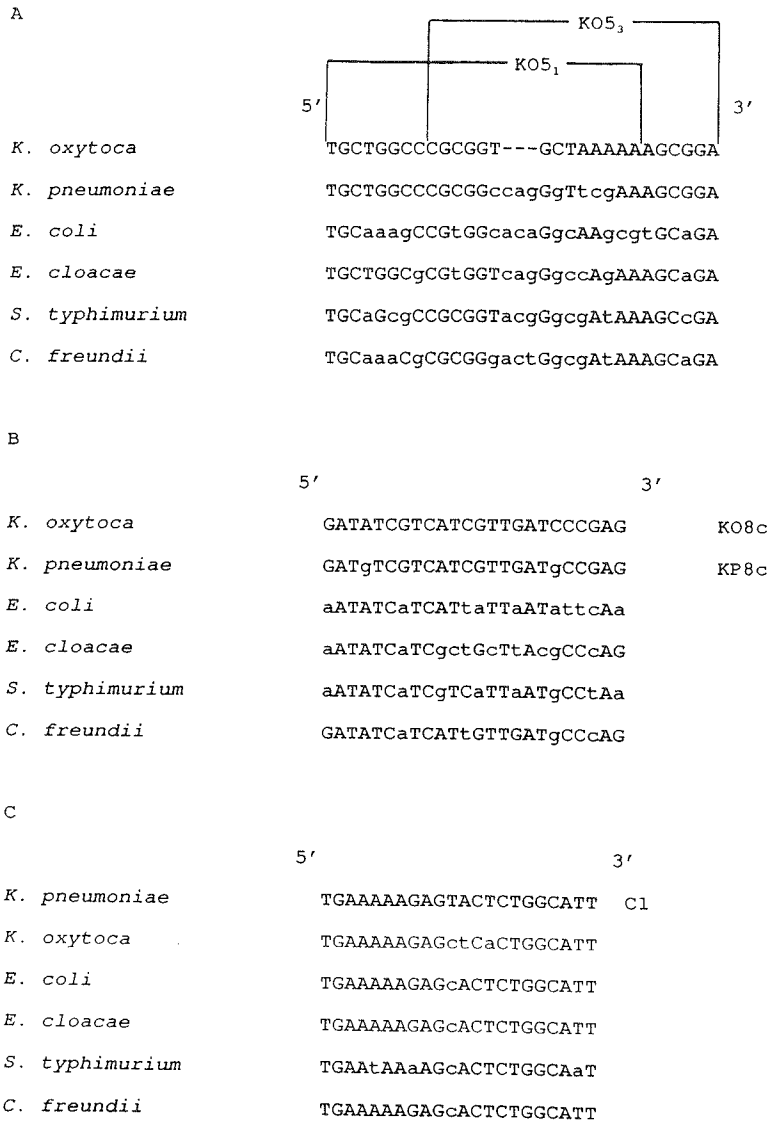


Fig. 3. Comparison of the nucleotide sequences of the *K. oxytoc* oligonucleotides (A, B) and the *K. pneumoniae* oligonucleotides (B, C) with th corresponding sequences of the other *phoE* genes. Dashes represent deletion of nucleotides. Capital and small letters indicate identical and differen nucleotides, respectively. $KO5_1$, $KO5_3$, and C_1 are based on the noncoding DN strand, whereas KO8c and KP8c are based on the coding DNA strands.

these strains no amplified product could be detected (data not shown). The sensitivity c the oligonucleotides was tested on *K. pneumoniae* strain CE1346 and on the 77 differen K antigen reference strains (9). With five out of the 77 K antigen reference strains, i.e K26, K29, K41, K66 and K70, the expected amplified product could be detected (Fig. 4)

An important characteristic of *K. oxytoca* is its ability to produce indole. Excep

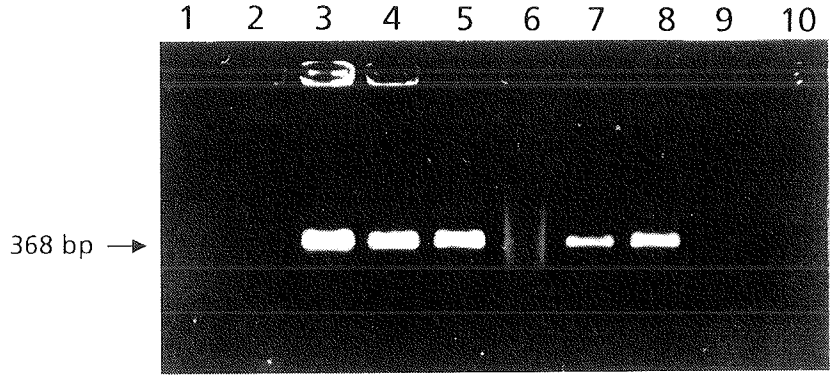


Fig. 4. Analysis of PCR products using primer-couple $KO5_1$/KO8c on a 1% agarose gel. Lane 1, *K. pneumoniae* strain CE1346; lane 2, K59; lane 3, K26; lane 4, K29; lane 5, K41; lane 6, K44; lane 7, K66; lane 8, K70; lane 9, K72; lane 10, K74. The position of the amplified products is indicated on the left.

for some *K. planticola* strains, all other *Klebsiella* species are negative in this assay (2). Of the K antigen reference strains, eight were positive in the indole-test, namely K26, K29, K41, K44, K66, K70, K72 and K74 (17). *K. oxytoca* strains can be distinguished from indole-positive *K.planticola* strains by their ability to degrade pectate. Based on this test K26, K29, K41, K66, K70 and K74 can be classified as *K. oxytoca* and K44 and K72 as *K. planticola* strains (data not shown). This classification is in agreement with that of Mori *et al.* (7) who tested the ability of the strains to degrade pectate, to produce pigment on gluconate-ferric citrate and to use gentisate or m-hydroxybenzoate as a sole carbon source. The PCR-results correlate well with the above mentioned classificastions. The only exception is strain K74. Therefore, an alternative for oligonucleotide $KO5_1$ was tested. This oligonucleotide, designated $KO5_3$ (Fig. 3A) overlaps with $KO5_1$ but has its 3'end in a more conserved part of the fifth cell surface-exposed region. When it is used together with KO8c as a primer-couple in PCRs, a 360 basepairs DNA fragment is expected to be amplified when the primers recognize their complementary sequences on a template. Primer-couple $KO5_3$/KO8c recognized all eight indole-positive *Klebsiella* strains (Fig. 5). No amplified product could be detected with the 72 indole-negative *Klebsiella* strains and with the non-*Klebsiella* strains tested (results not shown).

Development of a *Klebsiella*-specific PCR.

Whereas the DNA sequence corresponding to the fifth cell surface-exposed region of PhoE is apparently species-specific, we considered the possibility that the DNA sequence encoding the eighth cell suface-exposed region is genus-specific. To test this possibility, oligonucleotide KP8c (Fig. 3B) was used together with oligonucleotide C1 (Fig. 3C) in PCRs. C1 encodes a part of the signal peptide that is highly conserved in PhoE proteins. Both oligonucleotides are based on the *K. pneumoniae phoE* sequence. When C1 and KP8c recognize their complementary sequence in a template, a 1019/1022 basepairs DNA fragment is expected to be amplified. Primer-couple C1/KP8c correctly

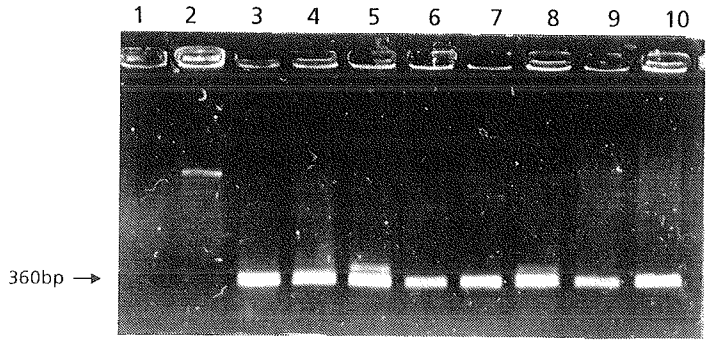


Fig. 5. Analysis of PCR products using primer-couple $KO5_3$/KO8c on a 1% agaros gel. Lane 1, *K. pneumoniae* strain CE1346; lane 2, K59; lane 3, K26; lane 4 K29; lane 5, K41; lane 6, K44; lane 7, K66; lane 8, K70; lane 9, K72; lane 1(K74. The position of the amplified prdoducts is indicated on the left.

recognized *K. pneumoniae* strain CE1346 and 76 out of the 77 K antigen reference strair (see Fig. 6 for examples). The only *Klebsiella* strain that gave a negative result wi C1/KP8c was *K. pneumoniae* strain K5. No amplified product could be detected with th 19 non-*Klebsiella* strains tested.

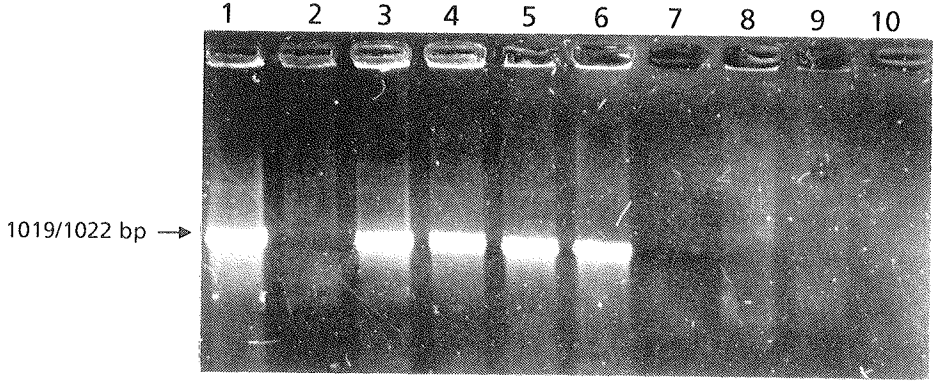


Fig. 6. Analysis of PCR products using primer-couple C1/KP8c on a 1% agaros gel. Lane 1, *K. pneumoniae* strain CE1346; lane 2, K5; lane 3, K26; lane 4 K59; lane 5, K65; lane 6, K74; lane 7, *E. coli* strain CE1195; lane 8, *E cloacae*; lane 9, *C. freundii*; lane 10, *S. typhimurium*. The position of th amplified products is indicated on the left.

DISCUSSION

The PhoE protein of *K. oxytoca* consists of a signal peptide and a mature domai of 21 and 328 amino acid residues, respectively. When the *K. oxytoca* PhoE sequence i compared with those of bacteria belonging to different genera, i.e. *E. coli*, *E. cloacae*, *C freundii* and *S. typhimurium*, four hypervariable regions can be discerned, all predicted t be cell surface-exposed. Only the fifth cell surface-exposed region shows also a hig variability when compared to that of *K. pneumoniae* PhoE. Two overlappin oligonucleotides, designated $KO5_1$ and $KO5_3$, based on this fifth surface-exposed regio

were used in PCRs together with KO8c, an oligonucleotide corresponding to part of th eighth surface-exposed region. Both primer-couples, i.e. $KO5_1$/KO8c and $KO5_3$/KO8c, di not react with the 19 non-*Klebsiella* strains and with the 70 indole-negative *Klebsiell* strains tested. Primer-couple $KO5_1$/KO8c reacted with five out of the eight tested indole positive *Klebsiella* strains, i.e. K26, K29, K41, K66 and K70, whereas primer-coupl $KO5_3$/KO8c also recognized the other three, i.e. K44, K72 and K74. Strains that ar positive in the indole-test belong either to the species *K. oxytoca* or *K. planticola*. Base on their ability to degrade pectate strains K26, K29, K41, K66, K70, and K74 ar classified as *K. oxytoca* strains. The specificity of primer-couple $KO5_1$/KO8c correlate well with this classification, the only exception being strain K74. A conclusion that ma be drawn from these results is that the PCR, using primers based on PhoE, is no infallible. The results obtained with primer-couple $KO5_3$/KO8c, that recognized all eigh indole-positive strains, however point into a different direction. These data indicate tha strain K74 may be more related to the indole-positive *K. planticola* strains than to *K oxytoca* but moreover, that the indole-positive *K. planticola* strains are more related to *K oxytoca* than to the indole-negative *K. planticola* strains.

The *phoE* gene was also used to develop a *Klebsiella*-specific PCR. Primers C1 an KP8c, both based on more conserved regions of the *K. pneumoniae phoE*, correctl recognized 77 out of the 78 tested *Klebsiella* strains, whereas no amplified product coul be detected with the 19 tested non-*Klebsiella* strains. The negative *Klebsiella* strain, K5 belongs to the species *K. pneumoniae* subspecies *ozaenae*. Recently, we have reported o the development of a *K. pneumoniae*-specific PCR (17). Also in that PCR, K5 was th only *K. pneumoniae* strain that was not recognized. These results, together with the fac that we were unable to detect a PhoE protein by SDS-PAGE after growth of this strai under phosphate-limitation (18), strongly suggests that this strain does not possess a *phoE* gene.

In conclusion, the *phoE* gene was succesfully used to develop species-specific an genus-specific *Klebsiella* oligonucleotides, with a high degree of selectivity and sensitivity We expect that these oligonucleotides will be very useful for the rapid detection an identification of *Klebsiella* strains. Furthermore, the genus-specific oligonucleotides ca be used to amplify and clone the majority of the *phoE* genes of indole-negative *K planticola* and *K. terrigena* strains, in order to develop specific oligonucleotides for thes species as well.

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GENERAL AND SUMMARIZING DISCUSSION

The detection and identification of members of the family *Enterobacteriaceae* important in a wide variety of studies, including fundamental and applied research in th medical, food, agricultural and environmental sectors. Existing detection and identificatio procedures are generally slow and laborious. A genetic procedure involving DN hybridizations, especially when combined with an amplification procedure like the PCI could potentially increase the precision and the rapidity and decrease the labou intensiveness of microbial detection and identification.

The first step in the development of such a detection and identification system the selection of specific oligonucleotides. In order to be specific for a group of organism the oligonucleotide has to recognize all strains and serotypes of this group, but it may n cross-react with other bacteria.

The aim of the investigations described in this thesis was to determine whethe DNA sequences corresponding to hypervariable cell surface-exposed regions of the outc membrane protein PhoE can be used as species-specific enterobacterial oligonucleotide

The PhoE proteins.

In *Escherichia coli* K-12, the *phoE* gene, which encodes a phosphate-limitation inducible pore-forming protein, is located at min 6 on the chromosomal map adjacent to the *proBA* operon (Fig. 1) (40). The PhoE protein is synthesized as a precursor composed

Fig. 1. Schematic representation of the *E. coli* chromosomal DNA in the *gpt* to *proBA* region. The arrows indicate the direction of transcription.

of a signal peptide and a mature domain of 21 and 330 amino acid residues, respectively (33). According to the proposed topology model (39, 42), the polypeptide traverses the outer membrane 16 times in an antiparallel ß-sheet structure having eight regions exposed at the cell surface. Comparison of the primary structures of OmpC, OmpF and PhoE showed that the membrane-spanning segments are conserved whereas the surface-exposed regions are hypervariable (39) (see Chapter 1, Fig. 4). The *phoE* genes of *Enterobacter cloacae* (41, 43), *Klebsiella pneumoniae* (41), *Salmonella typhimurium* (Chapter 4), *Citrobacter freundii* (Chapter 5) and *Klebsiella oxytoca* (Chapter 7) have been cloned by transferring the *proB*A regions of the corresponding genomes to *E. coli* K-12 followed by subcloning of the *phoE* genes in multicopy vectors. This cloning strategy however, appeared to be unsuccessful in case of the *Yersinia enterocolitica phoE* gene. Although plasmids were obtained that complemented *proBA* and *gpt* mutations in *E. coli*, the cloned DNA did not react in Southern blot analysis with *E. coli phoE* as probe (unpublished observation). Since a positive result was obtained in a Southern blot analysis with the chromosomal DNA of *Y. enterocolitica* and since a membrane protein of the expected molecular weight was induced when this strain was grown under phosphate-starvation (unpublished data), *Y. enterocolitica* seems to possess a *phoE* gene but this gene is apparently not located next to the *proBA* operon. This seems also to be the case in *Serratia marcescens* (30). Therefore another strategy is necessary to clone these *phoE* genes. A possibility is to take advantage of the observation that *E. coli* strains that lack OmpC and OmpF are sensitive to 3% sodium dodecyl sulphate, whereas expression of PhoE in such strains results in resistance to the detergent (22). However, this method only works if the foreign *phoE* gene is normally expressed in *E. coli* K-12. To bypass this necessity, hybridizations with *E. coli phoE* as probe can be used to screen a genebank. In this case attention should be paid to the fact that the probe will also recognize *E. coli ompC* and *ompF*. Therefore, it is advisable to use as acceptor an *E. coli* strain that lacks *ompC* and *ompF*.

The foreign *phoE* genes were normally expressed in *E. coli* K-12, except for the *S. typhimurium phoE* gene that had a low expression level. Nucleotide sequence analysis revealed differences between the *S. typhimurium* and *E. coli* K-12 sequences which may explain the low expression level (Chapter 4). One difference is located in a part of the

promoter sequence called the pseudo-*pho* box, whereas another one is located in t hydrophobic core of the signal sequence where a Gly residue is lacking (Chapter 4). T possible influence of these differences on gene expression can be studied by site-direct mutagenesis. Another difference, located in the 5' untranslated region, is the reduc ability of the *S. typhimurium* sequence to form stable hairpin structures. Recently, Emo *et al.* (13) have shown that a stable 5' terminal stem-loop structure stabilized the Omp mRNA in *E. coli*.

Interestingly, when three different *S. typhimurium* strains, including strain SJ235 and a *Salmonella panama* strain were grown under phosphate-limitation, also lo expression of PhoE protein was found (unpublished results). There can be tv explanations for the poor expression of *Salmonella* PhoE, i.e. (i) high expression of Phc is less important for *Salmonella* because these bacteria do not normally encount phosphate-limiting growth conditions, or (ii) the expression of PhoE is under the influen of a second regulatory system, suggesting that PhoE has an additional, yet unknow function. An important difference between *Salmonella* and *E. coli* is that *Salmonella* is a intracellular pathogen. Recently, a two-component regulatory system, *phoP/phoQ*, has bee described that regulates the expression of genes involved in virulence and macropha survival of *S. typhimurium* (25). However, when pST3, containing *S. typhimurium phoI* was introduced into *S. typhimurium* strain LT_2 and its $phoP_{12}$ derivative (17), no differenc in PhoE production could be detected suggesting that *phoE* is not under control of th system (unpublished results). Maybe important is to note that *Shigella flexneri*, i.e. anothe enterobacterial strain known to be an intracellular pathogen, also expresses PhoE only a a low level under phosphate-limitation (R. Janssen and J. Tommassen, unpublishe results).

The nucleotide - and deduced amino acid sequences (Fig. 2) showed that all Pho proteins are synthesized as precursors with an NH_2-terminal signal sequence of 21 amin acid residues but of 20 amino acid residues in case of the *S. typhimurium* PhoE. Like *E coli* PhoE, the mature domains of the *S. typhimurium* and the *C. freundii* PhoE protein contain 330 residues whereas the *E. cloacae*, *K. pneumoniae* and *K. oxytoca* PhoE protein contain a deletion of the Gln residue at position 72. The *K. oxytoca* PhoE has a additional deletion of one amino acid residue at position 203 and therefore contains onl 328 amino acid residues. The homologies in terms of identical amino acids between th *E. coli* PhoE and that of *C. freundii*, *E. cloacae*, *K. pneumoniae*, *K. oxytoca* and *S typhimurium* are 90%, 87%, 83%, 85% and 87%, respectively. The homology in terms o totally conserved amino acid residues in all six proteins is 72%. Interesting is th remarkably well conserved region between amino acid residues 90 and 140, whicl contains the postulated third cell surface-exposed region. This region probably correspond with the "eyelet" in the three dimensional structure of the *Rhodobacter capsulatus* porin as determined by Weiss *et al.* (45, 46) which is not really cell surface-exposed, bu extends in the pore interior. This region contains the Lys residue in position 125 that i very important for the anion-selectivity of the PhoE pores (3). Also the other amino acic residues reported to contribute to the selectivity of the *E. coli* PhoE pores, i.e. the Ly residue at position 18, 29 and 64 (3) and those important for the biogenesis of the protein i.e. Glu-2 (37), Gly-144 (10) and Phe-330 (38) are conserved among the six PhoE proteins. Inspection of the amino acid sequences of the 16 membrane-spanning segments

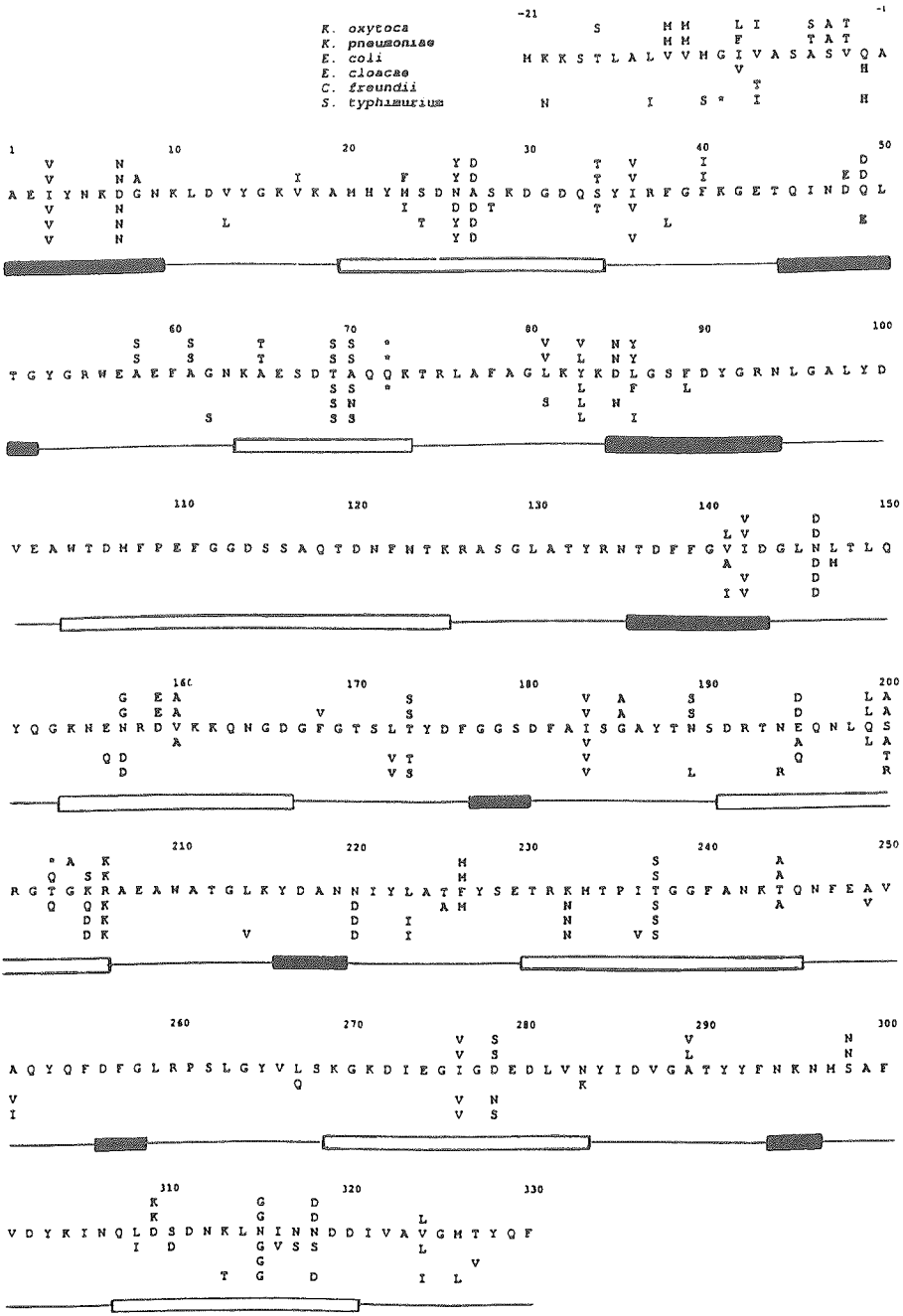

Fig. 2. Comparison of the primary structures of six enterobacterial PhoE proteins. The predicted amino acid sequences from *K. oxytoca*, *K. pneumoniae*, *E. cloacae*, *C. freundii* and *S. typhimurium* are compared with the *E. coli* sequence. Of the former five sequences only those residues that differ from the latter sequence are shown. Asterisks (*) represent deletions of amino acid residues. Residues -21/-20 to 1 represent the signal sequences. Included are the following features of the proposed model for PhoE topology; (□), region predicted to be exposed at the cell surface; (–), transmembrane ß-sheet strand; (■), reverse turn at periplasmic side.

of *E. coli* PhoE showed the remarkable frequent occurrence of a Tyr as the fourth resid at the hydrophobic side of the amphipathic ß-strands (39). Ten out of the eleven T residues at these positions appear to be conserved in the different PhoE proteins. The on exception is Tyr-83 which is replaced by a Val residue in case of the *K. oxytoca* PhoE by a Leu residue in case of the other PhoE proteins.

Although the overall homolgy between the PhoE proteins is high, fo hypervariable regions can be discerned. They correspond to the postulated cell surfac exposed regions one, two, five and eight and the corresponding homologies between t different PhoE proteins in these domains are 60%, 62%, 47% and 50%, respectively. T question why these regions are less conserved than the cell surface-exposed regions fou six and seven, having homologies of 69%, 75% and 80%, respectively, remai unanswered. No strong correlation can be found between hypervariability and regio described as phage receptor domains, i.e. regions two and four, or regions identified immunogenic domains, i.e. regions four, five, six and seven (39). Interesting and importa with respect to the development of species-specific oligonucleotides is the fact tha compared to the cell surface-exposed regions of two other outer membrane proteins, i. the maltose-inducible pore protein LamB (49) and the constitutively expressed Omp protein (5), the surface-exposed regions of PhoE are more conserved. The variability the cell surface-exposed regions of outer membrane proteins is probably due to the la of functional constraints on the amino acid content of these regions (1) and to selecti pressure imposed by phages, colicins and antibodies. The higher variability of the ce suface-exposed regions of OmpA might be explained by the fact that OmpA constitutively expressed, resulting in a more constant selective pressure than in the ca of PhoE. The expression of LamB however is, like that of PhoE, inducible. Possibl bacteria encounter more frequently conditions that lead to the induction of LamB tha phosphate-limitation. Alternatively, the higher variability of the exposed regions of Lam may be explained by the fact that these regions are larger and therefore more accessib than in the case of PhoE. Anyhow, it is remarkable that bacteriophages that recogni OmpA or LamB as their receptor can readily be isolated from sewage, whereas it is ve difficult to find phages that use PhoE as receptor (P. de Graaff and J. Tommasse personal communication).

Oligonucleotides based on *phoE*.

Comparison of the amino acid sequences of the PhoE proteins of bacteria belongi to different genera of the family *Enterobacteriaceae* (Fig. 2) revealed four hypervariab regions. Based on the idea that DNA sequences encoding (part of) the hypervariab regions could be species-specific, oligonucleotides were designed based on the fifth a eighth cell surface-exposed regions of the PhoE proteins of *E. coli* K-12 (Chapters 2, 3 *S. typhimurium* (Chapter 4), *C. freundii* (Chapter 5), *K. pneumoniae* (Chapter 6) and *oxytoca* (Chapter7). The specificity of the oligonucleotides, i.e. the ability to recognize a strains and serotypes of the species and to differentiate them from other bacteria, w tested in DNA hybridizations and PCRs. In Table 1, the PCR results of the five prime couples are summarized. They were all species-specific and they turned out to be ve reliable, i.e. no false-positive and only two false-negative results were obtaine Comparison of the amino acid sequences of the *K. pneumoniae* and the *K. oxytoca* Pho

proteins however revealed only the fifth surface-exposed region as a hypervariable domain. This suggests that the species-specificity of primer-couples KP5/KP8c and $KO5_1$/KO8c is due to the primers KP5 and $KO5_1$, whereas KP8c and KO8c are genus-specific. To test this possibility and to develop a *Klebsiella*-specific assay, KP8c was used together with oligonucleotide C1 in PCRs (Table 1). C1 encodes a part of the signal peptide of PhoE that is highly conserved among bacteria belonging to the different genera of the family of *Enterobacteriaceae*. The primer-couple was as expected specific for the genus *Klebsiella* and only failed to recognize the K antigen reference strain K5, which was also negative in the *K. pneumoniae*-specific PCR (Table 1). In addition, the specificity of a PCR using as primers C1 and C2c, another oligonucleotide based on a highly conserved region of PhoE, was tested. This primer-couple recognized the vast majority of strains tested from the species *E. coli/Shigella* and from the genera *Citrobacter, Enterobacter, Klebsiella* and *Salmonella* (Table 1). Only two strains were not recognized. Also in this assay, *Klebsiella* K antigen reference strain K5 and in addition *Salmonella* strain serovar *brookfield* failed to give a positive reaction. Combined with the observation that no PhoE could be detected by SDS-PAGE after growth under phosphate-limitation (unpublished observations), these results strongly suggest that the latter two strains do not possess a *phoE* gene. According to the above mentioned results, the *phoE* gene can not only be used to develop species-specific oligonucleotides but also oligonucleotides that have a specificity above the species-level. From the fact that only two out of the 355 tested strains probably do not have a *phoE* gene, it can be concluded that PhoE must be an important protein for the bacteria. Furthermore, since *S. flexneri* and all but one of the 133 tested *Salmonella* strains have a *phoE*, although they express the protein only at a low level when grown under phosphate-limitation, PhoE expression might have another inducing signal and the PhoE protein might have an additional function. This makes the discovery that the *phoE* gene of *Y. enterocolitica* and probably also that of *S. marcescens* (30) are located on different positions on the chromosomal maps as compared to the localization of *E. coli phoE*, even more interesting. Therefore, once the *phoE* gene of *Y. enterocolitica* is cloned it is important to sequence also the adjacent regions of *phoE*. If *phoE* turns out to be part of an operon an indication of its additional function might be found.

Can other outer membrane protein genes also be used for the development of species-specific oligonucleotides? OmpA is an outer membrane protein which is commonly present among enterobacterial strains (5). The N-terminal part of OmpA is also supposed to traverse the outer membrane repeatedly, mostly as amphipathic ß-strands (27, 44). Also in this case, sequence comparison of OmpA proteins of several members of the family *Enterobacteriaceae*, showed that the cell surface-exposed regions are hypervariable (5). The specificity of two oligonucleotides based on the first and third surface-exposed region of *S. typhimurium* OmpA have been tested in PCRs (unpublished results). No amplified product could be detected with the 13 tested non-*Salmonella* strains of which 9 belonged to the family of *Enterobacteriaceae*. The expected 285 bp DNA fragment was correctly amplified in 99 cases out of the 133 tested *Salmonella* strains, comprising the five subspecies. Although it should be noted that these results are based on a single experiment, they indicate that the sensitivity of this primer-couple is less than that of the primer-couple based on PhoE. This correlates well with the observations that the cell surface-exposed regions of OmpA, probably due to an intensive phage-selection pressure, are more variable than those of PhoE. The hypervariabilities of the variable surface-

TABLE 1: Overview of the specificities of the developed primer-couples.

primers / strains		EC5 EC8c$_2$	ST5 ST8c	CF5$_2$ CF8c$_2$	KP5 KP8c	KO5$_1$ KO8c	C1 KP8c	C1 C2c
Escherichia coli	+	21	0	0	0	0	0	21
	−	0	1	1	1	1	1	0
Shigella	+	14	0	−	0	0	0	14
	−	0	2	−	2	2	2	0
Salmonella	+	0	132	0	0	0	0	19
	−	20	1	18	4	4	4	1
Citrobacter freundii	+	0	0	5	0	0	0	6
	−	6	2	0	1	1	1	0
other *Citrobacters*	+	0	0	0	−	−	−	4
	−	4	1	5	−	−	−	0
Klebsiella pneumoniae	+	0	0	0	60	0	62	61
	−	62	1	1	1	63	1	1
Klebsiella oxytoca	+	0	−	−	0	5	6	6
	−	6	−	−	6	1	0	0
other *Klebsiellae*	+	0	−	−	0	0	9	9
	−	9	−	−	9	9	0	0
Enterobacter cloacae	+	0	0	0	0	0	0	1
	−	1	1	1	1	1	1	0
other *Entero-bacteriaceae*	+	0	0	0	0	0	0	0
	−	6	7	6	5	6	6	6
non-*Entero-bacteriaceae*	+	0	0	0	0	0	0	0
	−	4	4	4	5	4	4	4

exposed regions of LamB are even higher than those of OmpA. However Bej *et al.* (4 developed an *E. coli/Shigella* specific PCR using primers based on LamB. Interestingly the primers were based on two surface-exposed regions that, according to Werts *et al.* (49 are reasonable conserved. This suggests that when studying the possibility of using LamB for the development of enterobacterial species-specific oligonucleotides the focus should be on the more conserved surface-exposed regions rather than on the highly variable domains.

Also for other Gram-negative bacteria, i.e. *Chlamydia trachomatis* (50) and *Neisseria meningitidis* (23), it has been shown that variable cell surface-exposed regions of outer membrane proteins can be used to develop specific oligonucleotides. In both cases, the oligonucleotides were not species-specific, but recognized only specific serovars and suggestions were made to use them in epidemiological research. In conclusion, so far only PhoE has the right specificity in order to develop species-specific oligonucleotides whereas other outer membrane proteins seem to be either too variable, or in case of for instance the phospholipase PldA for which homologies have been found between the *E. coli* PldA and those of *S. typhimurium*, *K. pneumoniae* and *Proteus vulgaris* of 94%, 87% and 87%, respectively, (R. Brok, B. Verhey and J. Tommassen,unpublished results) are

probably too conserved.

Nucleotide sequences can also be based on random fragments, selected for thei specificity from a gene library as has been described for *Salmonella* (14, 29). The majo drawback of this method is that the found nucleotide sequences are large, i.e. ranging fron several 100 to several 1000 basepairs. The development of oligonucleotides that have th same specificity as the maternal sequence might be possible but is very laborious.

Another elegant approach in developing specific oligonucleotides is based o ribosomal RNA (rRNA) sequences. The rRNA molecules occur in all organisms and thei mosaic-like pattern, comprising highly conserved as well as more variable sequences allows the construction of oligonucleotides of defined specificities. Furthermore, they ar easily sequenced and provide high abundant targets for detection (19). They have alread proven their usefulness as taxonomic tools, especially in discerning the relationship between distantly related taxa. A question to be answered is however whether rRNAs ca be used to distinguish between closely related taxa, e.g. between the different specie belonging to the family of *Enterobacteriaceae*. The sequence of the 16S rRNA of *E. col* and *S. typhimurium* differs by only 2 to 3 % and therefore some scientists preclude thei use in fine-scaling positioning of taxa (7, 20). On the other hand reports have been mad on the development of *P. vulgaris* (15), *E. coli/Shigella*, *Salmonella* and *Y. enterocolitic* specific oligonucleotides (19) based on their 16S rRNA. However, the extent of non homogeneity in closely related rRNA sequences, particularly in the highly variabl regions, has only recently become evident, since large numbers of closely relate sequences have been inspected. For instance, sequence analysis of different highly variabl regions of the *Salmonella* 16S and 23S rRNA revealed that different sequence pattern occur among the *Salmonella* strains. The different hypervariable regions segregat *Salmonella* strains differently and exhibit different homologies to non-*Salmonella* strains Therefore, Lane *et al.*, concluded that the use of highly variable regions of rRNA sequenc for fine-scaling structure phylogenetic mapping of closely related species, while quit seductive, is a dangerous and unreliable practice (19). From the above discussion, it i clear that although the rRNAs are very suited for the development of specifi oligonucleotides they have their limitations. A comparitive evaluation of the rRNA an PhoE probes is, at this stage, not easy, simply because their specificities have not bee tested on the same set of strains. However, from the fact, that the homologies between th variable rRNA regions are significantly higher than compared to those of PhoE, it can b expected that PhoE is more suited to be used for the development of species-specifi oligonucleotides.

phoE based oligonucleotides as taxonomic tools.

Historically the classification of bacteria was based on similarities in phenotypi characteristics. The main disadvantage of this system is that it is not based on a singl definition but on subjective criteria. Depending on the taxonomists point of view differer importance is given to different biochemical properties. This resulted in classifications tha were often drastically revised by others who made different intuitive judgements (35). A example is *Enterobacter aerogenes* for which proposals have been made to rename *Klebsiella mobilis* since it became evident that it is more related to *Klebsiella* than t *Enterobacter* strains (31). At the moment, most taxonomists agree that a species definitio

which represents the basic taxonomic group, can be made from DNA relatedness da that are essentially equally applicable to all bacteria (7). The data are obtained comparing the ability of DNA from one microorganism to reassociate or hybridi specifically with DNA from another microorganism (11). In general, the definition of species is a group of strains in which DNA relatedness is 70% or greater at conditio optimal for reassociation, 60% or more relatedness at stringent reassociation criteria, a in which related sequences differ 5% or less (7). All species are assigned to a genu which is a well-defined group that is clearly separated from other genera. Howeve considerable subjectivity is involved at the genus level, because there is so far no gener agreement on the definition of the genus in bacterial taxonomy. Classification relationshi at the familial and ordinal levels are even less certain. Recent developments in taxonom such as the rRNA homology studies however have in several instances already been usef to justify the classification and increase its objectiveness (35). The family *Enterobacteriaceae* is a good example of how classification can change over a period time. In 1974, the family of *Enterobacteriaceae* contained 13 genera with 40 species. 1985 there were already 26 published genera containing some 115 species (7).

A first indication that oligonucleotides based on PhoE can be used as taxonom tools was obtained when the specificity of a PCR using primers based on *E. coli* Pho was tested (Chapter 3). The oligonucleotides correctly recognized all tested *E. coli* a *Shigella* strains given further proof to the fact that *E. coli* and *Shigella* belong to o single species (6). The PCR, using primers based on *S. typhimurium* PhoE (Chapter 4 recognized all, but one, tested *Salmonella* strains, comprising the five different subspecie is in agreement with the fact that the genus *Salmonella* consists of only a single specie (34). The PCR, using primers based on the *C. freundii* PhoE (Chapter 5), recognized onl the *C. freundii* strains, including the slow-lactose-fermenting strain, that previously w designated *S. bethesda*/*S. ballerup*. This supports the idea that there is no reason distinguish the *bethesda* group from *C. freundii* solely on the basis of slow-lactos fermentation (9). Because the PCRs were apperently very reliable, the possibility w tested to simplify the classification of *Klebsiella*, by using oligonucleotides based on *pneumoniae* and *K. oxytoca* PhoE. The genus *Klebsiella* consists of four species, i.e., *pneumoniae, K. oxytoca, K. planticola* and *K. terrigena* and proposals have been made transfer *E. aerogenes* to the genus as *K. mobilis* (31). The classification of *Klebsiella* in the different species is based on their biochemical properties which are in many cas difficult to interpret. Most difficulties occur in differentiating *K. pneumoniae* from t indole-negative *K. planticola* strains. Since there is no key discriminatory test in t biochemical classification a combination of an L-sorbose fermentation test and a hydrox L-proline utilization test is used to distinguish between the two species (2). Also, tw other tests are used, i.e. the faecal coliform reaction at 44,5 °C and growth at 10 °C (2 However, scientist have found different correlation percentages between the two tes combinations, i.e. 65% (26) and more than 90% (2), demonstrating the difficulties in t biochemical classification. A PCR, using primers based on part of the fifth and eighth ce surface-exposed regions of *K. pneumoniae* PhoE, correctly recognized 60 out of the 6 tested *K. pneumoniae* strains, whereas no amplified product could be detected with the 3 tested non-*K. pneumoniae* strains of which 15 belonged to the genus *Klebsiella*. G chromatographic analysis of the bacterial fatty acids confirmed that the *Klebsiella* strai that were recognized by the PCR were closer related to each other than to other *Klebsiel*

strains (Chapter 6). To distinguish between the indole-positive *K. planticola* strains and *K. oxytoca*, the ability of the strains to degrade pectate, to produce pigment on gluconate ferric citrate and to utilize gentisate as sole carbon source has to be tested (2). Two overlapping oligonucleotides, designated $KO5_1$ and $KO5_3$, based on the fifth surface exposed region of *K. oxytoca* PhoE were used together with KO8c, an oligonucleotide corresponding to part of the eighth surface-exposed region, in PCRs. Both primer-couples did not react with the 19 non-Klebsiella strains and with the 70 indole-negative *Klebsiella* strain tested (Chapter 7). Primer-couple $KO5_1$/KO8c reacted with five out of the eight indole-positive *Klebsiella* strains. All these PCR positive strains were positive in the pectate degradation test and were classified by others as *K. oxytoca* (26), whereas the PCR negative strains were negative in the pectate degradation assay and were classified as *K. planticola*. There was only one exception; K antigen reference strain K74, although classified as *K. oxytoca* was not recognized by $KO5_1$/KO8c. A conclusion that may be drawn from these results is that PCRs using primers based on PhoE are not infallible. The results obtained with primer-couple $KO5_3$/KO8c however, point into a different direction. This primer-couple recognized all eight indole-positive *Klebsiella* strains. These data suggest that strain K74 may be more related to the indole-positive *K. planticola* strains than to *K. oxytoca* but moreover, that the indole-positive *K. planticola* strains are more related to *K. oxytoca* than to the indole-negative *K. planticola* strains. Especially the latter point is very interesting and should be studied more accurately, for instance by DNA-DNA hybridization studies and of course by analysing the corresponding *phoE* genes. Sequence analysis revealed that the species-specificity of primer-couples $KO5_1$/KO8c and KP5/KP8c is probably located in the primers based on the fifth exposed regions, i.e. $KO5_1$ and KP5. Therefore the possibility was considered to use KP8c together with C1, which is based on a highly conserved region of PhoE, as a primer-couple in order to develop a genus-specific PCR. This approach was successful (Chapter 7) and is a first indication that PhoE can also be used to develop PCRs with a specificity above the species-level. Furthermore, it also has a practical application since it is now possible to amplify the *phoE* genes of the other *Klebsiella* species by PCR, simplifying our strategy to develop species-specific oligonucleotides.

Application for detection in food, water, clinical and environmental samples.

Diagnostical assays must be specific, sensitive, rapid, easy to perform and cheap. To meet these criteria, the developed oligonucleotides must be used in assays that contain amplification steps like PCR, Nucleic Acid Sequence Based Amplification (NASBA) (18) or Q_β replicase (21). Therefore, the specificities of the oligonucleotides have been evaluated in PCRs. Although the PCR is commonly seen as a very powerful technique there are still some points to be considered:

- Theoretically, a single copy of target DNA can be detected in PCRs and indeed detection-limits of one to ten colony forming units (cfu) have been reported, when the assay was performed on pure cultures (48). However, when performed on food (48), clinical (28, 32) or environmental (36) samples, detection-limits of not lower than 100 to 1000 cfu have been reported. Limiting factors are the recovery of the target sequence from the sample and the efficiency of the PCR. The enzyme, commonly used in PCRs, i.e. the thermostable *Taq*-polymerase is very susceptible to inhibitory compounds that may be

present in the sample (24, 28, 47, 48). Therefore, suitable DNA must be prepared a depending on the sample, this may involve treatment with enzymes and/or reagen filtration or centrifugation steps. Another option is to add a brief enrichment-culturing st prior to the actual detection (47). In this way not only more target organisms becor present in the sample, but also possible inhibitory compounds are diluted. It is importa to note that additional steps required to increase the sensitivity also increase the tin required to perform the assay. The Q_β-replicase system may have an advantage over t PCR because an amplification compatible target isolation procedure is integrated in t amplification process. The system relies on the ability of the enzyme Q_β-replicase amplify exponentially recombinant RNA molecules that contain the oligonucleoti sequence specific for the organism to be detected. It consists of three steps: hybridization of the recombinant RNA probe to the target (ii) separation and isolation the formed probe-target hybrids by use of a capture probe and paramagnetic particles ar (iii) amplification of the isolated recombinant RNA probe by Q_β-replicase.

- The equisite sensitivity of the PCR has also an important drawback, i.e. t synthesized PCR products, designated amplicons, can contaminate reagents or samp specimens and subsequently cause systematic errors. Therefore, extreme care is essenti when handling amplified samples in order to minimize carryover. In addition sterilizatic of the amplicons can be used to prevent them from acting as templates in subsequer PCRs. At two points in the PCR, a sterilization step can be implemented. Shortwav ultraviolet irridiation (12) can be performed just before the amplification. This pre-PC sterilization procedure sterilizes only carryover molecules present in the reaction mixtur Because the true target molecule must be introduced after the sterilization process, PC errors due to carryover still exist. This problem does not occur when the sterilization i performed after the amplification which results in the sterilization of all nucleic acid including PCR products. Examples of post-PCR sterilization are the (photo)chemical (8 and enzymatic (51) modification system. A promising enhancement of PCR is th homogeneous PCR assay described by Higuchi *et al.* (16). By adding ethidium bromid to the reaction-mixture and measuring the increase in fluorescence they were able to detec the amplified DNA sequences without opening the reaction-tube. This method only work if appropriate amplification conditions are used and no or only small amounts of non specific double stranded DNA is produced. Also the start amount of chromosomal DN should not be too high otherwise background fluorescence will become a problem. major advantage of this assay is that the amplification process can be continuousl monitored in order to follow its progress.

- The PCR method can not discriminate between life and dead cells. In some cases this can be disadvantageous. For instance, the presence of dead cells does not generall constitute a health hazard although their detection would provide useful informatio regarding the quality of food. A brief enrichment culturing step followed by dilutio would decrease the amount of these "false-positive" results, provided that the amount o dead cells is not too high. However, in that case also strains that are not culturable bu still viable will no longer be detected. Another option is to use messenger-RNA (m-RNA instead of DNA as the target molecule. To detect RNA, the PCR requires a precedin reverse transcriptase (RT) step, that "translates" the RNA into DNA and this complicate the procedure. The NASBA and Q_β-replicase system are more suited to be used for th amplification of RNA targets. NASBA is based on the simultaneous activity of thre

enzymes, i.e. AMV-reverse transcriptase (AMV-RT), RNAse H and T_7 RNA polymerase. The reaction starts with the hybridization of primer 1, containing the T_7 promoter sequence, to the mRNA and the subsequent "translation" of the RNA in DNA by AMV-RT. The RNAse H degrades the RNA strand in the formed RNA/DNA hybrid molecule. Primer 2 anneals to the resulting single stranded copy DNA and the second DNA strand is sythesized by the DNA-depedent DNA polymerase activity of the AMV-RT. This results in a double stranded DNA molecule that contains a T_7 RNA polymerase promoter sequence. The T_7 RNA polymerase gives a 100 to 1000 fold increase in specific RNA. This newly formed RNA enters the so called cyclic phase were the events are the same but the primers are incorporated in reversed order. A serious drawback of taking mRNA as startpoint of the detection is that it may not always be present. Because procaryotic mRNAs are unstable and therefore have a short lifetime cells in rest will not be detected. Other false-negative results are due to cells that, although containing the target gene do not express it. For instance, the PhoE mRNA will only be present when cells are grown under phosphate-limitation but for all target genes it remains a question whether the cells have encountered the conditions necessary to express them.

In conclusion, although several problems still have to be solved, the recent developments in amplifying genetic sequences of interest heralds a new area in diagnostic technology. The question what kind of assay performs best is at this moment not easy to answer and will propably depend on the organism to be detected and the sample in which it is present. One thing however is sure; ***no probe, no assay!***

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SAMENVATTING

De detectie en identificatie van diverse bacteriën, inclusief leden van de famil *Enterobacteriaceae,* is belangrijk in een groot aantal studies van zowel fundamentele a toegepaste aard in de medische, voedsel, agrarische en milieu sectoren. Bestaande detect en identificatie procedures, die voornamelijk gebruik maken van de klassiek microbiologische methoden, zijn arbeidsintensief en nemen veel tijd in beslag. DN hybridisatie, vooral wanneer gecombineerd met een amplificatie procedure zoals d polymerase ketting reactie (PCR), wordt in het algemeen gezien als een veelbelovend techniek om snelle, en daarom economisch belangrijke diagnostische methodieken t ontwikkelen.

De eerste stap in het ontwikkelen van detectie en identificatie methoden op bas van DNA hybridisaties is het vinden van DNA sequenties (probes) die uniek zijn voor ee groep van organismen. Deze probes moeten zowel sensitief als selectief zijn; dit w zeggen ze moeten reageren met alle stammen en serotypen behorend tot de groep maa ze mogen niet kruisreageren met andere bacteriën. Het doel van het in dit proefschri beschreven onderzoek was te bepalen of species-specifieke DNA probes ontwikkel kunnen worden op basis van de *phoE* genen die coderen voor het buitenmembraan eiw PhoE in diverse *Enterobacteriaceae.*

De buitenmembraan, die deel uitmaakt van de celenveloppe, beschermt de bacteri tegen schadelijke stoffen uit de omgeving. In de buitenmembraan bevinden zich eiwitte waaronder de porines, waardoor de noodzakelijke voedingsstoffen deze membraan kunne passeren. Onder standaard groeicondities synthetiseert *Escherichia coli* twee porie-eiwitter OmpC en OmpF. De synthese van een derde porine, het PhoE eiwit, wordt geïnduceer wanneer gekweekt wordt onder fosfaatlimitatie. De nucleotide-sequenties van de genen di coderen voor deze porines zijn bepaald en vergelijking van de afgeleide aminozuur sequenties leerde dat deze porines nauw aan elkaar verwant zijn: de homologie tussen dez drie eiwitten bedraagt ongeveer 60%. Er is een model voor de topologie van het Phol eiwit opgesteld. Volgens dit model wordt het eiwit voorgesteld als een polypetideketen di 16 maal de buitenmembraan passeert in de vorm van een anti-parallele ß-sheet structuu en daarbij acht gebieden aan het celoppervlak exposeert. Wanneer de primaire stucture van OmpC, OmpF en PhoE met elkaar vergeleken worden, blijkt dat d membraanoverspannende segmenten geconserveerd zijn, terwijl de aan het celoppervla geëxposeerde segmenten variabel zijn.

Naast het *phoE* gen van *E. coli* zijn ook de *phoE* genen van *K. pneumoniae* en *E cloacae* gekloneerd. Vergelijking van de primaire structuren van deze PhoE eiwitte toonde een onderlinge homologie van 81%. De vier hypervariabele gebieden di onderscheiden kunnen worden bleken allen aan het celoppervlak geëxposeerd te zijn Inmiddels zijn in dit onderzoek ook de nucleotiden-sequenties van de *phoE* genen va *Salmonella typhimurium* (Hoofdstuk 4), *Citrobacter freundii* (Hoofdstuk 5) en *Klebsiell oxytoca* (Hoofdstuk 7) bepaald. Bij vergelijking van alle nucleotide en aminozuu sequenties met elkaar bleek dat de PhoE eiwitten sterk geconserveerd zijn. De homolgiëi wat betreft identieke aminozuur residuen tussen het PhoE eiwit van *E. coli* en die van *C*

freundii, *E. cloacae*, *K. pneumoniae*, *K. oxytoca* en *S. typhimurium* bedragen respectievelijk 90%, 87%, 83%, 85% en 87%. De homologie in aminozuur residuen die geconserveerd zijn in alle zes de eiwitten bedraagt 72%. Ondanks de hoge homologie tussen de verschillende PhoE eiwitten konden er vier gebieden onderscheiden worden die minder geconserveerd zijn. Deze variabele gebieden corresponderen met regionen die in het gepostuleerde topologie model celoppervlak geëxposeerd zijn.

Uitgaande van het idee dat DNA sequenties die coderen voor (gedeelte van) hypervariabele regionen species-specifiek zijn, werden er oligonucleotiden ontwikkeld gebaseerd op het vijfde en het achtste celoppervlak geëxposeerde gebied van de PhoE eiwitten van *E. coli* K-12 (Hoofdstuk 2, 3), *S. typhimurium* (Hoofdstuk 4), *C. freundii* (Hoofdstuk 5), *K. pneumoniae* (Hoofdstuk 6) en *K. oxytoca* (Hoofdstuk 7). Wanneer deze oligonucleotiden werden gecombineerd als primerkoppels in PCR's bleken alle specifiek te zijn voor het species waartoe de bacteriën behoorden waaraan de sequenties werden ontleend. Onderlinge vergelijking van de PhoE sequenties van *K. pneumoniae* en *K. oxytoca*, beide behorend tot het genus *Klebsiella*, wees echter alleen het vijfde geëxposeerde gebied als variabel aan. Hieruit werd geconcludeerd dat de species-specificiteit van de *K. pneumoniae*- en *K. oxytoca*-specifieke primerkoppels gelocaliseerd is in de primers gebaseerd op de vijfde celoppervlak geëxposeerde gebieden terwijl de primers gebaseerd op de achtste celoppervlak geëxposeerde gebieden mogelijk genus-specifiek zijn. Dit werd getest door de laatsgenoemde primers te combineren in PCR's met een primer gebaseerd op een deel van *phoE* dat sterk geconserveerd is tussen bacteriën die tot verschillende genera behoren. Het koppel bleek inderdaad specifiek te zijn voor het genus *Klebsiella* (Hoofdstuk 7). Door twee primers gebaseerd op sterk geconserveerde gebieden van *phoE*, als primerkoppel te gebruiken werd tevens een PCR ontwikkeld die specifiek alle nauw aan elkaar verwante genera binnen de familie herkende, i.e. *E. coli/Shigella, Citrobacter, Enterobacter, Klebsiella en Salmonella* (Hoofdstuk 3).

Samenvattend kan geconcludeerd worden dat de *phoE* genen gebruikt kunnen worden voor het ontwikkelen van enterobacteriële species-specifieke oligonucleotiden, door uit te gaan van de hypervariabele, celoppervlak geëxposeerde gebieden. Verder blijkt het mogelijk te zijn om, door uit te gaan van meer geconserveerde gebieden van *phoE*, oligonucleotiden te ontwikkelen met een specificiteit boven het species-niveau.

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CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 9 juli 1959 te Beek en Donk geboren. In 1976 werd het H.A.V.O. diploma en in 1978 werd het V.W.O. diploma behaald aan de Philips van Horne Scholengemeenschap te Weert. In september 1978 werd begonnen met de studie Farmacie aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen werd behaald in juni 1981. Het doctoraalexamen, met als bijvak Microbiologie (begeleiding Dr. I.M. van Die en Prof.Dr. W.P.M. Hoekstra), werd in mei 1985 behaald. Op 1 juni 1986 werd een tijdelijke aanstelling verkregen als wetenschappelijk medewerkster en later als toegevoegd onderzoekster bij de Vakgroep Moleculaire Celbiologie van de faculteit Biologie aan de Rijksuniversiteit te Utrecht. Na een korte periode in tijdelijk dienstverband werkzaam te zijn geweest bij het Koninklijk Instituut voor de Tropen, is de schrijfster van dit proefschrift vanaf 29 september 1992 in dienst bij Applied Biosystems als "Molecular Biology Sales Specialist".

LIST OF PUBLICATIONS

1. Van Die, I., G. Spierings, I. van Megen, E. Zuidweg, W. Hoekstra and I Bergmans. Cloning and genetic organization of the gene cluster encoding F7 fimbriae of a uropathogenic *Escherichia coli* and comparison with the F7-2 ge cluster. (1985) *FEMS Microbiol. Lett.* **28**:329-334.

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